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DIFFERENTIAL REGULATION OF HIGH-AFFINITY AGONIST BINDING TO MUSCARINIC SITES IN THE RAT HEART, CEREBELLUM, AND CEREBRAL CORTEX

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The muscarinic agonist [^{3}H]cismethyldioxolane ([^{3}H]CD) was used to characterize the effects of regulators upon high-affinity agonist binding sites of the rat heart, cerebral cortex and cerebellum. Comparative studies with sodium ions (Na^+), magnesium ions (Mg^{++}), N-ethylmaleimide (NEM) and the guanine nucleotide Gpp(NH)p revealed tissue-specific effects. Mg^{++} preferentially enhanced while Gpp(NH)p and NEM reduced high-affinity [^{3}H]CD binding in the heart and cerebellum. By comparison NEM enhanced high-affinity agonist binding in the cerebral cortex while Gpp(NH)p and Mg^{++} had little or no effect. Kinetic studies support an allosteric mechanism for these effects and provide further evidence for muscarinic receptor subtypes in mammalian tissues.

Recent studies indicate that distinct subtypes of the muscarinic cholinergic receptor (M_1 and M_2) are present in some tissues(1-5). While previous studies of the drug binding properties (2-5) and physiological responses (3) mediated by these receptor subtypes have been based upon potency comparisons between muscarinic antagonists, very little is known about the interaction of muscarinic agonists with the M_1 and M_2 sites. Therefore, in order to directly characterize the agonist binding properties of muscarinic receptor subtypes, we have developed a highly-specific rapid filtration binding assay (6) for the potent muscarinic agonist [^{3}H]cismethyldioxolane ([^{3}H]CD). From our initial results in the heart, cerebellum and cerebral cortex, it appears that several known regulators of muscarinic binding differentially affect the high-affinity agonist binding properties of muscarinic receptor subtypes.

METHODS

For this study, tissues were obtained from male albino Sprague-Dawley rats (200-400 g). Following decapitation, brains were removed and cerebral cortices

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were separated from noncortical brain regions on a cold glass surface. Hearts were excised and perfused with 20ml of ice-cold 10mM sodium-potassium (Na/K) phosphate buffer, then minced and blotted dry. Homogenates of each tissue (2 percent, cortical; 2.5 percent, heart and cerebellum) were prepared in cold 10mM Na/K phosphate buffer (Na_2HPO_4, 8.1 mM and KH_2PO_4, 1.9 mM; pH 7.4) with a Brinkmann polytron (three 15 second bursts at setting 5.5 separated by 30 second intervals on ice). Cardiac homogenates were filtered through four layers of cheesecloth before use in binding assays.

For studies in N-ethylmaleimide (NEM)-treated tissues, all tissue homogenates were treated identically. One volume of NEM or buffer was incubated with 19 vol of tissue homogenate for 20 min at 37°C. The reaction was terminated by adding 50 vol of ice cold buffer and centrifuging the membranes at 48,000 x g for 10 min. Each sample was then washed twice with 50 vol of cold buffer to remove any residual NEM. Finally, membranes were resuspended in the original volume of buffer before use in binding assays.

[^{3}H]CD binding was measured by a rapid filtration technique(6). In all experiments, a 300µl aliquot of tissue homogenate (approximately 6 mg cortex or 7.5 mg heart or cerebellum) was incubated with [^{3}H]CD (L(+)-cis-[2-methyl-^{3}H]dioxolane, 38.1 Ci/mmol, New England Nuclear) in 2 ml of 10 mM Na/K phosphate buffer (pH 7.4). All binding measurements were done in duplicate or triplicate and binding displaced by 1 µM atropine sulphate was defined as specific tissue binding. Typically, steady-state binding experiments were carried out at 25°C for 120 min. The binding reaction was terminated by rapid filtration of samples through Whatman GF-B glass fiber filter strips (which had been presoaked for 60 to 90 min in 0.05 percent aqueous polyethylenimine) and five washes with 3 ml aliquots of ice-cold 10mM Na/K phosphate buffer. Filters were removed and allowed to dry before assaying filter bound radioactivity by liquid scintillation spectrophotometry in 6 ml of a Triton X-100 (1 liter): toluene (2 liters):Omnifluor (16g) mixture (45 percent counting efficiency). The concentration of protein in tissue homogenates was estimated (7) using standards of bovine serum albumin.

RESULTS

The effects of sodium ions (Na^+), magnesium ions (Mg^{++}), guanyl-5'-yl imidodiphosphate (Gpp(NH)p) and N-ethylmaleimide (NEM) upon high-affinity [^{3}H]CD binding sites were compared in three tissues. As shown in Table 1, Na^+ produced a concentration-dependent reduction of high-affinity [^{3}H]CD binding in the heart, cerebellum and cerebral cortex. No apparent tissue differences were evident for this effect. However, comparative studies of other regulators in these tissues revealed several distinct differences. The nonhydrolyzable GTP analogue Gpp(NH)p reduced high-affinity [^{3}H]CD binding with similar potencies in the heart and cerebellum (M_2 tissues) but was without effect in the cerebral cortex (Table 2). Similarly, Mg^{++} produced concentration-dependent changes in high-affinity [^{3}H]CD binding which were also tissue-dependent. As outlined in Table 1, Mg^{++} increased myocardial [^{3}H]CD binding at low concentrations (less than 1 mM) but reduced binding at

TABLE 1. EFFECTS OF Na^+ AND Mg^{++} ON HIGH-AFFINITY [^{3}H]CD BINDING IN RAT CEREBRAL CORTEX, HEART AND CEREBELLUM.

Addition	Final Conc(mM)	Cortex	Heart	Cerebellum
NaCl[a]	10	84 ± 8	86 ± 12	83 ± 14
	25	59 ± 6*	60 ± 4*	64 ± 10*
	50	42 ± 3*	37 ± 4*	41 ± 6*
	75	34 ± 4*	18 ± 2*	28 ± 9*
	150	22 ± 4*	11 ± 1*	21 ± 5*
	300	9 ± 1*	2 ± 1*	8 ± 3*
$MgCl_2$	0.01	111 ± 10	111 ± 3	135 ± 20
	0.05	108 ± 3	121 ± 4*	138 ± 10*
	0.10	105 ± 3	138 ± 3*	152 ± 6*
	1.0	109 ± 6	124 ± 8*	250 ± 21*
	5.0	86 ± 5	62 ± 3*	168 ± 9*
	10.0	79 ± 5*	44 ± 5*	104 ± 5

Each value in the table represents the mean ± S.E. specific binding (as a percent of control) from four or five experiments. Control specific binding (in fmoles/mg protein) was 15.2±2.1 (cortex), 6.5±1.6 (heart), and 6.9±2.4 (cerebellum).

a. Above 75mM NaCl, nonspecific [^{3}H]CD binding was significantly reduced in all three tissues.

* Significantly different from control ($p<0.05$, Mann-Whitney U test, two-tailed)

higher concentrations. A qualitatively similar inverted U-shaped concentration-effect curve was observed in cerebellar homogenates. By comparison, cortical [^{3}H]CD binding was unaffected by low concentrations of Mg^{++} and only slightly reduced at higher concentrations (Table 1).

The mechanism of binding alterations induced by Mg^{++} or Gpp(NH)p were studied kinetically. As shown in Figure 1a, 30 μM Gpp(NH)p signficantly

TABLE 2. EFFECT OF Gpp(NH)p ON HIGH-AFFINITY [^{3}H]CD BINDING IN RAT CEREBRAL CORTEX, HEART AND CEREBELLUM.

Gpp(NH)p (μM)	Cortex	Heart	Cerebellum
0.01	104 ± 7	101 ± 6	103 ± 7
0.1	100 ± 8	90 ± 4	88 ± 7
1.0	101 ± 5	54 ± 4*	51 ± 6*
10.0	92 ± 6	18 ± 6*	20 ± 7*
30.0	88 ± 7	4 ± 2*	6 ± 3*
100.0[a]	87 ± 4	3 ± 1*	4 ± 2*

Each value in the table represents the mean ± S.E. specific binding (as a percent of control) from three to six experiments.

a. Significantly reduced nonspecific [^{3}H]CD binding in the heart and cerebellum.

* Significantly different from control ($p<0.05$, Mann-Whitney U test, two-tailed).

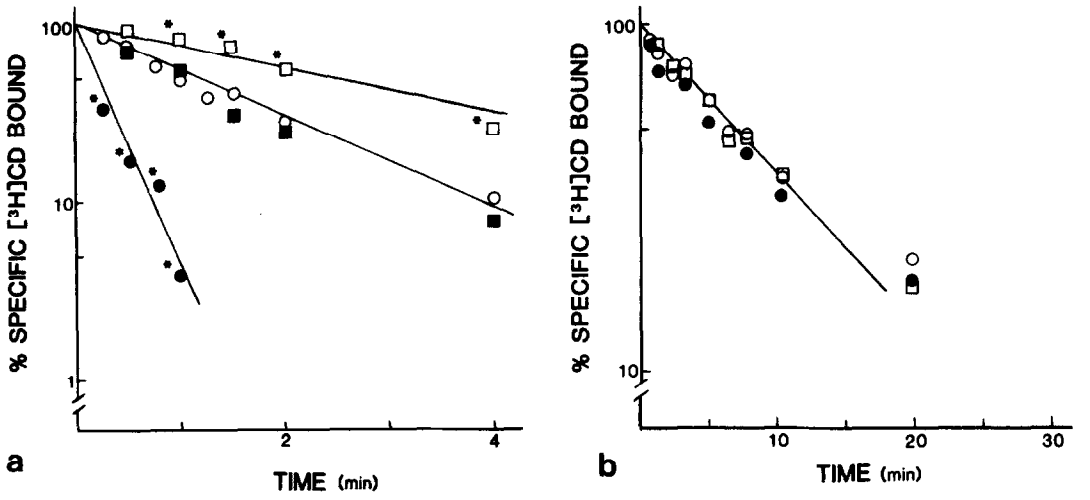

FIGURE 1. EFFECTS OF Mg^{++} AND Gpp(NH)p ON [^{3}H]CD DISSOCIATION RATES IN THE RAT HEART AND CEREBRAL CORTEX. Homogenates of rat heart (panel a) or cerebral cortex (panel b) were equilibrated with 0.6 nM [^{3}H]CD for 2 hr. at 25°C. Immediately thereafter, dissociation was initiated with (in final conc.)1μM atropine (open circles) or 1 μM atropine containing 30 μM Gpp(NH)p (closed circles), 0.1 mM $MgCl_2$ (open squares) or 5 mM $MgCl_2$ (closed squares).

* Significantly different from atropine control (Student's paired t test).

enhanced the rate of [^{3}H]CD dissociation from cardiac membranes but had no effect in the cerebral cortex (Figure 1b). By comparison, 0.1 mM Mg^{++} significantly reduced the rate of [^{3}H]CD dissociation from myocardial sites while 5 mM Mg^{++} had little effect. However like Gpp(NH)p, Mg^{++} had no effect on [^{3}H]CD dissociation in the cerebral cortex (Figure 1b).

The effects of NEM (a sulfhydryl alkylating agent) were also compared in the heart, cerebellum, and cerebral cortex. As shown in Figure 2, NEM pretreatment elicited a concentration-dependent reduction of high-affinity [^{3}H]CD binding in the heart with virtually complete reduction by 1 mM NEM. In cerebellar homogenates, NEM (1mM) also reduced (by 95 percent) high-affinity [^{3}H]CD binding (data not shown). Parallel studies in the cerebral cortex, however, revealed that NEM-pretreatment had little or no effect in this tissue except at a concentration of 1mM where high-affinity [^{3}H]CD binding was enhanced (Figure 2).

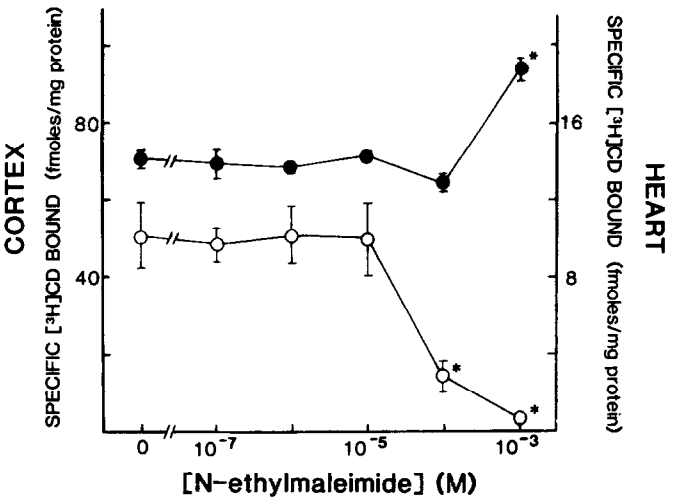

FIGURE 2. EFFECTS OF NEM ON HIGH-AFFINITY [^{3}H]QCD BINDING IN THE RAT HEART AND CEREBRAL CORTEX. Homogenates of each tissue were incubated with the indicated concentration of NEM and washed as described in "Methods". Symbols and vertical bars represent the mean ± S.E. specific binding from four experiments in duplicate (closed circles-cortex; open circles-heart). NEM did not alter nonspecific binding.

* Significantly different from corresponding zero NEM-treatment group ($p<0.05$, Student's t test, two-tailed). ANOVA $F_{5,15}$ (cortex)=4.31 ($p<0.025$); $F_{5,15}$ (heart)=8.89 ($p<0.001$).

DISCUSSION

These results directly demonstrate for the first time a differential regulation of high-affinity muscarinic agonist binding sites in the rat heart, cerebellum and cerebral cortex. Our main conclusions from this study are: (1) Gpp(NH)p selectively reduces high-affinity agonist states of the M_2 subtype by an allosteric mechanism; (2) NEM reduces high-affinity agonist binding states of the M_2 subtype but increases high-affinity M_1 agonist binding states; (3) submillimolar concentrations of Mg^{++} selectively enhance high-affinity M_2 agonist binding states by an allosteric mechanism; and (4) Na^+ and possibly high concentrations of Mg^{++} (>10 mM) nonselectively reduce high-affinity agonist binding to both M_1 and M_2 subtypes. The novelty of these results lies in the high degree of specificity for these regulator-induced changes as well as their consistency with the emerging M_1/M_2 subclassification of muscarinic receptors (1).

It is important to note here that the results of this study should be viewed in terms of the high-affinity agonist binding state of muscarinic recep-

tors. Under the described assay conditions, [^{3}H]CD labels a saturable high-affinity (K_d= 1.5 - 2.0 nM) low capacity muscarinic site in both the heart and cerebral cortex (manuscript in preparation). Lower affinity states, which are labelled by [^{3}H]CD using a centrifugation assay (8), presumably are not detected due to their rapid dissociation during the filtration process. Therefore, many of the apparent discrepancies between our results and results previously published from this and other laboratories are most likely due to the select population of high-affinity agonist binding states which are detected by this assay technique. Within this context, the specificity of the effects reported here are more easily understood. For example, previous direct and indirect studies of guanine nucleotide regulation of muscarinic agonist binding have been reported (9-13). While the results of these studies indicated some differences between tissues, we now demonstrate that Gpp(NH)p has nearly complete selectivity (for M_2 sites) when the high-affinity agonist site is studied (Table 2). This selectivity is even more apparent if one considers that a small population of M_2 sites may be present in the predominantly M_1 tissue cerebral cortex (4,5) and may account for the slight reduction in cortical binding observed here (Table 2). The lack of an effect at M_1 sites and our kinetic evidence for an allosteric mechanism in the heart (M_2 sites) (see Figures 1a and 1b) are consistent with a guanine nucleotide binding protein (G-protein) being functionally coupled to the high-affinity agonist state of M_2 but not M_1 receptor sites.

Like Gpp(NH)p, the sulfhydryl alkylating agent NEM also promotes different changes in the high-affinity agonist states of M_1 and M_2 sites. As previously noted (14), earlier studies of NEM-induced changes in muscarinic agonist binding have produced conflicting results. However, these apparent discrepancies and our results (Figure 2) are entirely consistant with the observation that NEM differentially alters agonist affinity states of M_1 and M_2 sites. If the changes produced by NEM are actually due to sulfhydryl group modification, then our results demonstrate that sulfhydryl groups are intimately yet oppositely involved in the regulation of M_1 and M_2 receptors. The basis

of this difference, while presently unknown, clearly suggests basic chemical differences between M_1 and M_2 subtypes.

Finally, magnesium ions were also found to have selective effects upon high-affinity agonist binding to M_2 sites. As previously reported by Wei and Sulhake (15,16), Mg^{++} (as well as calcium ions) enhance the binding site affinity for muscarinic agonists. Our results (Table 1) directly confirm this indirect observation and for the first time demonstrate that the change is a selective allosteric effect which occurs at all M_2 sites and not only myocardial sites.

In summary, we have obtained direct evidence that Mg^{++}, NEM and Gpp(NH)p differentially regulate the high-affinity agonist binding states of muscarinic receptor subtypes. These results, while providing further support for distinct muscarinic receptor subtypes, demonstrate that different allosteric sites regulate the ability of M_1 and M_2 sites to bind agonists.

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