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Muscarinic Receptors in Porcine Caudate Nucleus

II. Different Effects of *N*-Ethylmaleimide on [^{3}H]*cis*-Methyldioxolane Binding to Heat-Labile (Guanyl Nucleotide-Sensitive) Sites and Heat-Stable (Guanyl Nucleotide-Insensitive) Sites

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SUMMARY

Heat treatment of membranes from porcine caudate nucleus (50° for 7 min) caused a marked decrease in [^{3}H]*cis*-methyldioxolane ([^{3}H]CD) binding without affecting seriously the binding of [^{3}H]3-quinuclidinyl benzilate ([^{3}H]QNB). Approximately 20% of the [^{3}H]CD binding at 5 nM [^{3}H]CD remained after the heat treatment. The remaining binding was not affected by 0.1 mM guanylyl-5′-imidodiphosphate (GppNHp) or by nickel or other cations at concentrations below 10 mM. Treatment of the membranes with trypsin (30 μg/mg of protein) at 20° for 20 min also caused a marked decrease in [^{3}H]CD binding without affecting seriously the binding of [^{3}H]QNB. About 20% of the original [^{3}H]CD binding remained in the presence of trypsin at a high concentration of protein (90 μg/mg). *N*-Ethylmaleimide (NEM) affected [^{3}H]CD binding in two different ways: (a) preincubation of the membranes with NEM caused a marked reduction in heat- and GppNHp-sensitive [^{3}H]CD binding, and (b) treatment with NEM caused an enhancement of heat-, GppNHp-, and trypsin-insensitive [^{3}H]CD binding. Neither of the NEM effects required the coexistence of agonists. The concentration of NEM required for the first effect was 10 times lower than that for the second effect, indicating the existence of two NEM-binding sites with different affinities for NEM. The equilibrium dissociation constant (K_d) for [^{3}H]CD after NEM treatment was 33 nM and was not affected by GppNHp, Ni^{2+}, or heat treatment; the K_d was only 4 times higher than that (8 nM) without NEM treatment. These findings indicated the existence of two kinds of [^{3}H]CD binding sites with high affinities for agonists: one is sensitive to guanyl nucleotide and is abolished by NEM and the other is induced by NEM and insensitive to guanyl nucleotide.

INTRODUCTION

In the preceding paper (1), we demonstrated that approximately 10% of muscarinic receptors in porcine caudate nucleus had high affinities for agonists and were specifically labeled with an agonist, [^{3}H]CD.[1] In addition, [^{3}H]CD binding was sensitive to guanyl nucleotide with the same specificity as has been shown for other receptors. The sites (or states) with high affinities for agonists have been reported to be susceptible to treatment with heat, proteases, and NEM as well as to guanyl nucleotide for various kinds of receptors (2–11), including muscarinic receptors (12–19). The similarity of these effects among different receptors has suggested the existence of a common mechanism. However, some differences have been reported between *beta*-adrenergic and cardiac muscarinic receptors as to the effect of NEM (17, 19). Detailed analyses of the effects of treatments with heat, proteases, and NEM may be required for elucidation of the function of receptors which are thought to couple with the GTP binding proteins.

In the present study, two kinds of NEM effects on [^{3}H]CD binding were separately observed by combining NEM treatment with treatment with heat or trypsin. One effect was abolishment of the guanyl nucleotide-sensitive high-affinity binding of [^{3}H]CD and was very similar to that reported for anterior pituitary dopaminergic (3) and cardiac muscarinic receptors (19). The other was enhancement of the GppNHp-, heat-, and trypsin-insensitive high-affinity binding of [^{3}H]CD and seems to be unique to muscarinic receptors.

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[1] The abbreviations used are: [^{3}H]CD, [^{3}H]*cis*-methyldioxolane; [^{3}H]QNB, [^{3}H]3-quinuclidinyl benzilate; NEM, *N*-ethylmaleimide; GppNHp, guanylyl-5′-imidodiphosphate

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EXPERIMENTAL PROCEDURES

Materials. NEM, GppNHp, trypsin (bovine pancreas Type XI), and soybean trypsin inhibitor were obtained from Sigma Chemical Company (St. Louis, Mo.). [^{3}H]CD (38.1 Ci/mmole) and [^{3}H]QNB (33.1 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). Sources of other materials were as described in the preceding paper (1).

Membrane preparations and standard [^{3}H]CD and [^{3}H]QNB binding assay. Preparation of synaptic membranes from porcine caudate nucleus and assay of [^{3}H]CD and [^{3}H]QNB binding were carried out as described in the preceding paper (1).

Heat treatment. Membranes (3 mg of protein per milliliter) were preincubated at 50° for 7 or 30 min in 20 mM Tris-maleate buffer (pH 7.5 at 20°) and then chilled on ice for 5 min followed by the [^{3}H]CD or [^{3}H]QNB binding assay at 20°. The pH of the Tris-maleate buffer at 50° was 7.0, but [^{3}H]CD binding was unaffected by the change in pH from 6.0 to 8.0.

Trypsin treatment. Membranes (3 mg of protein per milliliter) were preincubated with trypsin (10–100 μg/mg of protein) in 20 mM Tris-maleate buffer (pH 7.5) at 20°. After 20 min, soybean trypsin inhibitor (200 μg/mg of protein) was added, and the preincubation was continued for an additional 5 min at 20° before placing the incubation tubes on ice. The soybean trypsin inhibitor did not affect [^{3}H]CD and [^{3}H]QNB binding at the concentration of 200 μg/mg of protein.

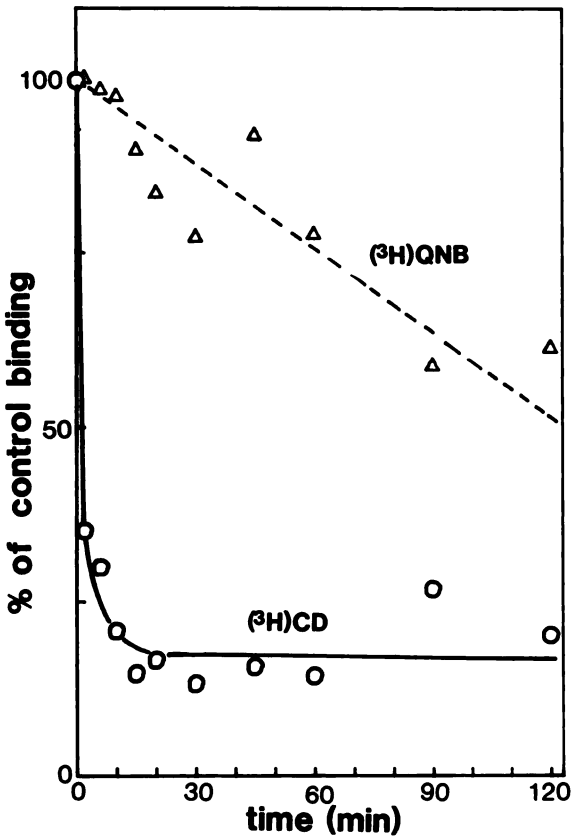

FIG. 1. *Heat stability of [^{3}H]CD and [^{3}H]QNB binding*

Membranes in 20 mM Tris-maleate buffer (pH 7.5) were incubated at 50° for the periods indicated on the abscissa and then subjected to assay of [^{3}H]CD and [^{3}H]QNB binding as described under Experimental Procedures. Specific binding was determined as the difference between binding in the absence and presence of 10 μM atropine. O——O, Binding of [^{3}H]CD; Δ- - -Δ, binding of [^{3}H]QNB. The values are the means of duplicate determinations in one of two similar experiments; 100% binding was 1700 ± 15 cpm for [^{3}H]CD and 2800 ± 10 cpm for [^{3}H]QNB (mean ± standard deviation).

TABLE 1

Effects of GppNHp, Ni^{2+}, and NEM on [^{3}H]CD binding to heat-stable and heat-labile sites

Membranes were treated at 50° for 7 min or 30 min followed by preincubation at 20° for 20 min in the presence or absence of 1 mM NEM as described under Experimental Procedures. The membranes (1 mg of protein/ml) were then incubated with 5 nM [^{3}H]CD in 20 mM Tris-maleate buffer (pH 7.5) in the presence or absence of 1 mM $NiCl_2$ and/or 0.1 mM GppNHp plus 2 mM $MgCl_2$ at 20° for 30 min. The heat-labile [^{3}H]CD binding (Δ) was calculated by subtracting the [^{3}H]CD binding to heat-treated membranes (+) from the [^{3}H]CD binding to heat-untreated membranes (−). [^{3}H]CD binding to membranes without heat and NEM treatment and without addition of any reagents was taken as 100% (1583 ± 10 cpm; mean ± standard deviation). The data shown are the means of duplicate determinations from two experiments.

Addition or treatment	Heat treatment					
	−	50°, 7 min +	Δ	−	50°, 30 min +	Δ
	%					
None	100	28	72	100	6	94
+ GppNHp	25	14	11	17	5	12
$NiCl_2$	225	28	197	240	6	234
+ GppNHp	25	9	16	29	6	23
NEM	78	70	8	84	49	35
+ GppNHp	55	55	0	66	38	28

Pretreatment of membranes with NEM. Membranes (3 mg of protein per milliliter) were preincubated with 1 mM NEM in 20 mM Tris-maleate buffer (pH 7.5) at 20° for 20 min in a total volume of 0.1 ml. The preincubation was followed either by the standard binding assay with 5 nM [^{3}H]CD or 0.5 nM [^{3}H]QNB, or by centrifugation at 100,000 × *g* for 30 min to remove NEM. In the latter case, the precipitated membranes were washed twice by resuspension in 10 volumes of 20 mM Tris-maleate buffer (pH 7.5) followed by centrifugation. The final membrane pellet was suspended in 0.1 ml of the buffer, and the standard binding assay was started. Similar effects of NEM on [^{3}H]CD binding were obtained by either method.

RESULTS

Effects of heat treatment on [^{3}H]CD binding. The effect of preincubation of membranes at 50° on [^{3}H]CD and [^{3}H]QNB binding is shown in Fig. 1. [^{3}H]CD binding decreased very rapidly during the first 10 min, but the decline of [^{3}H]QNB binding occurred slowly with a half-time of more than 2 hr. However, a certain portion (usually 20%) of [^{3}H]CD binding remained intact even after the incubation at 50° for 2 hr. This indicated the existence of two kinds of [^{3}H]CD binding sites: a heat-labile site and a heat-stable site. [^{3}H]CD binding to heat-treated membranes (50°, 7 min) was inhibited by atropine and unlabeled CD (IC_{50} values of 3 nM and 35 nM, respectively). The IC_{50} for atropine was almost identical with, and the IC_{50} for unlabeled CD was twice, the respective values obtained from the inhibition of the [^{3}H]CD binding to heat-untreated membranes, indicating that the heat-stable binding site of [^{3}H]CD had a property of muscarinic receptors.

Effects of GppNHp, nickel ion, and NEM on [^{3}H]CD binding before and after heat treatment. Table 1 summarizes the effects of various reagents on [^{3}H]CD binding before and after heat treatment at 50° for 7 and 30 min.

[^{3}H]CD binding to the heat-labile site was calculated from the difference between [^{3}H]CD binding before and after heat treatment. [^{3}H]CD binding to the heat-labile site was increased by 2- to 3-fold by Ni^{2+} and reduced to about 10% by GppNHp in both the presence and absence of Ni^{2+}. On the other hand, the [^{3}H]CD binding after heat treatment for 30 min was not affected by both Ni^{2+} and GppNHp. The slight effect of GppNHp that remained after heat treatment for 7 min was probably due to incomplete inactivation of the heat-labile site.

An unexpected finding was that [^{3}H]CD binding in the presence of 1 mM NEM was apparently not affected by heat treatment for 7 min, and about 60% of the binding was observed even after 30 min of heat treatment (Table 1). In this experiment, membranes were treated at 50° for 7 min or 30 min followed by the NEM treatment, and then [^{3}H]CD was added for the binding assay. The same results were obtained when NEM was removed by centrifugation before the assay or when NEM treatment preceded heat treatment. [^{3}H]QNB binding activity was not affected by treatment with heat, GppNHp, $NiCl_2$, or NEM (Table 2).

Two different effects of NEM on GppNHp-sensitive and GppNHp-insensitive sites. The membranes were treated with various concentrations of NEM followed by incubation with [^{3}H]CD in the presence or absence of GppNHp or Ni^{2+}. The heat-treated membranes were also incubated with various concentrations of NEM before [^{3}H]CD binding was determined (Fig. 2). [^{3}H]CD binding in the presence of GppNHp and binding by the membranes treated with heat were similarly increased by treatment with increasing concentrations of NEM. The concentration of NEM required for the half-maximal increase was approximately 200 μM. On the other hand, [^{3}H]CD binding in the presence of Ni^{2+} was decreased to about 50% by treatment with NEM. The concentration of NEM required for 50% inhibition was about 20 μM and was 10 times lower than the concentration of NEM giving half-maximal enhancement of GppNHp- and heat-insensitive binding. When membranes were not treated with heat and neither Ni^{2+} nor GppNHp was added, the effect of NEM on [^{3}H]CD binding was biphasic (Fig. 3, *solid line*). As the concentration of NEM was increased, [^{3}H]CD binding decreased initially and then increased. Minimal binding of [^{3}H]CD was observed at an NEM concentration of about 70 μM. [^{3}H]CD binding determined was assumed to be the sum of the binding to the GppNHp-sensitive and GppNHp-insensitive sites. The *dotted line* in Fig. 3 shows [^{3}H]CD binding in the presence of GppNHp and represents the effect of NEM on the binding of [^{3}H]CD to the GppNHp-insensitive sites. The *broken line* shows the difference between [^{3}H]CD binding in the absence and presence of GppNHp and represents the effect of NEM on [^{3}H]CD binding to the GppNHp-sensitive sites. The IC_{50} of NEM for [^{3}H]CD binding to the GppNHp-sensitive sites was calculated from the curve to be about 30 μM. This value was comparable to the IC_{50} of NEM for [^{3}H]CD binding in the presence of Ni^{2+} (20 μM). These results are compatible with the interpretation that there are two kinds of NEM binding sites and that the binding of NEM to one site leads to an appearance of heat- and GppNHp-insensitive [^{3}H]CD binding; binding to another site leads to a dis-

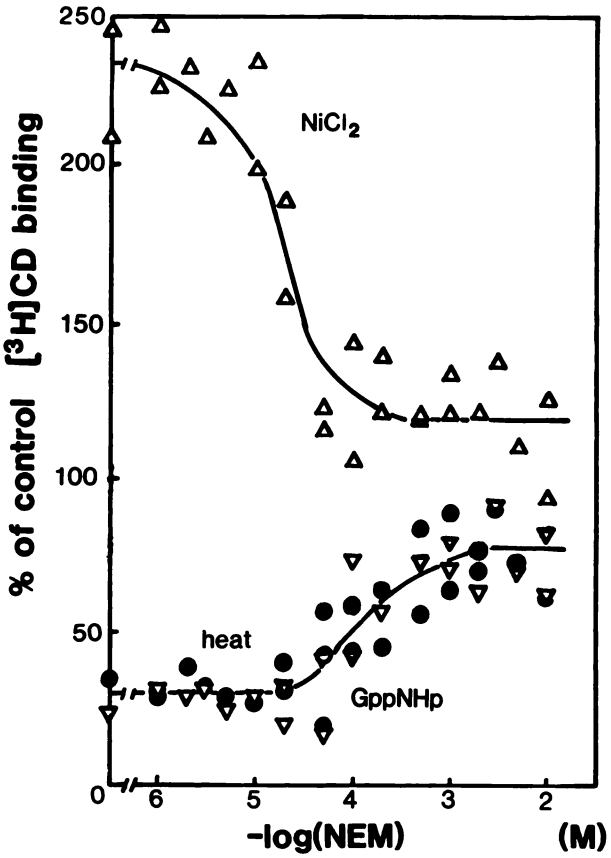

FIG. 2. *Effects of various concentrations of NEM on [^{3}H]CD binding in the presence of Ni^{2+} or GppNHp*

In one experiment, membranes were incubated with various concentrations of NEM as indicated on the abscissa and then subjected to the [^{3}H]CD binding assay in the presence of 1 mM $NiCl_2$ (△——△) or 0.1 mM GppNHp plus 2 mM $MgCl_2$ (▽——▽) as described under Experimental Procedures. In another experiment, membranes were incubated at 50° for 7 min and then incubated with different concentrations of NEM followed by the [^{3}H]CD binding assay in the absence of both Ni^{2+} and GppNHp (●——●). The data shown are the means of duplicate determinations from three experiments; 100% binding was 1500 ± 30 cpm (mean ± standard deviation).

TABLE 2

Effects of GppNHp, $NiCl_2$, NEM, and heat treatment on [^{3}H]QNB binding

Membranes were treated at 50° for 7 min followed by a preincubation at 20° for 20 min in the presence or absence of 1 mM NEM. The membranes (0.2 mg protein/ml) were then incubated with 0.5 nM [^{3}H]QNB in 20 mM Tris-maleate buffer (pH 7.5) in the presence or absence of 1 mM $NiCl_2$ or 0.1 mM GppNHp plus 2 mM $MgCl_2$ at 30° for 30 min. [^{3}H]QNB binding to membranes without heat treatment and without addition of any agents was taken as 100% (2838 ± 25 cpm; mean ± standard deviation). The data shown are the means of triplicate determinations from two experiments.

Addition	Heat treatment (50°, 7 min)	
	−	+
	%	
None	100	100
GppNHp	101	98
$NiCl_2$	97	99
NEM	101	99

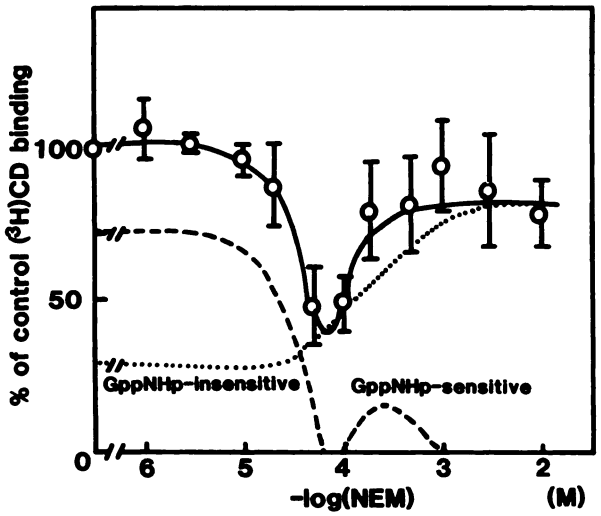

FIG. 3. *Effects of various concentrations of NEM on [^{3}H]CD binding in the absence of Ni^{2+} and GppNHp*

Membranes were incubated with various concentrations of NEM as indicated on the abscissa followed by the [^{3}H]CD binding assay in the absence of both Ni^{2+} and GppNHp (O——O). Data are expressed as means ± standard deviation (n = 3). [^{3}H]CD binding in the presence of GppNHp was taken from the data in Fig. 2 and is represented by the *dotted line*. The difference between [^{3}H]CD binding in the absence (O——O) and the presence (·····) of GppNHp is shown by the *broken line* and represents the expected [^{3}H]CD binding to the GppNHp-sensitive site; 100% binding was 1500 ± 30 cpm (mean ± standard deviation).

appearance of heat- and GppNHp-sensitive [^{3}H]CD binding.

[^{3}H]CD binding to membranes treated with heat and then with NEM was inhibited by atropine and carbachol (IC_{50} values of 3.5 nM and 32 nM, respectively) (data not shown). The IC_{50} for atropine was almost identical with, and the IC_{50} for carbachol was approximately twice, the respective values obtained for membranes that were not treated with heat and NEM. This indicated that the [^{3}H]CD binding site increased by the NEM treatment had a characteristic property of muscarinic receptors.

Scatchard analysis. The Scatchard plot of the saturation binding data (Fig. 4) showed that the k_d of the NEM-treated membranes for [^{3}H]CD was 33 nM and approximately 4-fold higher than that without NEM treatment or in the presence of Ni^{2+} (8–9 nM). Similar k_d values for NEM-treated membranes were obtained irrespective of the presence or absence of Ni^{2+} and GppNHp and whether or not the membranes were treated with heat. The B_{max} of NEM-treated membranes was 410 fmoles/mg of protein and was comparable to that without the treatment. [^{3}H]CD binding remaining after heat treatment was roughly equivalent to that in the presence of 0.1 mM GppNHp, but accurate estimation of the K_d and B_{max} was not possible because of the low specific binding. [^{3}H]CD binding after heat treatment was greatly increased by NEM treatment. The B_{max} was 330 fmoles/mg of protein. GppNHp did not affect significantly the [^{3}H]CD binding to heat- and NEM-treated membranes. These results confirmed that the [^{3}H]CD binding induced by NEM treatment had a high affinity for [^{3}H]CD and was not regulated by guanyl nucleotide.

Effect of trypsin treatment on [^{3}H]CD binding. [^{3}H]CD binding was markedly decreased by pretreatment of membranes with trypsin with the half-maximal effect at approximately 8 μg/mg of protein (Fig. 5). On the other hand, [^{3}H]QNB binding was much more resistant to trypsin treatment. A certain portion (about 15%) of [^{3}H]CD binding remained after trypsin treatment, even at a high concentration of protein (100 μg/mg).

[^{3}H]CD binding after trypsin treatment at a concentration of 35 μg/mg of protein was also increased by NEM in a way similar to that observed after heat treatment (data not shown). The concentration of NEM required for the half-maximal effect was 200 μM and was comparable to that required for half-maximal enhancement of [^{3}H]CD binding in the presence of GppNHp or after heat treatment.

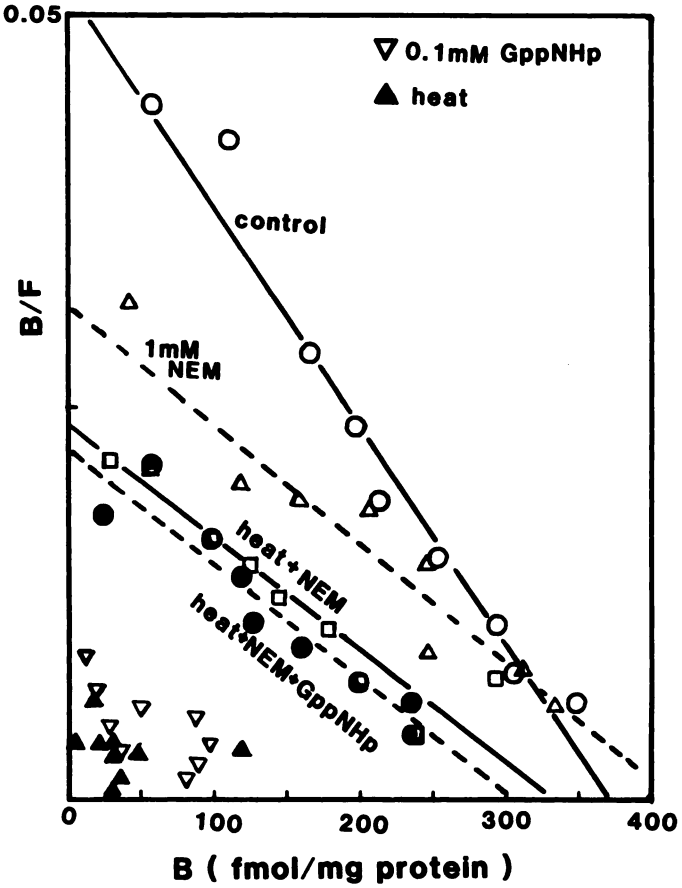

FIG. 4. *Scatchard plot of [^{3}H]CD binding to membranes that were treated with heat and/or NEM*

Treatment with heat (50°, 7 min) and NEM (1 mM) and the subsequent binding assay in the absence or presence of 0.1 mM GppNHp were performed as described in the legend to Fig. 2. Symbols and lines to represent respective experimental conditions are as follows: [^{3}H]CD binding to membranes without any treatment and without addition of GppNHp (O——O); binding in the absence of GppNHp to membranes treated with NEM (Δ- - -Δ), to those treated with heat (▲) and to those treated with heat and NEM (□- - -□); binding in the presence of 0.1 mM GppNHp to membranes without any pretreatment (▽) and to membranes that were treated with heat and NEM (●- - -●). The values shown are the means of duplicate determinations in one of two similar experiments.

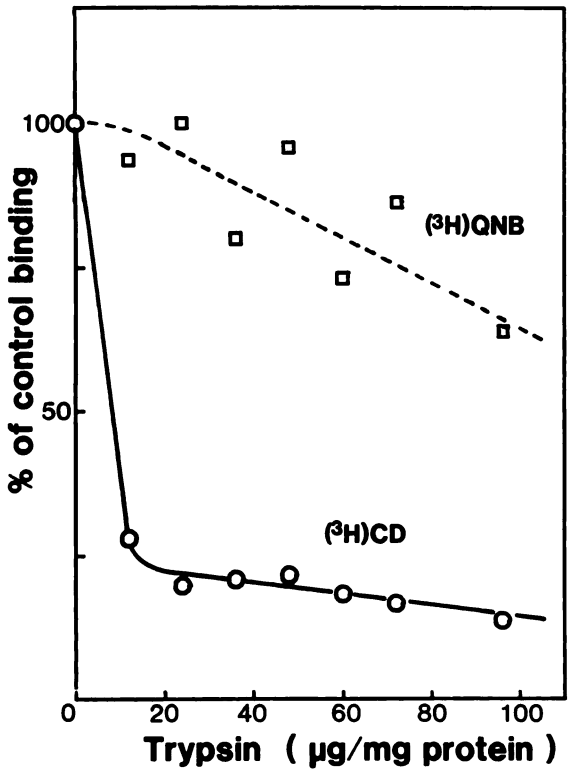

FIG. 5. *Effects of treatment with different concentrations of trypsin on [^{3}H]CD and [^{3}H]QNB binding*

Trypsin treatment and subsequent binding assays were performed as described under Experimental Procedures and legend to Fig. 1. ○——○, Binding of [^{3}H]CD; □- - -□, binding of [^{3}H]QNB. The values shown are the means of duplicate determinations in one of two similar experiments; 100% binding was 1410 ± 15 cpm for [^{3}H]CD and 2810 ± 23 cpm for [^{3}H]QNB (mean ± standard deviation).

DISCUSSION

Heat treatment of membranes from porcine caudate nucleus (50° for 7 min) allowed the distinction of heat-labile and heat-stable [^{3}H]CD binding sites among the high-affinity binding sites for [^{3}H]CD. [^{3}H]CD binding was reduced to about 20% with heat treatment, whereas [^{3}H]QNB binding was scarcely affected at 50° for 7 min. These findings suggested that some mechanisms causing the receptor to have high affinity for [^{3}H]CD were labile to heat rather than that a portion of the total receptor sites had the heat-labile property. [^{3}H]QNB binding was gradually decreased by further incubation at 50° indicating that the receptor itself was slowly inactivated at this temperature.

[^{3}H]CD binding to the heat-stable sites was not affected by GppNHp, whereas the binding to the heat-labile site was greatly decreased by GppNHp. The displacement curve by CD of [^{3}H]QNB binding (data not shown) and the Scatchard plot of [^{3}H]CD binding in the presence of GppNHp were similar to those observed with the heat-treated membranes. In addition, heat treatment as well as GppNHp enhanced the dissociation of [^{3}H]CD from the membranes which had been preincubated with [^{3}H]CD (data not shown). These findings indicated that heat treatment mimicked GppNHp in its effects on [^{3}H]CD binding. Gurwitz and Sokolovsky (12) demonstrated that both treatment at 50° for 5 min and the addition of GTP reduced the affinities for agonists of the muscarinic receptors in rat medulla-pons and cerebellum. In dopaminergic receptors, heat treatment (53°) was reported to mimic guanyl nucleotide in its inhibitory effect on agonist binding with a half-time of 2–8 min for the inactivation (2, 3). Ross and Gilman (20) reported that the GTP-binding protein solubilized with Lubrol PX was labile at 50° with a half-time of 2–8 min for the inactivation. The temperature susceptibility of the three different kinds of receptors is very similar. It seems plausible to assume that GTP-binding proteins, irrespective of their stimulatory or inhibitory action on adenylate cyclase, are inactivated by heat treatment.

The effect of trypsin treatment on [^{3}H]CD and [^{3}H]QNB binding was very similar to that of heat treatment. In addition, the amount of the [^{3}H]CD binding remaining in the presence of guanyl nucleotide and after treatment with heat and trypsin was always very similar, accounting for approximately 20% of the original binding. After these three different treatments, [^{3}H]CD binding was increased by NEM with a half-maximal effect at the same concentration of 200 μM. These findings indicate that guanyl nucleotide and treatments with heat and trypsin affect a common site.

NEM reduced the high-affinity binding of [^{3}H]CD to the heat-, trypsin-, and GppNHp-sensitive site and increased another kind of the high-affinity binding that is insensitive to heat, trypsin, and GppNHp. The possibility could be considered that NEM stabilizes the heat-stable site at the expense of the interconvertible heat-labile site. However, this possibility seems unlikely because the concentrations of NEM effective for the first and second effects were different by a factor of 10. These two effects of NEM appeared to be due to the binding of NEM to two different sites.

NEM mimicked GppNHp in its effect of decreasing heat-labile [^{3}H]CD binding. The loss of the effect of GTP or GppNHp by treatment with NEM has been shown for cardiac muscarinic receptors by using the agonist/[^{3}H]QNB competition binding (15, 19). There has been controversy whether the receptors have a high affinity for agonist after the NEM treatment or are converted into the lower affinity state. Aronstam *et al.* (14) and Wei and Sulakhe (15) reported that the receptors had high affinities for agonists after NEM treatment, but Harden *et al.* (19) reported that after NEM treatment the affinity of the receptor for agonist became as low as that in the presence of guanyl nucleotides. In the present study, the decrease in GppNHp-sensitive binding of [^{3}H]CD occurred with low concentrations of NEM with a half-maximal effect at 20 μM, and the increase in GppNHp-insensitive [^{3}H]CD binding was brought about with higher concentrations of NEM with the half-maximal effect at 200 μM. The K_d for [^{3}H]CD in GppNHp-sensitive binding was calculated to be 7–8 nM and that of GppNHp-insensitive binding enhanced by NEM was 33 nM. In the light of these findings, it seems that in the experiments by Harden *et al.* (19) only the heat-labile site was affected by NEM, but in those by Aronstam *et al.* (14) and Wei and Sulakhe (15) both the heat-labile and heat-stable sites were affected. Ehlert *et al.* (16) reported that the

increase caused by NEM in the super high affinity binding of [^{3}H]CD corresponded to 3.5% of the total receptors. This can be considered to represent the difference between the increase in the heat-stable site and the decrease in the heat-labile site.

The effect of NEM on muscarinic receptors appears to be distinct from that of NEM on *beta*-adrenergic receptors, where agonists seem to be locked in the receptor-GTP binding protein complex (*RN* complex) after the NEM treatment (9, 10). The effect of NEM on the muscarinic receptor did not require the coexistence of agonists, in contrast with the case of *beta*-adrenergic receptors. Recently, Andre *et al.* (21) reported that the treatment of *beta*-adrenergic receptors with a higher concentration (10 mM) of NEM exerted an effect similar to that of guanyl nucleotide and that the coexistence of agonists was not required for this effect of NEM. NEM also mimicked guanyl nucleotide in dopaminergic (D_2) receptor (3). The effect of NEM on dopaminergic receptors did not require the coexistence of agonists and the effective concentration of NEM was comparable to that in the present study. The GTP binding site of *beta*-adrenergic receptors seems to be less susceptible to NEM than that of muscarinic or D_2 receptors. The difference in the NEM effects between *beta*-adrenergic and muscarinic/D_2-dopaminergic receptors could reflect the difference in the GTP binding protein. The ability of NEM to increase agonist binding has not been reported in other receptors and appears to be unique to muscarinic receptors.

In conclusion, there seem to be at least two kinds of effects of NEM on muscarinic receptors: (a) an abolishment of guanyl nucleotide-sensitive high-affinity sites (states) and (b) an induction of high-affinity and guanyl nucleotide-insensitive sites (states).

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