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TEMPERATURE EFFECT ON THE DETECTION OF MUSCARINIC RECEPTOR–G PROTEIN INTERACTIONS IN LIGAND BINDING ASSAYS

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Abstract—The ability of guanine nucleotides to lower agonist binding affinity provides a convenient indication of receptor–G protein coupling: guanine nucleotides convert muscarinic receptors from high-affinity states for agonists to low-affinity states. We studied the influence of assay temperature on the demonstration of this coupling in rat brainstem and atrium. Agonist affinity of brainstem receptors increased as temperature was lowered, reflecting a greater proportion of receptors in high-affinity conformations. The influence of 5′-guanylylimidodiphosphate, a stable analog of GTP, on agonist binding, determined directly (using [^{3}H]oxotremorine-M) or indirectly (in [^{3}H]*N*-methylscopolamine/carbamylcholine competition studies), was greatest from 16 to 20°. Guanine nucleotide sensitivity was much reduced at 0–4° and 37°. Brainstem and atrial muscarinic receptors were similarly affected by temperature. We suggest that high-affinity receptor–G protein complexes are unstable at high temperatures, thereby decreasing agonist affinity and masking the guanine nucleotide effect. At low temperatures, the receptor–G protein complex is stabilized and fails to dissociate in the presence of guanine nucleotides. The optimum temperature for monitoring receptor–G protein interactions in binding assays was 16–20°.

Muscarinic receptor control of several cellular processes, including adenylate cyclase activity, potassium channel conductance, and phosphatidylinositol metabolism, are mediated by guanine nucleotide-dependent transducer proteins (G proteins) [1]. One useful index of receptor–G protein coupling is the influence of guanine nucleotides on agonist binding properties. Guanine nucleotides convert muscarinic receptors from a state of high affinity for agonists to a state of low affinity for agonists, presumably by binding to G proteins and engendering their dissociation from the receptor [2].

Receptor–G protein coupling is affected by a number of conditions and agents, including pH [3], protein modification [4, 5], proteases [6, 7], ions [8], and volatile anesthetics [9]. It is possible that the coupling of G proteins to muscarinic receptors plays a role in the physiological control of cholinergic transmission. We have been interested in how this coupling is affected in development and disease states and by drugs. To gain insight into the coupling process, we examined the effect of temperature on the ability of a guanine nucleotide to affect agonist binding to brainstem and atrial muscarinic receptor in standard binding assays. A major influence of temperature on whether evidence of receptor–G protein interactions can be obtained in binding experiments is demonstrated.

METHODS

Adult male Wistar rats were killed by decapitation, and their brainstems (medulla-pons-midbrain) and atria were removed and homogenized separately in 10 vol. of 50 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$ and 100 μM phenylmethylsulfonyl fluoride (to prevent proteolysis). The homogenates were spun at 20,000 *g* for 20 min at 4°. The pellets were resuspended in Tris–magnesium buffer and used without further treatment. Protein content was estimated by a modification of the method of Lowry *et al.* [10].

[^{3}H]*N*-Methylscopolamine ([^{3}H]MS; 84 Ci/mmol, New England Nuclear) binding was measured using a filtration procedure [4]. Assay conditions were as follows: incubation time, 90 min; buffer, 50 mM Tris–HCl, pH 7.4, with 2 mM $MgCl_2$; volume, 2 or 5 ml; [^{3}H]MS concentration, 0.032 or 0.1 nM; protein content, 75 μg (brainstem) or 125 μg (atrium). Nonspecific binding was determined in the presence of 10 μM atropine.

Carbamylcholine binding was inferred from its ability to inhibit 0.032 nM [^{3}H]MS binding [11]. The volume of the assay medium was increased to 5 ml so that maximum tissue binding (specific and nonspecific) represented less than 7% of the added radioactivity. Under these assay conditions, the maximum fraction of receptors occupied by [^{3}H]MS was less than 25%. Equilibrium binding data were fitted by iterative nonlinear regression analyses to mass action expressions for two or three populations of independent binding sites, as follows:

$$B = B_1 \cdot [C]/([C] + K_1) + B_2 \cdot [C]/([C] + K_2) + B_3 \cdot [C]/([C] + K_3)$$

where B is binding, $[C]$ is the concentration of carbamylcholine, and B_1, B_2, and B_3 are the fractions of receptors displaying dissociation constants of K_1,

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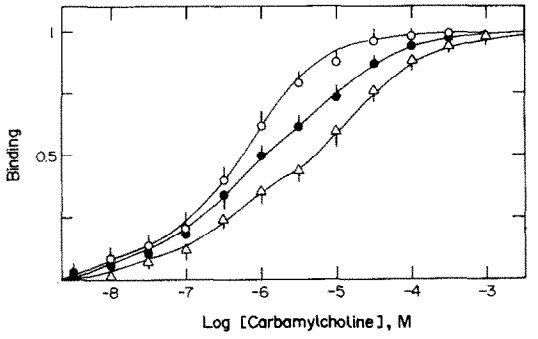

Fig. 1. Influence of temperature on carbamylcholine binding to brainstem muscarinic receptors. Carbamylcholine binding was determined in competition experiments using 0.032 nM [^{3}H]MS. Specific binding was measured at 0° (○), 20° (●) and 37° (△). Each point and bar represent the mean and standard deviation from three experiments, each performed in triplicate. Lines are drawn according to nonlinear regression fits of the data to models incorporating two or three independent binding sites, which revealed the parameters listed in Table 1.

K_2 and K_3 respectively. Nonlinear analyses were performed using procedures available in the SPSS system. The appropriateness of a 2 or 3 population fit was determined by the F distribution from the extra sum of squares test as described by Munson and Rodbard [12].

High-affinity [^{3}H]oxotremorine-M (85 Ci/mmol, New England Nuclear) binding was measured using a centrifugation assay. Membranes were incubated with 3 nM [^{3}H]oxotremorine-M for 15 min in 1 ml of 50 mM Tris–2 mM $MgCl_2$ buffer, pH 7.4, and then spun at 460 *g* for 3 min. The pellets were rinsed once with cold buffer, and the tubes were inverted and allowed to dry for 2 hr. The tips were then cut off, and their radioactivity content was determined by liquid scintillation counting.

Receptor–G protein coupling was indicated by the ability of 5′-guanylylimidodiphosphate (Gpp(NH)p), a stable analog of GTP, to either shift carbamylcholine/[^{3}H]MS competition curves to the right or to eliminate high-affinity [^{3}H]oxotremorine-M binding. Both actions reflect a conversion of receptors from a high-affinity (G-protein coupled) to a low-affinity (G-protein uncoupled) state.

RESULTS

Carbamylcholine affinity for brainstem muscarinic receptors increased as the temperature was lowered (Fig. 1; Table 1). Three components of carbamylcholine binding could be detected at 20° and 37° (comparing the 2- and 3-site models, $F = 3.4$ and 3.7, respectively; $P < 0.05$): a low-affinity component with a K_D from 19 to 24 μM, a high-affinity component with a K_D from 0.52 to 0.85 μM, and a small, super-high affinity component with a K_D from 0.017 to 0.020 μM (Table 1). As the temperature was decreased from 37° to 20° to 0°, the fraction of receptor displaying low-affinity binding decreased and the fraction of receptors displaying high-affinity binding increased (Table 1). There were no significant changes in the population of receptors displaying super-high-affinity carbamylcholine binding, or in the dissociation constants associated with each of the binding states. At 0°, carbamylcholine binding was best described by a model encompassing only high- and super-high-affinity receptors in a ratio of 83:17. Thus, the major influence of lowering temperature appears to be to convert muscarinic receptors from a state characterized by low affinity for agonists to a state characterized by high affinity for agonists.

The influence of temperature on guanine nucleotide modulation of agonist binding to brainstem muscarinic receptors is illustrated in Fig. 2. At 4°, carbamylcholine/[^{3}H]MS competition curves were unaffected by 10 μM Gpp(NH)p. At 20° and 37° the curves were shifted to the right 11- and 3-fold respectively.

The inhibition of 0.1 nM [^{3}H]MS binding by 1 μM carbamylcholine was measured at several temperatures in the presence and absence of 10 or 100 μM Gpp(NH)p (Fig. 3). The ability of Gpp(NH)p to depress carbamylcholine inhibition of [^{3}H]MS binding served as an indication of guanine nucleotide modulation of agonist binding. The greatest nucleotide effect was observed at 15–20°. The

Table 1. Carbamylcholine binding to brainstem muscarinic receptors at three temperatures

	Binding parameters*					
	Super-high		High		Low	
Temperature	K_D	%	K_D	%	K_D	%
0°	0.017 ± 0.008	17 ± 3	0.85 ± 0.21	83 ± 7		
20°	0.018 ± 0.009	16 ± 4	0.61 ± 0.31	50 ± 10	19 ± 7	34 ± 8
37°	0.020 ± 0.010	10 ± 4	0.52 ± 0.21	34 ± 7	24 ± 8	54 ± 11

* Carbamylcholine binding was resolved into two or three components, as appropriate, using models incorporating independent binding sites. K_D, dissociation constant in μM; %, percent of total receptor population displaying binding characterized by the indicated K_D. Means and standard deviations from three experiments are shown. The only significant differences ($P < 0.05$) in carbamylcholine binding at the different temperatures were in the fractions of receptors displaying high- and low-affinity agonist binding.

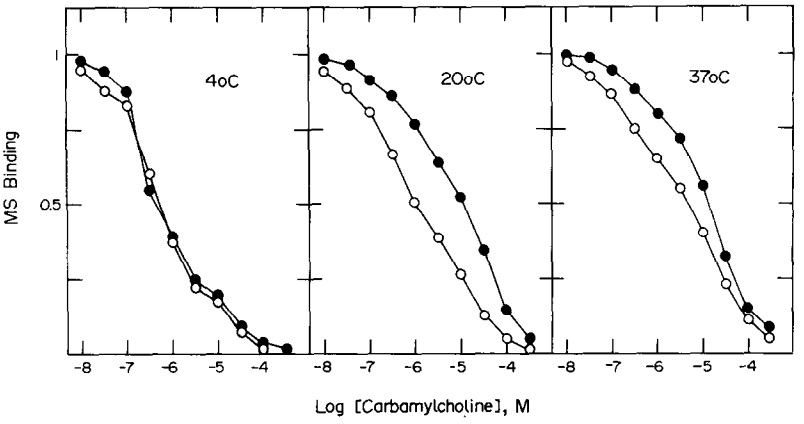

Fig. 2. Influence of temperature on guanine nucleotide modulation of carbamylcholine binding. The binding of 0.1 mM [^{3}H]MS to brainstem muscarinic receptors was measured in the presence of the indicated concentrations of carbamylcholine at 4° (left), 20° (center) and 37° (right). Binding was measured in the absence (○) and presence (●) of 10 μM Gpp(NH)p. Each point represents the mean from three experiments.

nucleotide effect fell sharply below 15° and more gradually at temperatures greater than 20°. The nucleotide effect was eliminated almost completely at 0° and was depressed 36% at 37°.

The guanine nucleotide sensitivity of agonist binding to muscarinic receptors from rat atrium was similarly affected by temperature (Fig. 4): the guanine nucleotide effect was greatest from 15 to 20°. The depression of the guanine nucleotide effect at low temperatures, however, was not as great as with brainstem receptors.

[^{3}H]Oxotremorine-M (3 nM) was used to label muscarinic receptors with high affinity for agonists (Fig. 5). This binding was eliminated by guanine nucleotides as they converted receptors to conformational states characterized by lower agonist affinity (low-affinity [^{3}H]oxotremorine-M binding cannot be detected with the assay employed). Guanine nucleotide elimination of high-affinity [^{3}H]oxotremorine-M binding to both brainstem and atrial muscarinic receptors was greatest at 20°. No inhibition was observed at either 0–4° or at 37°.

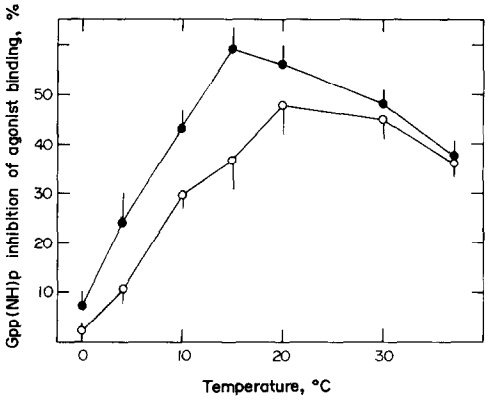

Fig. 3. Influence of temperature on guanine nucleotide modulation of carbamylcholine binding to brainstem muscarinic receptors. [^{3}H]MS binding (0.1 nM) was measured in the presence and absence of 1 μM carbamylcholine. The fraction of this inhibition which was eliminated by including 10 (○) or 100 (●) μM Gpp(NH)p in the assay medium was then determined and is plotted as a function of assay temperature. Each point and bar represent the mean and standard deviation from three experiments.

DISCUSSION

The present work demonstrates that (1) agonist affinity increased as temperature was lowered, reflecting a greater proportion of receptors in a high-affinity conformation, and (2) the ability of guanine nucleotides to lower the affinity of muscarinic receptors (brainstem and atrium) for agonist was decreased at high temperatures (above 30°) and was decreased or eliminated at low temperatures (below

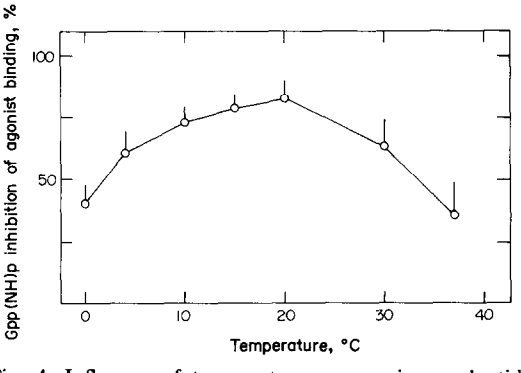

Fig. 4. Influence of temperature on guanine nucleotide modulation of carbamylcholine binding to atrial muscarinic receptors. [^{3}H]MS binding (0.1 μM) was measured in the presence and absence of 1 μM carbamylcholine. The portion of this inhibition that was eliminated by including Gpp(NH)p (10 μM) in the assay medium was then determined and is plotted as a function of assay temperature. Each point and bar represent the mean and standard deviation from three experiments.

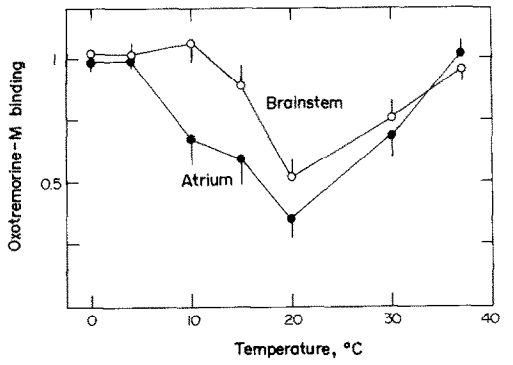

Fig. 5. Influence of temperature on the guanine nucleotide sensitivity of 3 nM [^{3}H]oxotremorine-M binding to brainstem (○) and atrial (●) muscarinic receptors. [^{3}H]Oxotremorine-M (3 nM) binding was measured in the absence and presence of 100 μM Gpp(NH)p. The fraction of [^{3}H]oxotremorine-M binding remaining in the presence of 100 μM Gpp(NHH)p was measured at the temperatures indicated on the abscissa. Each point and bar represent the mean and standard deviation from three experiments.

10°). Temperatures from 16 to 20° appear optimal for demonstrating receptor–G protein interactions in agonist binding assays. The influence of temperature on agonist binding affinity is in agreement with the work of others (e.g. Refs. 13 and 14).

The precise sequence of events involved in signal transduction by muscarinic receptors is incompletely understood, but is believed to involve the following [2]: (1) binding of an agonist promotes the formation of a receptor–G protein complex, (2) a conformational change in the G protein promotes a GTP–GDP exchange, (3) the G protein dissociates into its *alpha* and *beta-gamma* subunits, which then influence various cellular processes. Subsequent to GTP hydrolysis by the alpha subunit, the G protein subunits reassociate and the G protein is available for interaction with a receptor–agonist complex.

Populations of receptors with high and low affinity for agonists have been observed in binding experiments [15], and inclusion of a guanine nucleotide promotes the conversion of receptor from a high-affinity to a low-affinity form [16]. This led to the proposal that receptors with high affinity for agonists are coupled to G proteins, whereas receptors with low affinity for agonists are uncoupled. Thus, both the fraction of receptors displaying high-affinity agonist binding and the ability of guanine nucleotides to convert receptors to states of low affinity provide information concerning receptor association with G proteins. It is interesting that both of these properties are influenced by (1) drugs (e.g. volatile anesthetics [9]), (2) disease states (e.g. diabetes [17]), (3) development [18–20] and (4) alterations of membrane composition [21].

It is not clear, however, to what extent binding properties measured *in vitro* mirror the physiological situation. While the agonist–receptor–G protein complex is believed to be energetically stable [2], the intracellular concentration of GTP is high (1 mM). Under these conditions the half-life of high-affinity receptor complexes would be short; high-affinity agonist binding may be largely a consequence of the low GTP concentration frequently used in binding experiments. Nonetheless, the apparent distribution of receptors among high- and low-affinity forms follows predictable regional [4, 22] and developmental [18–20] patterns and is affected in a consistent manner by drugs and physical conditions. Accordingly, it is assumed that this distribution *in vitro*, as well as the ability of guanine nucleotides to affect this distribution, provides information concerning the native condition of the receptor complex.

The interactions of brainstem and atrial muscarinic receptors with G proteins were similarly affected by temperature. Subtle differences in their responses to temperature may reflect differences in membrane composition. Greater membrane fluidity, for example, may explain the relative insensitivity of atrial receptors to low temperature. The predominant receptor subtype in both of these tissues is M2. Both M1 and M2 receptor subtypes (which are apparently different gene products [23, 24]) display multiple agonist binding affinities, although G protein coupling is more readily demonstrated with M2 receptors.

The effects of temperature on agonist binding determined in [^{3}H]oxotremorine-M and [^{3}H]MS/carbamylcholine binding studies were somewhat different. For example, the guanine nucleotide sensitivity of [^{3}H]oxotremorine binding was eliminated completely at 4°, whereas carbamylcholine binding sensitivity was depressed only partially. These differences are probably related to the fact that [^{3}H]oxotremorine-M selectively labels only a fraction of the high-affinity receptors, whereas the entire receptor population is sampled in [^{3}H]MS/carbamylcholine competition studies.

We suggest the following explanations for temperature effects on the guanine nucleotide sensitivity of agonist binding to muscarinic receptors. At high temperatures receptor–G protein complexes are relatively unstable, even in the absence of guanine nucleotides, decreasing the apparent proportion of receptors in a high-affinity binding state and rendering the guanine nucleotide effect on binding less noticeable. At very low temperatures (0–10°) high-affinity receptor–G protein complexes are much more stable and fail to dissociate even in response to guanine nucleotide binding. This may reflect alterations in the physical properties (e.g. fluidity) of the membrane or in the proteins themselves. At intermediate temperatures (10–25°), receptor–G protein complexes have a relatively long half-life (i.e. the proportion of high-affinity binding is greater than at 37°) but are still capable of dissociating in response to guanine nucleotide binding. Thus, two processes are envisioned to play a role in the *in vitro* demonstration of guanine nucleotide sensitivity of agonist binding: (1) the stability of the receptor–G protein complex, and (2) the ability of the complex to dissociate in response to nucleotide binding. The optimal temperature with regard to these two processes appears to be 16–20°.

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