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FIRST FATTY ACYLATED DIPEPTIDES TO AFFECT MUSCARINIC RECEPTOR LIGAND BINDING

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Abstract: Fatty acylated dipeptides homologous to $G_i\alpha$ N-termini affect ligand binding to muscarinic acetylcholine receptors. Myristylglycine-serine containing dipeptides decrease antagonist binding at both M_1 and M_2 muscarinic receptors. Palmitate on the serine analogous to native palmitoylated cysteine affords dipeptide which selectively decreases the number of high affinity agonist binding sites at M_2 but not M_1 receptor. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction: G-proteins and G-protein coupled receptors (GPCRs) play a critical role in intercellular communication, intracellular signal transduction, and cell proliferation.[1,2] Selective control of G-proteins may be useful in the treatment of various cancers and neurological disease states. The N-terminus of G protein α subunits have been implicated in G-protein coupling to receptor, association of α subunits with $\beta\gamma$ dimers, and regulation of effector activation.[3] $G_i\alpha$ subunits have myristylglycine-palmitoylcysteine at their N-termini. The myristate is irreversibly, co-translationally attached by amide to glycine while palmitate is reversibly, post-translationally linked by a thioester bond to cysteine. We have hypothesized that fatty acylated peptides analogous to $G\alpha$ N-temini, such as myristylglycine-palmitoylserine benzyl ester **1**, can be used to discover both structural motifs and G-protein properties useful for generation of new disease treatment strategies. The main difference between a cysteine analog of the $G\alpha$ N-termini and **1** is the substitution of a thioester by a more stable ester bond. The functional importance of palmitate can be assessed by comparing the biological activity of **1** with a non-palmitoylated analog such as **2**. The specificity of peptide interaction with GPCRs can be evaluated by comparing the effects of **1** and **2** on a receptor that activates $G\alpha$ subunits with N-terminal myristylglycine with one which does not.

In the present study, lipophilically modified peptides **1** and **2**, analogous to the $G_i\alpha$ N-terminus, were synthesized and assayed for effects on radioligand binding at human M_1 and M_2 muscarinic acetylcholine receptors which selectively interact with G_q and G_i, respectively. The data obtained supports the hypothesis that the N-terminus of $G_i\alpha$ and the presence of a palmitate group could be important determinants in receptor binding selectivity and receptor/G-protein interactions.

Chemistry: Myristylglycine-palmitoylserine benzyl ester **1** can be synthesized in five steps and greater than 60% overall yield from myristoyl chloride (Scheme 1). Addition of myristoyl chloride **3** to glycine in THF and aqueous 1 M NaOH gave myristylglycine **4**. Treatment of **4** in THF with N-hydroxysuccinamide and DCC afforded the activated ester **5**. Recrystallization of **5** from mixtures of THF and 2-propanol was required to remove uncharacterized contaminants which interfered with peptide bond forming reactions. Addition of **5** in THF to unprotected serine, sodium carbonate, and a minimum amount of water produced a homogeneous

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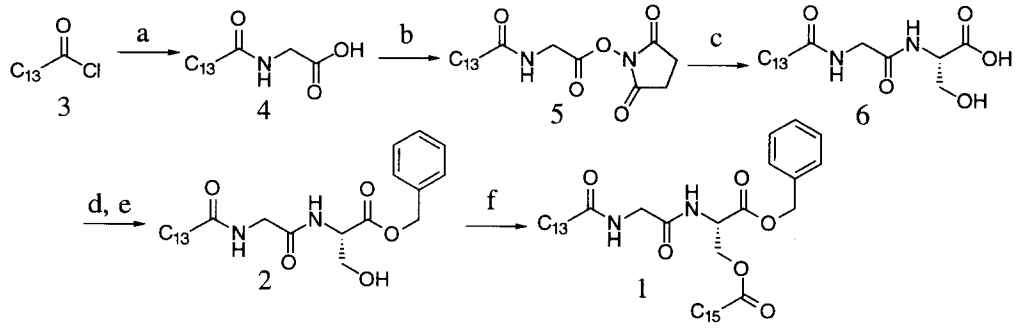
mixture in which myristylglycine-serine **6** was afforded in good yield. Compound **6** was found unchanged when subjected to most acylation and esterification conditions. However, the cesium salt of **6** could be converted into the benzyl ester **2**. Thus, a suspension of **6** in methanol was treated with 1.2 equiv of 20% w/v aqueous cesium carbonate.[4,5] After 15 min, solvents were removed under reduced pressure and the residue lyophilized. The cesium salt residue was mixed with 3 equiv of benzyl bromide in anhydrous DMF for 24 h to afford **2**. The synthetic sequence of coupling **6** with serine, cesium salt formation, and benzylation afforded **2** of similar optical purifty to that obtained by coupling of **5** with serine benzyl ester {$[a]^D$ = +8.5 vis +8.1 (CH_2Cl_2) at 21°C and 23°C, respectively}. The coupling of **5** with serine benzyl ester was achieved in 82% yield and 12 h using 1.2 equiv of Na_2CO_3 in THF with a minimum amount of water. *The advantage of the cesium carbonate sequence is the ability to use inexpensive unprotected serine in the peptide synthesis.* The alcohol **2** in chloroform was mixed with palmitoyl chloride and triethylamine to produce palmitate **1**.[6]

Scheme 1. Synthesis of myristylglycine-palmitoylserine **1** and myristylglycine-serine **2** benzyl esters.



Reagents and conditions (**a**) glycine, NaOH, THF, H_2O, 1 h, 93%. (**b**) NHS, DCC, THF -10°C --> 0°C --> rt, 22 h, 85%. (**c**) serine, Na_2CO_3, THF, H_2O, 12 h, 87%. (**d**) Cs_2CO_3, MeOH, H_2O, 15 min (**e**) benzyl bromide, DMF, 24 h (89% yield for two steps). (**f**) palmitoyl chloride, Et_3N, $CHCl_3$, 6 h, 99%. Reactions at room temperature (rt) unless otherwise noted.

Biological Evaluation: The dipeptides **1** and **2** were tested in receptor binding and ligand inhibition assays in 96-well plates suitable for high throughput screening. CHO cells overexpressing muscarinic receptor subtypes were used to obtain membrane bound receptor. To calculate maximal receptor binding in the absence of agonist, various concentrations of ^{3}H-QNB (Quinuclidinyl Benzilate) were used to measure saturation binding at the receptors (Figure 1). In the saturation binding assay, the maximum amount (B_{max}), of [^{3}H]-QNB bound to HM_2 receptor was 2.4 pmol/mg with a dissociation constant, K_d, of 0.17 nM. In the presence of 20 μM dipeptide **1**, a B_{max} of 1.8 pmol/mg and K_d of 1.8 nM were observed. These data indicate that **1** causes a 26% drop in B_{max} and a tenfold increase in the K_d for QNB at saturation. While **1** also decreased maximal binding at HM_1 receptor, no significant change in K_d was observed. Interestingly, the non-palmitoylated peptide **2** also caused a comparable decrease in B_{max} at both receptor subtypes with no change in K_d values.

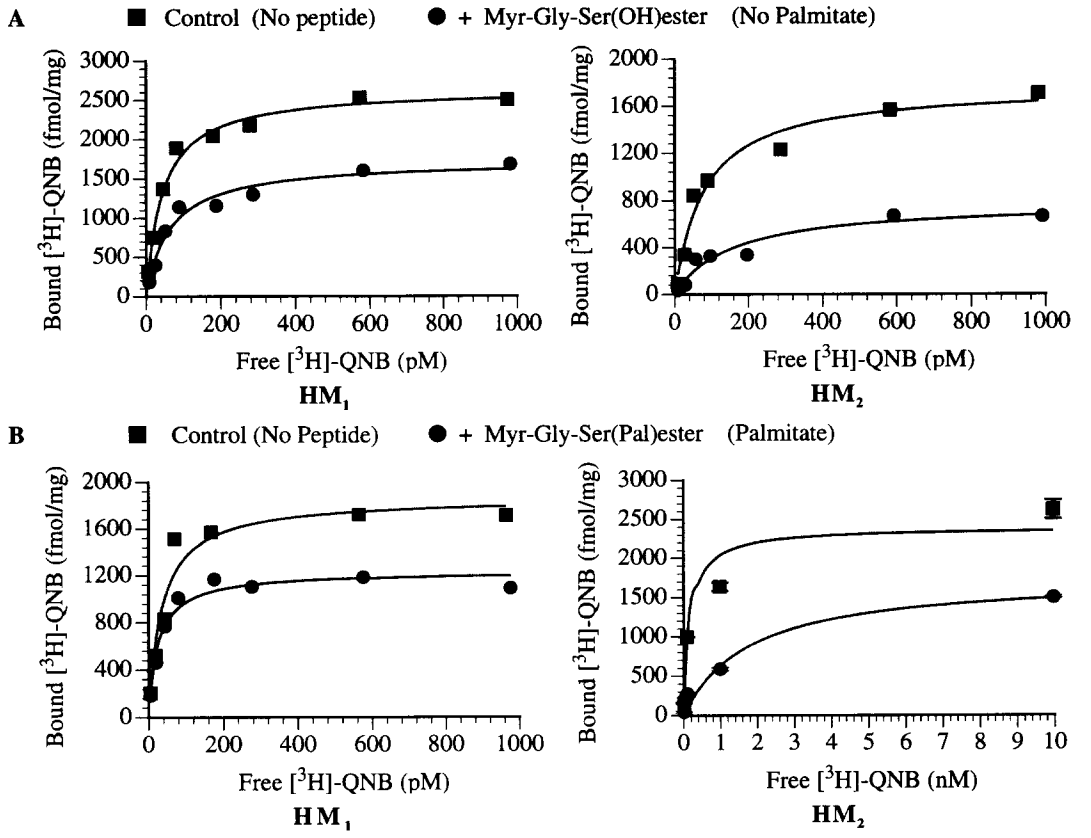


Figure 1. Antagonist binding profiles at HM_1 and HM_2 receptors. (**A**) Shows the decrease in maximal binding in the presence of non-palmitoylated peptide. (**B**) Shows the change in maximal binding in the presence of palmitoylated peptide. Concentrations of ^{3}H-QNB used ranged from 10 pM to 10 nM. The peptides were pre-incubated with the receptor for one half to one hour to ensure equilibrium of peptide with receptor. Briefly, membranes were incubated with various concentrations of [^{3}H]-QNB in the presence of binding buffer for 2 h in a 96-well plate. Total volume of reaction was 1 mL. Reaction was terminated by transferring the reaction mixture onto a GF/B filter followed by washing with ice-cold buffer. Bound radioactivity was measured using scintillation techniques on a TopCountNT^R (Packard Instruments). Data are representative of three to five experiments each performed in triplicate ($P < 0.05$).

An agonist binds preferentially to receptor coupled to G-protein (high affinity site) even at low concentrations. This property of agonist was used to determine the effect of the peptides on the high affinity state of the receptor. To measure changes in agonist binding to HM_2 receptor, the influence of dipeptide on the ability of agonist acetylcholine to displace [^{3}H]-QNB was evaluated (Figure 2). The same membrane preparation with overexpressed HM_2 receptor was used in all experiments to minimize variability while paired sets of control and test experiments were run on different days to confirm reproducibility. Reported data were reproducible using different membrane preparations as well. The concentration of dipeptide was maintained at 20 μM while concentrations of acetylcholine were varied (for details see text of Figure 2). For test experiments, total binding of QNB in the presence of peptide was considered as 100% binding to compensate for the peptides' negative effect on QNB binding. This procedure ruled out the possibility of decreased antagonist binding leading to an apparent

change in agonist binding. Normalization ensured that any changes in QNB binding were solely due to displacement by the agonist acetylcholine. The normalized changes in binding were fitted to curves based on analysis of F distribution values. For ligand displacement assays, data was analyzed with curve fitting to a two or three site model.[7,8] Using SuperANOVA™ program for Macintosh, standard variance between the data sets was determined ($P < 0.05$).

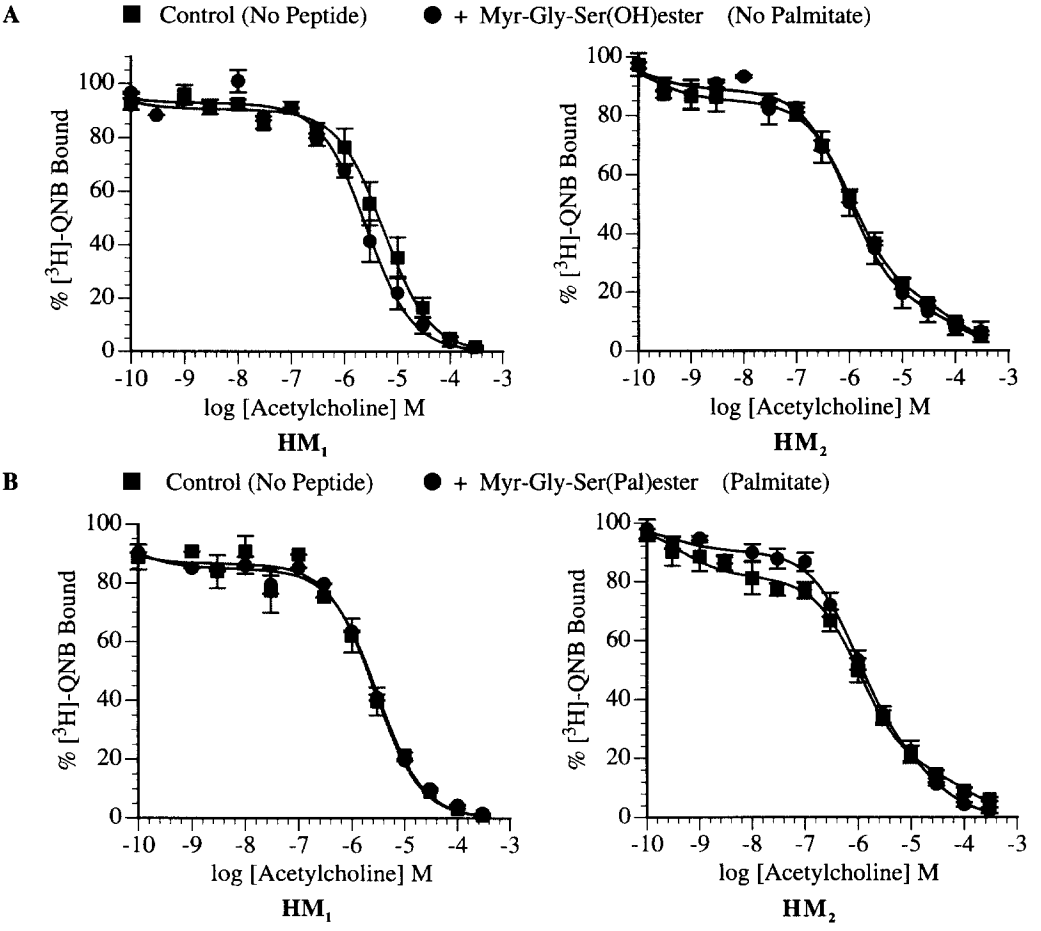


Figure 2. Agonist displacement of bound radioactive antagonist at HM_1 and HM_2 receptors. (**A**) Comparison of acetylcholine displacement of [^{3}H]-QNB in the presence of the non-palmitoylated dipeptide **2**. (**B**) Agonist displacement profiles at HM_1 and HM_2 in the presence of the palmitoylated dipeptide **1**. Concentrations of acetylcholine ranged from 100 pM to 0.3 mM. A fixed concentration (100 pM) of [^{3}H]-QNB was used to determine the displacement of antagonist by increasing concentrations of acetylcholine. Data represent the mean ± S.E of three to six experiments, each performed in triplicate.

We observed that dipeptide **1** lowered the number of high affinity agonist binding sites at HM_2 receptor. Acetylcholine displaced QNB with pK_i of -9.59 ($K_i = 10^{-9.59}$ M) and -9.64 in the absence and presence of dipeptide, respectively (Table 1). These comparable K_i values (test for significance, $P = 0.59$) indicate that the

population of high affinity HM_2 receptors analyzed in each experiment was the same. The total of high affinity receptors was 18.54% and 9.75% in the absence and presence of **1**, respectively. The 47% reduction in high affinity acetylcholine binding sites caused by dipeptide was found to be significant ($P < 0.05$).

Table 1. Dipeptide Effects on Agonist and Antagonist Ligand Binding

		Saturation Binding Assay		Ligand Inhibition Assay					
		[^{3}H]- QNB Binding		**Acetylcholine Displacement of [^{3}H]- QNB**					
Peptide	Pal*	**B_{max}** (pmol /mg)	**K_d** (nM)	**High affinity sites** (%)	pK_i [M] (High)	**Medium affinity sites** (%)	pK_i [M] (Med)	**Low affinity sites** (%)	pK_i [M] (Low)
HM_1 Receptor									
Control	No	2.6	0.04	9.54	-10.90	--	--	90.46	-5.79
2	No	1.7	0.04	7.17	-11.10	--	--	92.83	-6.12
Control	Yes	1.9	0.04	13.54	-11.14	--	--	86.16	-6.11
1	Yes	1.2	0.03	15.22	-10.89	--	--	84.78	-6.15
HM_2 Receptor									
Control	No	1.7	0.08	14.96	-10.14	67.26	-6.33	17.78	-4.31
2	No	0.8	0.15	11.34	-10.12	73.40	-6.25	15.26	-4.19
Control	Yes	2.4	**0.17**	**18.54**	-9.59	65.67	-6.16	15.79	-4.09
1	Yes	1.8	**1.78**	**9.75**	-9.64	67.85	-6.07	22.40	-4.65

Maximal binding was calculated by measuring saturation binding of [^{3}H]-QNB to receptor. Agonist displacement of [^{3}H]-QNB was used to measure the changes in high affinity receptor sites (G-protein bound receptor). For the latter, data was fit to a three site model where permitted (F-value analysis). In the case of HM_1 receptor, a 2-site model provided the best fit. pK_i values are shown to indicate that similar populations of receptors were targeted in control and test experiments. Values are averages of mean ± S.E for three to six experiments each performed in triplicate. * Pal = palmitate.

Results and Discussion: *We report for the first time the synthesis and biological evaluation* of fatty acylated analogs of a Gα N-terminus. Analysis of the experimental data suggests that a $G_i\alpha$ N-terminal mimetic can exhibit specificity in interaction with a G-protein coupled receptor. The interactions of both **1** and **2** with HM_1 and HM_2 receptors results in marked decreases of antagonist binding as seen by decreases in B_{max}. Antagonists bind to GPCRs irrespective of their G-protein association such that they bind non-selectively to all affinity states of a given receptor. While the K_d of QNB with HM_2 receptor was increased in the presence of **1**, the intrinsic affinity of HM_2 receptor for agonist was not since the K_i for acetylcholine displacement of QNB was unchanged. The association of a G-protein with a GPCR is considered to correlate with that receptor's high affinity state for agonist. The interaction of G-protein with HM_2 receptor appeared to be inhibited by **1** since the number of HM_2 receptor high affinity sites for agonist was decreased. There are several possible interpretations of the data. One hypothesis is that the fatty acylated dipeptide **1** acts at the same site and manner as the N-terminus of $G_i\alpha$. Other G-protein factors such as the Gα C-terminus are expected to contribute to the formation of high affinity receptor

sites.[1] Dipeptide interactions with receptor are probably not sufficient to produce a receptor in a high affinity state for agonist but could be sufficient to interfere with optimal G-protein interaction with the receptor. The disruption of the G-protein coupling to HM_2 receptor by **1** would thus account for the loss of high affinity sites for agonist. The loss of antagonist binding in the absence of agonist is evidence of direct interaction of **1** and **2** with HM_1 and HM_2 receptors and is expected to be independent of G-protein activity. The loss of antagonist binding is presumably caused by dipeptide induced conformational changes in the receptors. While **1** interacted with both receptor subtypes, only its interaction with HM_2 receptor affected receptor affinity for agonist.

Palmitate on serine was not essential for decreasing receptor antagonist binding but was necessary for decreasing the number of high affinity sites for agonists. Both palmitoylated and non-palmitoylated myristylated dipeptides caused a decrease in maximal binding independent of HM_1 or HM_2 receptor subtype. In contrast, **1** induced a 47% decrease in the high affinity sites at HM_2 receptor while the non-palmitoylated **2** showed no effect. Thus, we have observed that compounds that are similar in structure to the N-terminus of a Gα N-terminus and as small as a dipeptide can interact with G-protein coupled receptors in a receptor subtype specific manner. Furthermore, the presence of a lipophilic fatty acid group can serve as a determinant for the specific interaction of such compounds with receptor. These and related compounds may therefore be used to increase our understanding of G-protein and G-protein coupled receptor properties and perhaps afford new strategies for regulating G-protein activities.

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6. Data for **1**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (t, J = 6.3 Hz, 6H), 1.23 (br s, 44H), 1.50 (m, 2H), 1.61 (m, 2H), 2.18 (m, 4H), 3.96 (m, 2H), 4.33 (dd, 11.4, 3.3 Hz, 1H), 4.47 (dd, J = 7.7, 3.8 Hz, 1H), 4.82 (m, 1H), 5.17 (m, 2H), 6.06 (m, 1H, NH), 6.71 (d, J = 7.7 Hz, 1H, NH), 7.33 (br s, 5H); Anal. Calcd for $C_{42}H_{72}N_2O_6$: C, 71.96; H, 10.35; N, 4.00. Found: C, 71.77; H, 10.48; N, 3.86.

   Data for **2**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (t, J = 6.3 Hz, 3 H), 1.23 (br s, 20 H), 1.62 (m, 2H), 2.21 (t, J = 7.5 Hz, 2H), 3.24 (br s, 1H), 3.98 (m, 4H), 4.66 (m, 1H), 5.20 (s, 2H), 6.34 (m, 1H, NH), 7.16 (d, J = 7.3 Hz, 1H, NH), 7.33 (br s, 5H); $[\alpha]_D$+8.5 (c = 4.0, CH_2Cl_2); Anal. Calcd for $C_{26}H_{42}N_2O_5$: C, 67.50; H, 9.15; N, 6.06. Found: C, 67.70; H, 9.23; N, 5.90.
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