Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 41-46

POTENTIATION OF MONOVALENT CATION EFFECTS ON LIGAND BINDING TO CARDIAC MUSCARINIC RECEPTORS IN N-ETHYLMALEIMIDE TREATED MEMBRANES

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Received December 29, 1982

Guanine nucleotides and monovalent cations decrease the affinity of cardiac muscarinic receptors for agonists and are required for muscarinic receptor mediated inhibition of adenylate cyclase. N-ethylmaleimide abolished the effects of Gpp(NH)p on the ability of the agonist oxotremorine to inhibit the binding of the antagonist [^{3}H]quinuclidinyl benzilate to purified chick heart membranes. However, the effects of NH_4^+ to decrease the IC_{50} for oxotremorine were retained in N-ethylmaleimide treated membranes. The N-ethylmaleimide treatment mimicked the effects of Gpp(NH)p and the oxotremorine inhibition curves obtained with treated membranes in the presence of NH_4^+ were identical to those obtained in control membranes in the presence of NH_4^+ and Gpp(NH)p. The results suggest that monovalent cation effects on muscarinic receptors are mediated at a site distinct from effects produced by guanine nucleotides and are greater on free receptors than on receptors coupled to guanine nucleotide binding proteins.

The cardiac muscarinic receptor is coupled to inhibition of adenylate cyclase (1, 2). Guanine nucleotides and monovalent cations are required to demonstrate this inhibition (1) and also decrease the affinity of the receptor for agonists (3). The effects of guanine nucleotides and monovalent cations on receptor affinity are additive or synergistic (3). Guanine nucleotides are thought to reduce receptor affinity by promoting the uncoupling of receptors (R) from a guanine nucleotide binding protein (G-protein)[1] believed to be the putative regulatory subunit of the adenylate cyclase complex (4-6). However, the nature of the site responsible for monovalent cation effects on receptor affinity is not known. Previous studies have shown that N-ethylmaleimide (NEM) treatment of membranes inactivates the G-protein(s) (7) and several studies have shown that NEM mimics the effects of guanine nucleotides on receptor affinity (8-11). In order to determine whether monova-

[1] The abreviations used are G-protein, guanine nucleotide binding protein; NEM, N-ethylmaleimide; QNB, quinuclidinyl benzilate; R, free receptors; RG, receptor/G-protein complexes.

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lent cation effects are produced at a site similar to or distinct from the G-protein, we have examined the effects of monovalent cations ± Gpp(NH)p on the affinity of chick heart muscarinic receptors in control and NEM -treated membranes.

EXPERIMENTAL PROCEDURES

Materials: [^{3}H]quinuclidinyl benzilate (QNB) was purchased from Amersham/Searle and had a specific activity of 34-36 Ci/mmole. Oxotremorine was from ICN; Gpp(NH)p was from P-L Biochemicals. N-ethylmaleimide and L-histidine were from Sigma. Fertilized White Leghorn eggs were obtained from SPAFAS, Inc. Chicks were sacrificed 7-10 days after birth and cardiac membranes prepared according to Method 1 as previously described (2).

Ligand binding assays: The binding of [^{3}H]QNB to membrane preparations was performed as described previously (12, 13) except that assay buffer was 10 mM L-histidine, pH 7.5. All assays contained 10 mM Mg^{2+} and 1 mM EDTA. Where used, Gpp(NH)p was 10 μM and NH_4Cl was 0.1 M. The average concentration of [^{3}H]QNB used was 78 pM.

NEM-treatment: The membranes (7.5 to 10 mg/ml) were incubated with 1 mM NEM for 30 min at 4 °C in a volume of 100 μl. The treatment was stopped by the addition of 1 μl of 100 mM dithiothreitol and centrifuging the sample at 11,000 xg for 45 min. The control membranes were incubated in the absence of NEM at 4 °C for 30 min and centrifuged similar to the treated membranes. No dithiothreitol was added to the control samples. The precepitated membranes were resuspended in 10 mM histidine to a final protein concentration of 2 to 5 μg/assay tube.

RESULTS

The concentration dependency of NEM to mimic the effects of Gpp(NH)p on the ability of the agonist oxotremorine to inhibit [^{3}H]QNB binding was determined. Effects were half-maximal at approximately 0.1 mM NEM and maximal between 0.3 - 1.0 mM NEM. These results were similar to those observed previously with NEM pretreatment of rat cardiac membranes (10). All further studies were performed with 1 mM NEM.

We previously determined that NH_4^+ was more potent than Na^+, K^+ and Rb^+ in regulating the affinity of cardiac muscarinic receptors for agonists [2]. The effects of Gpp(NH)p ± NH_4^+ on oxotremorine inhibition of [^{3}H]QNB binding to control and NEM -treated membranes are illustrated in Fig. 1. The IC_{50} values for oxotremorine from these experiments are summarized in Table I. In control membranes, Gpp(NH)p or NH_4^+ alone shifted the curves to the right and decreased the IC_{50} values an average of 4 and 8 -fold, respectively, whereas

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[2] Hosey, M. M., submitted for publication.

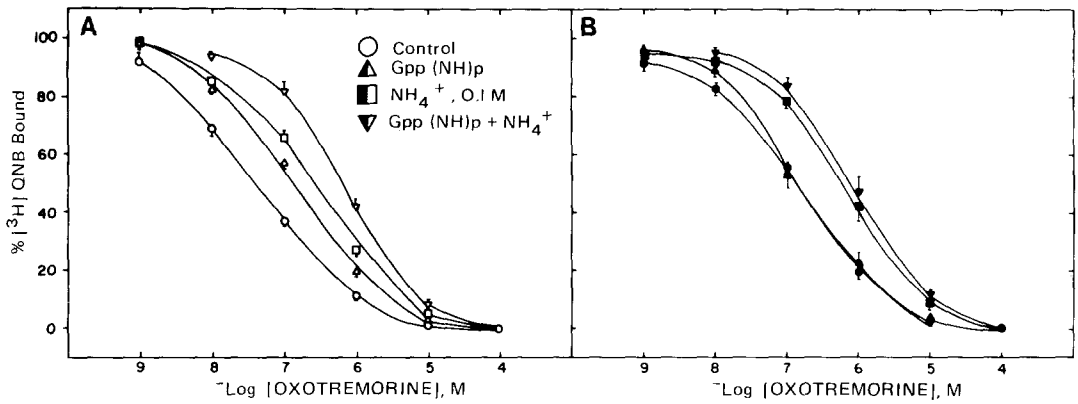

Fig. 1. Effects of Gpp(NH)p and NH_4^+ on oxotremorine inhibition of [^{3}H]QNB binding in untreated (A) and NEM-treated (B) membranes. Assays contained 2 to 5 μg membrane protein and other ingredients as indicated.

the combination of Gpp(NH)p and NH_4^+ decreased the IC_{50} value approximately 24-fold (Fig. 1A, Table 1).

In NEM-treated membranes, the IC_{50} value for oxotremorine in the absence of Gpp(NH)p or NH_4^+ (control) was increased to and indistinguishable from that observed in untreated membranes containing Gpp(NH)p (Fig. 1B, Table 1). As expected, Gpp(NH)p alone caused no further reduction of affinity for oxotremorine in NEM-treated membranes. In contrast, the effects of NH_4^+ were fully retained and potentiated in NEM-treated membranes (Fig. 1B). IC_{50}

TABLE 1 Effects of Gpp(NH)p and NH_4^+ on oxotremorine inhibition of [^{3}H]QNB binding in untreated and NEM-treated chick heart membranes

	Untreated			NEM-treated		
Addition	IC_{50}, μM	n_H	N	IC_{50}, μM	n_H	N
Control	0.039 ± 0.002	0.61 ± 0.04	8	0.15 ± 0.02	0.71 ± 0.04	8
Gpp(NH)p	0.15 ± 0.02	0.62 ± 0.05	8	0.18 ± 0.03	0.75 ± 0.03	7
NH_4^+	0.30 ± 0.06	0.73 ± 0.05	6	0.80 ± 0.19	0.81 ± 0.02	6
NH_4^+ and Gpp(NH)p	0.91 ± 0.20	0.82 ± 0.12	6	0.97 ± 0.20	0.80 ± 0.06	6

values for oxotremorine in the presence of NH_4^+ in NEM-treated membranes were indistinguishable from those obtained with the combination of NH_4^+ plus Gpp(NH)p in untreated membranes (Table 1). Furthermore, Gpp(NH)p did not enhance the effect of NH_4^+ in NEM-treated membranes. Thus, NEM pretreatment mimics the effects of Gpp(NH)p in the presence and absence of NH_4^+. Similar results were obtained using acetylcholine instead of oxotremorine, and using varying concentrations of NH_4^+.

We previously observed that Gpp(NH)p has reciprocal effects on agonist and antagonist binding to cardiac muscarinic receptors (13). Gpp(NH)p increases the binding of [^{3}H]QNB by increasing the affinity of chick heart muscarinic receptors by a factor of 1.5 -2 fold (13). In the present experiments we found that [^{3}H]QNB binding at zero or low oxotremorine concentrations was consistently increased in NEM-treated membranes. The NEM induced increase in [^{3}H]QNB binding averaged 27.9 $\pm$ 6.2% while the Gpp(NH)p induced increase in control membranes averaged 28.5 $\pm$ 7.5%. Thus NEM also appears to mimic the effects of Gpp(NH)p on antagonist binding.

DISCUSSION

The results suggest that monovalent cations and guanine nucleotides regulate the affinity of cardiac muscarinic receptors at distinct sites. NEM abolished but mimicked the effects of Gpp(NH)p without diminishing the effect of NH_4^+. That NEM mimicked the effect of Gpp(NH)p is expected because NEM inactivates the G-protein(s) (7) while Gpp(NH)p acts to dissociate receptor/G-protein (RG) complexes (4-6). Thus, Gpp(NH)p and NEM have functionally similar actions to create free receptors. Others have also observed that NEM mimicked the effects of Gpp(NH)p. However, it is not clear why one group observed no change in agonist IC_{50} after NEM treatment of rat cardiac membranes (15). These investigators did observe that NEM increased the pseudo--Hill coefficients for agonist competition curves to a value of 1.0. In contrast, in our studies, while there was a tendency for the pseudo-Hill coefficients to approach a value of 1.0 (Table 1), all the values were less than 1.0. This agrees with our previous observations that the pseudo-Hill coeffi-

cients only approach a value of 1.0 in the presence of high (0.4 - 0.8 M) concentrations of NH_4^+ plus Gpp(NH)p (2). Wei and Sulakhe (14) also did not observe potentiation of monovalent cation effects on agonist competition curves, most likely because NEM did not right shift their inhibition curves (14).

It is interesting that the synergistic effects of NH_4^+ + Gpp(NH)p were mimicked by NH_4^+ alone in NEM-treated membranes. This argues against the synergistic effects of NH_4^+ + Gpp(NH)p in untreated membranes being due to an effect of NH_4^+ to enhance the action of Gpp(NH)p on the G-protein. Rather the results suggest that the effect of NH_4^+ is independent of the G-protein and on the receptor itself. It follows therefore that the "synergistic" effects of NH_4^+ and Gpp(NH)p on untreated membranes reflect a more efficient action of NH_4^+ on free R than on RG complexes. The synergistic effects of Gpp(NH)p and NH_4^+ could be interpreted as a reflection of the ability of Gpp(NH)p to create more free R susceptible to NH_4^+. It also follows that the site of action of NH_4^+ may be at a site on R which is involved in the coupling to the G-protein. Other evidence obtained with the α_2-adrenergic receptors in platelets also suggests that the monovalent cation site is not on the G-protein (15). Solubilized preparations of platelet α_2-adrenergic receptors retained sensitivity to Na^+ but not guanine nucleotides (15). A further understanding of regulation of receptors coupled to inhibition of adenylate cyclase by monovalent cations and guanine nucleotides awaits purification of the components themselves.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Deborah Rymut for her expert technical assistance. This work was supported by a grant from the Chicago Heart Association.

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