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Spontaneous Coupling of the β-Adrenergic Receptor to N_s in Mammalian Cardiac Membranes

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SUMMARY

The β-adrenergic receptor in membranes from cat, rat, and guinea pig hearts appears to undergo spontaneous coupling to the stimulatory nucleotide-regulatory protein, N_s. This was demonstrated by incubating cardiac membranes from acutely reserpinized animals with the alkylating reagent *N*-ethylmaleimide (NEM), which is known to "freeze" the β-receptor N_s-complex. The concentration of norepinephrine in these membrane preparations was less than 0.1 nM. Cat heart membranes were preincubated for 10 min at 30° with NEM (10^{-7}–10^{-3} M) and subsequently incubated with (−)-[^{125}I]iodopindolol (IPIN) (18 min, 30°). NEM caused a concentration-dependent decrease in specific IPIN binding with a maximal reduction of about 20% at 0.1 mM. This decrease occurred even in the absence of $MgCl_2$ (0.5 mM EDTA added as well). A comparable reduction was also observed in myocardial membranes from rat and guinea pig. This fall reflects a decrease in the number of β-adrenergic receptor sites, as demonstrated by saturation binding experiments with IPIN. The equilibrium dissociation constant of the radioligand for the remaining receptors was not affected. When increasing concentrations of GTP were included in the preincubation mixture, it resulted in a dose-dependent protection of NEM-induced decrease in IPIN binding. The protection was complete at 0.1 mM GTP. In addition, GTP reversed the NEM effect when added to the incubation mixture 10 min after NEM. The apparent reduction in cardiac β-adrenergic receptor number by NEM (in the absence of β-receptor agonist) is compatible with a model in which part of the receptor population is able to undergo spontaneous coupling to N_s.

The first molecular events in β-adrenergic receptor stimulation of the adenylate cyclase system consist of the binding of an agonist (hormone) molecule (H) to a β-adrenergic receptor (R) and coupling of the agonist-receptor complex (H·R) to a stimulatory guanine nucleotide-regulatory protein, designated N_s (1).

Several reports have described the ability of catecholamines to cause an apparent decrease in the number of β-adrenergic receptors (i.e., radiolabeled antagonist binding sites) in, for instance, frog erythrocyte (2) and rat heart membrane preparations (3). Using the agonist ^{3}H-hydroxybenzylisoproterenol as radioligand, Williams *et al.* (4) clearly demonstrated that this phenomenon is due to tight binding of the agonist as a result of H·R·N_s-complex formation. However, in turkey erythrocyte membranes, tight agonist binding is only persistent in the presence of the alkylating reagent, NEM (5, 6). This observation gave rise to a model in which H·R·N_s-complex involves the conformational modification of both membrane components; the receptor adopts a slow agonist-dissociating conformation, wherease one or more sulfhydryl groups become exposed at the surface of N_s.

Alkylation of these groups by NEM is then responsible for the "freezing" of the H·R·N_s-complex (5). These effects are not observed in membrane preparations in which the H·R·N_s-complex cannot take place, for example, in membranes from desensitized cells or from the S49 lymphoma cell mutants *cyc*$^-$ and *unc* (7). Neither do these phenomena occur when the H·R·N_s-complex has only a short life time, as in the presence of guanine nucleotides known to dissociate the complex (8).

In a previous report we have shown that β-adrenergic agonists can undergo tight binding to part of the β-adrenergic receptor population in rat heart membranes (3). This phenomenon could only be investigated properly using membranes from reserpinized animals, since membrane preparations from control animals contained enough endogenous catecholamine to bring about its spontaneous onset. In the present study, we demonstrate that NEM in the absence of agonist is able to provoke a decrease of approximately 20% in the β-adrenergic receptor number in cardiac membranes from reserpinized cats, guinea pigs, and rats. On account of the ability of GTP to

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ABBREVIATIONS: N_s, adenylate cyclase-stimulating regulatory component; NEM, *N*-ethylmaleimide; IPIN, (−)-[^{125}I]iodopindolol; NE, norepinephrine; EDTA, ethylenediaminetetraacetic acid; N_i, adenylate cyclase-inhibiting regulatory component.

prevent and also to reverse this effect, it is suggested that part of the cardiac β-adrenergic receptor population can undergo coupling to N_s in the absence of agonists.

Materials and Methods

Chemicals. NEM, (−)-isoproterenol hydrochloride, and GTP were from Sigma (St. Louis, MO). IPIN (>2000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All chemicals were of the purest grade commercially available.

Treatment of the animals with reserpine. Male Sprague-Dawley rats (250–300 g) from Møllegaard, Denmark, male Duncin Hartley guinea pigs (300–400 g) from Sahlins, Sweden, and cats of either sex (2.5–3.0 kg) from Hässle, Sweden, were used. To deplete tissue catecholamines, the rats and guinea pigs were treated with reserpine intraperitoneally, 2.0 mg/kg, 18 hr, and 0.5 mg/kg, 4 hr before the animals were killed by a blow on the neck. The cats were treated intraperitoneally with 2.0 mg/kg of reserpine 18 hr before administration of pentobarbital (30 mg/kg) and exanguination. During the reserpine treatment all animals were kept in cages kept at a temperature of 25–28°.

Membrane preparations. The left ventricular free wall was thoroughly rinsed in ice-cold buffer A (20 mM Tris-HCl, pH 7.4). The muscle was freed of valvular connective tissue, weighed, and homogenized in 15 ml of buffer A with a Brinkman Polytron homogenizer (setting 7 for 15 sec). The homogenates were filtered through double layers of nylon mesh and centrifuged three times at 30,000 × g, 4°. The pellets were then resuspended in ice-cold buffer A and 10% (w/v) glycerol and kept frozen at −85° until further use.

Protein was measured by the method of Lowry *et al.* (9) using bovine serum albumin as the standard.

Catecholamine assay. Aliquots (1 ml) from the membrane suspension were transferred to small vials with HCl to obtain a pH of 3–4 and then put in a freezer (−85°) for later analysis of the NE content (10). Catecholamines were isolated by adsorption onto alumina and desorbed by elution with perchloric acid. The catecholamines were separated with ion-exchange liquid chromatography and were electrochemically detected. The sensitivity of the method for NE is 0.1 nM.

Preincubation with NEM. The preincubations were performed in buffer containing 20 mM Tris-HCl (pH 7.4 at 30°), 10 mM $MgCl_2$, and 154 mM NaCl (buffer B) except for one set of experiments, where 0.5 mM EDTA was added instead of 10 mM $MgCl_2$. Membranes were preincubated with increasing concentrations of NEM (10^{-7}–10^{-3} M), or with 10^{-4} NEM, in the absence or presence of GTP (10^{-6}–10^{-3} M) for 10 min at 30°. When indicated, the preincubation was terminated by centrifugation for 1 min in an Eppendorf centrifuge (15,000 × g) at room temperature (18–20°). After removal of supernatant, the precipitated membranes were resuspended in buffer B and centrifuged for 1 min. This washing step was repeated twice.

Preincubation with NE. Membranes were preincubated with increasing concentrations of NE (10^{-8}–10^{-3} M) for 10 min at 30° in the absence or presence of GTP (10^{-3} M). The preincubation was terminated by centrifugation and washing, as described above.

Binding of IPIN. Aliquots of membrane suspension (70–150 μg of protein) were incubated with IPIN (18 min, at 30°) in a final volume of 0.25 ml. The membranes and drugs were all diluted in buffer B. The binding reaction was terminated by the addition of 10 ml of buffer C (10 mM Tris-HCl, 2 mM $MgCl_2$ in 154 mM NaCl, pH 7.4) and the samples were immediately filtered through Whatman glass fiber filters (GF/C). Each filter was washed with an additional 10 ml of buffer C. The radioactivity remaining on the filters was determined in a Kontron gamma counter. The specific binding of IPIN was defined as the amount of radioligand bound in the absence minus the amount bound in the presence of 5×10^{-5} M (−)-isoproterenol. This concentration of (−)-isoproterenol occupies approximately 99% of the receptors. At the IPIN concentration used in these experiments (~100 pM), specific binding constituted 95% of the total binding, which itself was always less than 5% of the total amount of IPIN in the incubation mixture. Binding was always performed in triplicate.

To determine the density of binding sites, B_{max}, the amount of specifically bound IPIN was determined at nine concentrations of IPIN (20–400 pM). The data were analyzed according to the method of Scatchard (11) to provide a value for the density of receptors and equilibrium dissociation constant (K_d) for IPIN.

Thin layer chromatography. IPIN (~100 pM) was incubated in the presence or absence of 10^{-4} M NEM under radioligand binding conditions. Aliquots (25 μl) were applied on a plate coated with silica gel (Merk, 20 × 20 cm). After elution with ethyl acetate/methanol/H_2O, 1:2:1, the plate was autoradiographed. There was no difference in the R_F value for IPIN in the presence or absence of NEM.

Statistics. Results are expressed as mean values ± standard deviation. Data were compared by paired Student's *t* test and a *p* value of less than 0.05 was the criterion for statistical significance.

Results

The alkylating reagent NEM decreased IPIN binding to myocardial membrane preparations from reserpinized cats, rats, and guinea pigs. In the initial experiments, cat heart membranes were preincubated for 10 min with 0.1 mM NEM, washed free of reagent, and incubated with IPIN. In an alternative protocol, the washing step was omitted. These treatments resulted in a respective decrease of 20.1 ± 17.6% (n = 16) and 20.3 ± 5.9% (n = 11) in IPIN binding. Both protocols resulted in decreases that were highly significant ($p < 0.001$), but due to the smaller variation in the results when the washing was omitted, the second procedure was retained for the ensuing experiments. The action of NEM appears to be irreversible under our incubation conditions, since it is persistent after washing. It was also verified by thin layer chromatography that NEM does not cause a chemical modification of the radioligand (see Materials and Methods).

β-Adrenergic receptors from cat, rat, and guinea pig myocardium displayed similar IPIN saturation binding characteristics (Table 1). In Fig. 1 the representative example for the cat shows that the Scatchard plot is linear (r = 0.991). In myocardial membrane preparations from five cats, B_{max} equalled 37.6 ± 11.61 fmol/mg of protein and the K_d was 158 ± 49.6 pM. After preincubation of the membrane preparations with 0.1 mM NEM, the B_{max} decreased to 30.4 ± 9.74 fmol/mg of protein ($p < 0.01$) without any effect on the K_d of IPIN (150 ± 52.8 pM) ($p > 0.50$). These data indicate that the NEM-mediated decrease in IPIN binding corresponds to an apparent decrease in β-receptor number. As summarized in Table 2, 0.1 mM NEM provoked a similar decrease of approximately 20% in IPIN binding in myocardial membranes from cat, rat, and guinea pig. The NEM-mediated decrease in IPIN binding in cat heart membranes was concentration dependent in the absence as well as in the presence of 10 mM $MgCl_2$ (Fig. 2). Under both conditions maximal effect was attained at 0.1 mM NEM.

TABLE 1
IPIN saturation binding characteristics

Tissue	n^a	K_d	B_{max}
		pM	*fmol/mg of protein*
Cat left ventricle	5	158 ± 49.6[b]	37.6 ± 11.6
Rat left ventricle	6	118 ± 40.9	26.3 ± 10.0
Guinea pig left ventricle	4	79 ± 33.3	52.4 ± 21.3

[a] *n*, number of animals.
[b] Values are means ± SD.

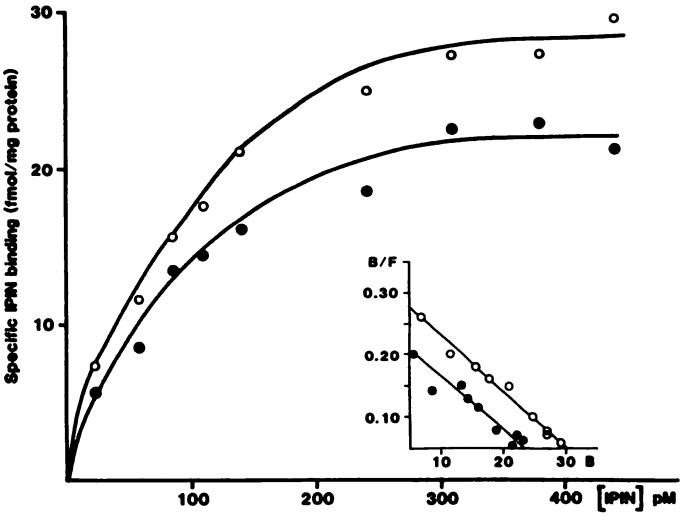

Fig. 1. The effect of NEM on IPIN binding to heart membranes from reserpinized cat. Membranes were preincubated with buffer only (○) or with 0.1 mM NEM (●) and subsequently incubated with increasing concentrations of IPIN. *Inset:* Scatchard plots of the saturation binding data. *B* is specific binding in fmol/mg of protein and *B/F* is the ratio of bound to free radioligand. In the representative experiment B_{max} equalled 34.5 and 28.3 fmol/mg of protein, and K_d was 102 and 107 pM for control and NEM-treated membranes, respectively.

TABLE 2

NEM effect in heart membranes

Heart membranes from reserpine-treated cats, rats, and guinea pigs were preincubated with NEM (10^{-4} M) in the absence or presence of GTP (10^{-3} M) for 10 min, and then immediately assayed for IPIN binding. Control refers to preincubation with buffer only. Values are means ± SD.

Preincubation	% Binding of control		
	Cat	Rat	Guinea pig
NEM (10^{-4} M)	83 ± 6.6[a] (n = 6)[b]	83 ± 4.2[a] (n = 6)	77 ± 7.4[a] (n = 7)
NEM (10^{-4} M) + GTP	101 ± 3.3 (n = 6)	103 ± 6.4 (n = 6)	99 ± 8.1 (n = 7)

[a] $p < 0.001$ versus control.
[b] n, number of animals.

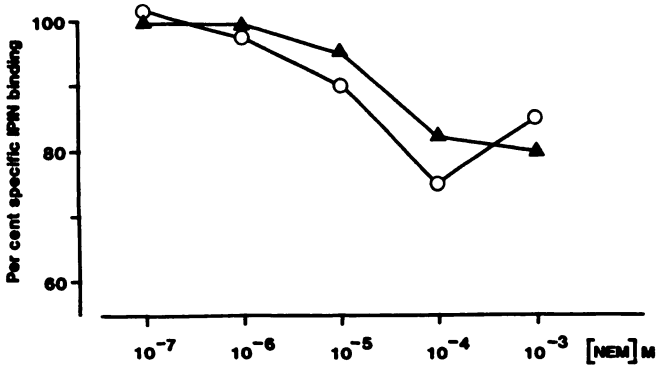

Fig. 2. Effect of NEM in heart membranes from reserpinized cat. Heart membranes were preincubated with increasing concentrations of NEM after which binding of IPIN was measured. Preincubation and incubation buffer contained either 10 mM $MgCl_2$ (○) or 0.5 mM EDTA (▲). Control binding (100%) was measured in the presence of the corresponding buffer only. Data shown are the mean values of three to six experiments.

Co-incubation with GTP (0.1 mM) prevented the effect of NEM in all three species (Table 2). The protection by GTP was concentration dependent. Fig. 3 shows IPIN binding to cat myocardial membranes after 10-min preincubation with 0.1 mM NEM together with increasing concentrations of the nucleotide. Concentrations of GTP exceeding 1 μM caused a dose-related protection of the receptor sites with full protection at 0.1 mM.

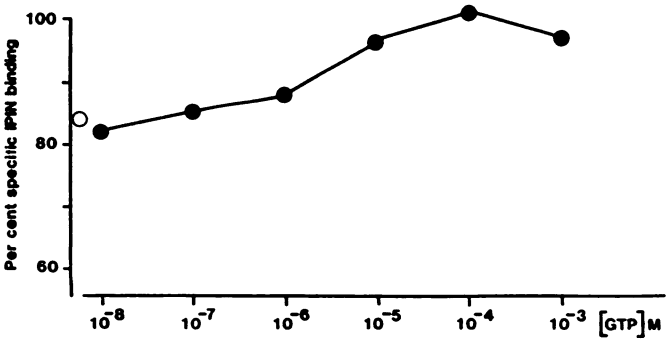

Fig. 3. Dose-dependent GTP protection of NEM-mediated decrease in IPIN binding. Cat heart membranes were incubated with 0.1 mM NEM alone (○) or in the presence of increasing concentrations of GTP (●) for 15 min at 30°. Control binding (100%) was always measured in the presence of buffer only. Data shown are the mean of three experiments.

TABLE 3

GTP reversal of the NEM effect

Cat heart membranes prepared from three different animals (cats I, II, and III) were preincubated with NEM (10^{-4} M) or buffer at 30° for 10 min. The binding of IPIN was immediately assayed in the presence or absence of GTP (10^{-3} M). Control binding refers to preincubation with buffer only followed by incubation with IPIN only.

Preincubation	Incubation	% Binding of control		
		Cat I	Cat II	Cat III
NEM		86	67	78
NEM	GTP	106	91	99
	GTP	106	101	99

Moreover, GTP was also capable of reversing the NEM-mediated decrease in receptor number. When 0.1 mM GTP was added to the incubation mixture 10 min after NEM, the original receptor number was restored (Table 3).

It has been reported previously that NEM alone did not affect the number of β-adrenergic receptor sites in turkey erythrocyte and S49 lymphoma cell membranes, but that there was a 50 and 65% decrease, respectively, when agonists were concomitantly present (7). However, several observations in the present study indicate that endogenous agonists were not involved in the NEM-mediated decrease in the number of cardiac β-adrenergic receptors. All membrane preparations from the reserpinized animals used in this study contained less than 0.1 nM NE, which is the limit of detection for NE with the method used. As depicted in Fig. 4, this concentration is at least 3 orders of magnitude below the one required to achieve a significant reduction in the number of binding sites due to tight agonist binding. Moreover, 10^{-5}–10^{-3} M NEM failed to affect the number of turkey erythrocyte β-adrenergic receptors in the presence of the supernatant of concentrated myocardial membrane suspensions from reserpinized cat (Table 4).

Discussion

The alkylating reagent NEM has been proven to be a valuable tool for investigating both the structure of α- and β-adrenergic receptors and their interaction with other components in the plasma membrane. In this study, we report the ability of the reagent to decrease the number of β-adrenergic receptors in

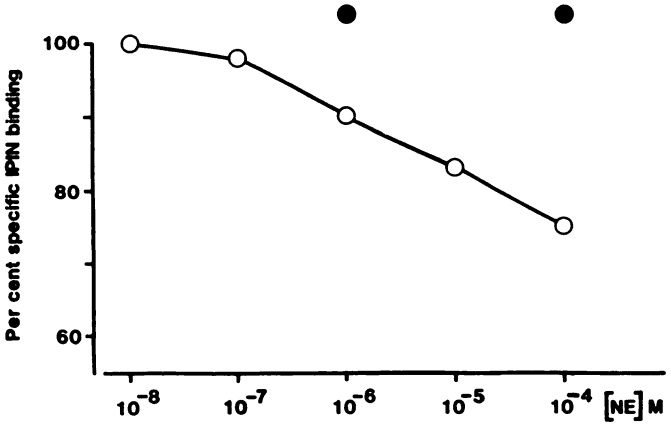

Fig. 4. Tight binding of NE to cat heart membranes. Membranes were preincubated with increasing concentrations of NE either in the absence (○) or in the presence (●) of 1 mM GTP for 10 min at 30°. Membranes were washed three times and IPIN binding was measured as described in Materials and Methods. Control binding corresponds to membrane incubated with buffer only. Values are the means of two to three experiments.

TABLE 4

Turkey erythrocyte membrane

Membranes from turkey erythrocytes (final concentration 1 mg/ml) were incubated with the supernatant from reserpinized cat cardiac membrane preparations (from two different animals). This mixture was preincubated with NEM (10^{-6}–10^{-3} M) and with GTP (10^{-4} M) for 10 min at 30°, and immediately assayed for IPIN binding. Control refers to preincubation with buffer only.

Preincubation	% Binding of control	
	Cat I supernatant	Cat II supernatant
GTP	101	102
NEM (10^{-5} M)	99	97
NEM (10^{-4} M)	99	95
NEM (10^{-3} M)	100	99
NEM (10^{-3} M) + GTP	98	99

cardiac membrane preparations and the ability of GTP to reverse this effect. These data cannot be reconciled with any of the three action mechanisms of NEM reported previously.

Chemical modification of the receptor. NEM and another alkylating reagent, *p*-chloromercuribenzoate, allowed the detection of cysteinyl residues within the ligand-binding site of α_2-adrenergic receptors (12, 13). However, chemical modification of the β-adrenergic receptor itself is highly improbable, since β_1- and β_2-adrenergic receptors have been reported to be insensitive to NEM at concentrations up to 10^{-3} M. These studies were performed on membranes from a variety of sources, such as turkey erythrocytes (14) and rat fat cells (15) (β_1-receptors), frog erythrocytes (16) and S49 lymphoma cells (7) (β_2-receptors), and rat lung (β_2- and β_1-receptors) (6). Moreover, GTP is able to reverse the reagent-mediated decrease in cardiac receptor number. Since alkylation by NEM is a covalent process, the possibility of reactivation implies that NEM does not inactivate the β-adrenergic receptors via steric hindrance at the ligand-binding site (6).

Alkylation of uncoupled N_s. β- and α_2-adrenergic receptors interact with the stimulatory (N_s) and the inhibitory proteins (N_i) of the adenylate cyclase system, respectively (17). Both regulatory components can be directly alkylated by NEM and are then no longer able to undergo functional receptor coupling (14, 16, 18). This mechanism cannot explain any change in the total receptor number but might be responsible for the fact that β-adrenergic receptors display only low agonist affinity in NEM-pretreated membranes (14, 16).

Locking of the H·R·N_s-complex. The sensitivity of N_s towards NEM increases considerably upon association with an agonist-bound β-adrenergic receptor (H·R) (14). However, in this case, the reagent provokes the freezing of the H·R·N_s-complex and hence tight binding of the agonist (5). The original number of antagonist-binding sites can be recovered when agonists/NEM-treated membranes are subsequently subjected to a reactivation procedure, such as incubation with GTP (5, 6). The ability of this nucleotide to prevent and even to reverse the NEM-mediated decrease in β-adrenergic receptor number in cardiac membranes suggests that freezing of the H·R·N_s-complex has taken place.

However, as outlined below, agonist molecules, which are a prerequisite for such a mechanism, are not present at effective concentrations in the cardiac membrane preparations used. Whereas cardiac membrane suspensions from control animals still contain appreciable amounts of endogenous NE under radioligand binding conditions (i.e., approximately 10 nM), this catecholamine can no longer be detected electrochemically (i.e., less than 0.1 nM) in membrane suspensions from the reserpinized animals used in this study. This concentration is 3–4 orders of magnitude below the one causing significant tight agonist binding (Fig. 4). The involvement of endogenous NE is further weakened by the lack of NEM effect on turkey erythrocyte β-adrenergic receptors in the presence of the supernatant of a concentrated cardiac membrane preparation from reserpinized cat.

Since pindolol has been described to be a weak partial agonist (19), the radioligand IPIN itself might have undergone tight binding in those experiments in which NEM was still present during the incubation phase. Being nondisplaceable by an excess of cold (−)-isoproterenol, this tightly bound radioligand might have been inadvertently interpreted as "nonspecific" binding instead of receptor binding. However, there are two arguments against this assumption. First, NEM was able to cause a comparable (20%) decrease in receptor number, irrespective of whether the reagent was washed away prior to the IPIN incubation phase. Second, in contrast to pindolol (20), the iodinated analogue does not cause any significant β_1-receptor-mediated rate increase in isolated rat right atrium or β_2-receptor-mediated relaxation in isolated rat uterus.[1] This finding suggests that IPIN has a very low efficacy toward β-adrenergic receptors and can thus be regarded as a pure β-receptor antagonist.

Using purified components, Cerione *et al.* (21) observed that the GTPase and the GTP-γ-*S* binding activity of N_s-containing phosphatidylcholine vesicles was significantly higher when β-adrenergic receptors were also present. These authors interpreted the results in terms of spontaneous receptor N_s association, whereas Pedersen and Ross (22) suggested that the association might have been induced by dithiothreitol present in the incubation medium. However, our data might be an independent confirmation of Cerione's hypothesis. The NEM effect on cardiac β-adrenergic receptors is compatible with a model in which part (15–30%) of the receptor population is able to undergo spontaneous association with N_s. The receptor N_s-complex formation might coincide with a conformational

[1] V. Nerme and T. Abrahamsson, manuscript in preparation.

change of both membrane components, involving the exposure of one or more sulfhydryl groups at the surface of N_s (i.e., N_s-SH) and the distortion of a reduction in accessibility of the binding site of the receptor (i.e., R′). Here again, NEM should be able to "freeze" the complex by alkylating N_s–SH. This model is schematically represented as follows:

$$R + N_s \rightleftharpoons R' \cdot N_s - SH \xrightarrow{NEM} R' \cdot N_s - S - NEM$$

The data presented in Fig. 2 suggest that Mg^{2+} is not strictly required for receptor N_s-complex formation, nor for its stabilization by NEM. These findings are compatible with our previous demonstration that "freezing" of the H·R·N_s-complex by NEM is Mg^{2+} independent in turkey erythrocyte membranes (23). The discrepancy between these results and the absolute requirement of Mg^{2+} for the purpose of tight agonist binding (4) suggests that Mg^{2+} and NEM have a similar stabilizing effect on H·R·N_s. Whereas the experiments reported by Cerione *et al.* (21) involved co-insertion of receptors and N_s of different cellular origins (i.e., guinea pig lung and human erythrocytes, respectively) in synthetic phospholipid vesicles, our data indicate that spontaneous receptor-N_s association can also occur in membrane preparations from tissues such as the heart.

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