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CYSTEAMINE SELECTIVELY ENHANCES NEUROPEPTIDE Y_2 RECEPTOR BINDING ACTIVITY

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SUMMARY: Affinity labeling of [^{125}I]NPY to the bovine hippocampal NPY receptor has revealed a 50 kDa specific binding protein, the Y_2 receptor. Cysteamine (10 μM - 10 mM) specifically enhanced NPY specific labeling of the Y_2 receptor without affecting cross-linking efficiency. Several structurally related agents, including reduced glutathione, cysteine, ß-mercaptoethanol and ethanolamine, were without effect on receptor binding. The enhancement of binding by cysteamine could be reversed by washing the membranes. These studies suggest that cysteamine may change the conformation of the NPY Y_2 receptor and increase its binding activity.

Neuropeptide Y is an amidated 36-amino acid peptide that is widely distributed in the central and peripheral nervous systems (1-4). NPY coexists with neurotransmitters such as norepinephrine (5) and has numerous proposed physiological functions in the peripheral and central nervous systems (6). NPY is a member of the PP-fold family of peptides, which consists of NPY, peptide YY and pancreatic polypeptide (PP). These three peptides have considerable sequence homology and differ from most other small and medium size peptides in having a surprisingly rigid conformation in aqueous solution (7, 8).

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Abbreviations: NPY, neuropeptide Y; PMSF, phenylmethylsulfonyl fluoride; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate.

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Specific binding sites for NPY have been described in brain and peripheral tissues from various species (9-11). Multiple NPY receptors have been characterized (12) and classified as Y_1 and Y_2 subtypes (13). The receptor in porcine and rat hippocampus has been characterized as a Y_2 receptor subtype and identified as a 50 kDa binding protein (14-17). We also characterized and identified NPY receptors in both human (15) and bovine hippocampus (18) as 50 kDa binding proteins fitting the Y_2 receptor subtype classification. During the course of our studies on bovine hippocampus we observed that the tetramine disulfide, benextramine, inhibited NPY binding and reduced glutathione reversed this inhibition (19). We have obtained evidence demonstrating that one or more benextramine-sensitive sulfhydryl groups are important for NPY binding to the Y_2 receptor (20). In the present study, we utilized affinity labeling in combination with binding studies to demonstrate the specific effect of cysteamine, a nucleophilic sulfhydryl compound, on Y_2 receptor binding activity.

MATERIALS AND METHODS

Materials: Synthetic NPY was purchased from Bachem Inc., Torrance, CA. Monoiodinated NPY was prepared by the Chloramine T method and purified by HPLC, with a specific activity of 1080 Ci/mmol. DSS was obtained from Pierce, Rockford, IL. Other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Membrane binding: The methods for membrane preparation and binding were the same as previously described (15). Briefly, bovine hippocampus (freshly obtained from a local slaughterhouse) was minced and homogenized (Teflon pestle in glass) in 0.3 M sucrose solution containing 10 mM HEPES (pH 7.4) and 0.1 mM PMSF. The homogenate was centrifuged at 6,000 x g for 20 min. The resultant supernatant was centrifuged at 100,000 x g for 60 min. The pellet was washed twice with binding buffer containing 50 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF and 1 mg/ml bacitracin. Membrane protein (40 μg) was incubated with [^{125}I]NPY in the absence or presence of 1 μM unlabeled NPY in the binding buffer with additional protease inhibitors (10 μg/ml leupeptin, 10 μg/ml antipain, 80 μg/ml benzamidine, 20 μg/ml aprotinin and 10 μg/ml pepstatin A) for 90 min at 24°C. [^{125}I]NPY degradation during the incubation was determined by HPLC to be less than 12%. The membranes were centrifuged, washed and pelleted. Radioactivity remaining in the pellets was determined with a gamma counter.

Affinity labeling: Membranes (200 μg) were incubated with 250 pM [^{125}I]NPY under the same condition as described in Membrane binding. The membranes were centrifuged, washed and resuspended in the binding buffer. The affinity labeling method was the same as previously described (21). DSS (1 mM) was added to initiate the cross-linking reaction and membranes were incubated at 4°C for 30 min. The cross-linking reaction was stopped by the addition of 0.5 ml of 0.1 M Tris-HCl (pH 7.4). The membranes were pelleted and analyzed by SDS-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) followed by autoradiography as described previously (22).

RESULTS AND DISCUSSION

Affinity labeling demonstrated that [^{125}I]NPY binds to the 50 kDa receptor protein since the labeling was reduced by the addition of 1 μM unlabeled NPY (Fig. 1A). [^{125}I]NPY labeling of the 58 and 62 kDa proteins was non-specific since the addition of unlabeled NPY did not reduce labeling. Cysteamine at concentrations from 10 μM - 10 mM enhanced NPY labeling of the 50 kDa Y_2 receptor, but not the non-specific binding proteins (Fig 1A). In contrast increasing concentrations of dithiothreitol did not enhance NPY labeling (Fig. 1B). Cysteamine and dithiothreitol, at higher concentrations (100 mM), reduced the labeling of the 50 kDa protein. These results demonstrate that cysteamine at low concentrations (10 μM -

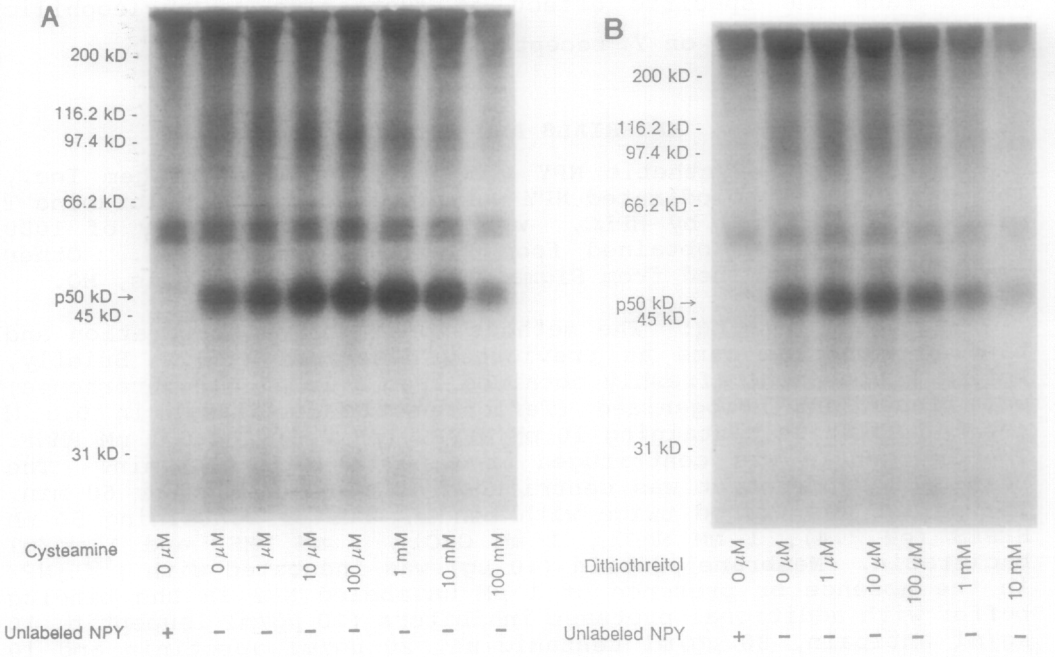

Figure 1. **(A) Autoradiogram of [^{125}I]NPY affinity labeling of the NPY receptor in bovine hippocampus membranes with increasing concentrations of cysteamine.** Membranes (200 μg/ml) were incubated with 250 pM [^{125}I]NPY for 90 min at 24°C with increasing concentrations of cysteamine as indicated. The membranes were then pelleted, washed and cross-linked with 1 mM of DSS followed by electrophoresis as described in Materials and Methods. ROD values for the 50-kDa bands were as follows: *lane 1, 0.069; lane 2, 0.538; lane 3, 0.587; lane 4, 0.753; lane 5, 0.861; lane 6, 0.986; lane 7, 0.904; lane 8, 0.419.* **(B) Autoradiogram of [^{125}I]NPY affinity labeling of the NPY receptor with increasing concentrations of dithiothreitol.** ROD values for the 50-kDa bands were as follows: *lane 1, 0.059; lane 2, 0.883; lane 3, 0.888; lane 4, 0.850; lane 5, 0.648; lane 6, 0.445; lane 7, 0.245.* Experimental methods were the same as described in Fig. 1A.

10 mM) enhances Y_2 receptor binding activity. The inhibitory effect of high concentrations (100 mM) of cysteamine and dithiothreitol on receptor binding may be due to the disruption of disulfide bonds important for receptor conformation.

[^{125}I]NPY also specifically labeled a diffuse band migrating at 95 - 120 kDa. The labeling of these proteins was enhanced by cysteamine (Fig. 1A) and inhibited by dithiothreitol (Fig. 1B) in parallel with the 50 kDa protein. In other studies, we found that benextramine (19), N-ethylmaleimide and p-chloromercuriphenylsulfonic acid (20), which alkylate sulfhydryl groups, also inhibited NPY labeling of the 50 kDa Y_2 receptor and 95 - 120 kDa binding proteins in parallel. These results suggest that the 50 kDa binding protein and 95 - 120 kDa binding proteins have the same binding site conformation. The exact relationship between these proteins and the 50 kDa Y_2 receptor is unknown. One possibility is that the 95 - 120 kDa binding proteins may be covalently cross-linked complexes of the 50 kDa binding protein and other closely-associated membrane proteins.

Cysteamine enhancement of NPY labeling of the 50 kDa protein could be due to either an enhancement of Y_2 NPY receptor binding activity or an increased cross-linking efficiency. These possibilities were examined by comparing the results of binding and cross-linking experiments carried out under the same conditions. We observed that cysteamine caused 233% and 225% increases in binding and affinity cross-linking of NPY with the 50 kDa species, respectively (Table I). Moreover, the affinity cross-linking efficiency between [^{125}I]NPY and the 50 kDa receptor (1.39% in control and 1.35% after cysteamine treatment) was not influenced by 1 mM cysteamine (Table I). These data suggest that the effect of cysteamine is to enhance Y_2 NPY receptor binding activity.

Several other reagents with similar structures were also examined for their effects on the [^{125}I]NPY labeling of the 50 kDa receptor. Reduced glutathione and cysteine, which are sulfhydryl containing compounds, did not enhance receptor binding. Moreover, ß-mercaptoethanol and ethanolamine, which differ from cysteamine only in that both contain a hydroxyl in place of either a primary amine or a sulfhydryl group, respectively, did not enhance Y_2 receptor binding (Fig. 2). The enhancement of binding was specific for cysteamine. These results suggest that cysteamine possesses a unique arrangement of a sulfhydryl and an amine group which enhances 50 kDa Y_2 receptor binding activity.

Table I. **Cysteamine enhancement of NPY binding to the 50 kDa Y_2 receptor**

Treatment		Quantitative Analysis*		
		Membrane pellet		
		dpm ± S.D.	Net dpm	% Change
Control	Non-specific binding	24137 ± 3309		
	Total binding	39990 ± 1523[a]	15853	100%
Cysteamine	Non-specific binding	22384 ± 310		
	Total Binding	59277 ± 857[ab]	36893	233%
		50 kDa Band[c]		
		dpm ± S.D.	Net dpm	% Change
Control	Non-specific binding	177 ± 19		
	Total binding	398 ± 6[a]	221	100%
Cysteamine	Non-specific binding	140 ± 8		
	Total Binding	638 ± 12[ab]	498	225%

The conditions for membrane binding with or without 1 mM cysteamine are the same as in Fig. 1. Membranes were divided into two groups and incubated under the same conditions with [^{125}I]NPY in the presence or absence of 1 μM unlabeled NPY. One group was washed and the membrane pellets counted in a gamma counter. The second group was dealt with similarly and then treated with DSS as described in MATERIALS AND METHODS. The 50 kDa band was cut out using the autoradiogram as a template and counted in the gamma counter.

* n=3
a: $p<0.005$, Total binding versus non-specific binding.
b: $p<0.005$, Total binding with cysteamine versus total binding without cysteamine.
c: Cross-linking efficiency was 1.39% in control and 1.35% in cysteamine samples.

We further examined whether the effect of cysteamine is reversible. Labeling of the 50 kDa protein was enhanced by the addition of cysteamine (1 mM) to the binding reaction (Fig. 3). However, binding activity was not changed if the cysteamine was removed by washing the membranes prior to the addition of [^{125}I]NPY demonstrating the reversibility of cysteamine action.

In conclusion, cysteamine has the unique ability to enhance NPY binding to the bovine hippocampal Y_2 NPY receptor in a reversible fashion. This effect is specific for cysteamine since analogues and other closely related compounds do not share this action. Cysteamine may be a useful agent for revealing the nature of the interaction of NPY with the Y_2 receptor.

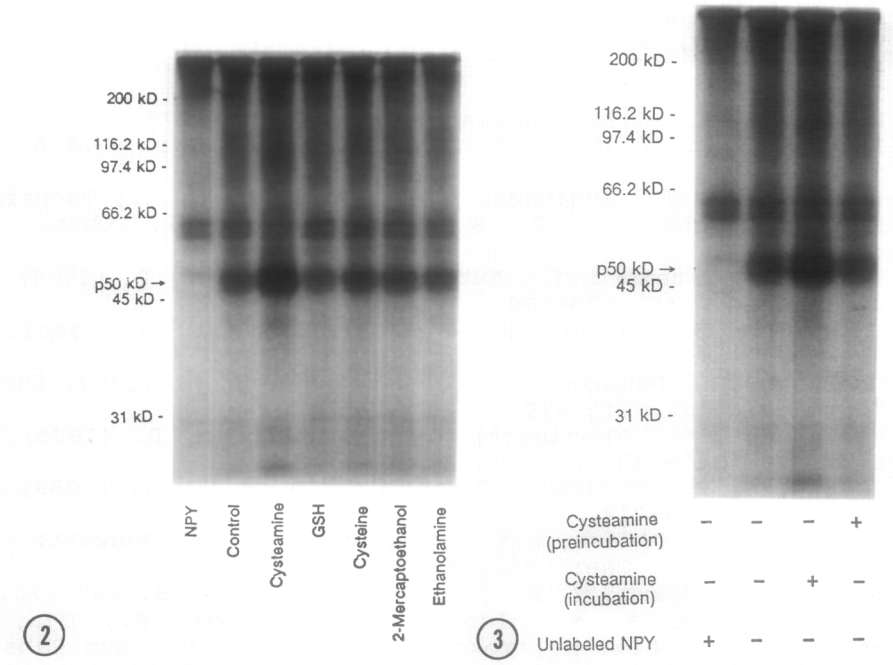

Figure 2. **Comparison of cysteamine with other related reagents.** Membranes were incubated with [^{125}I]NPY for 90 min at 24°C in the presence of various reagents (final concentration 1 mM) as indicated. Experimental methods were the same as described in Fig. 1.

Figure 3. **The reversibility of cysteamine enhancement of Y_2 receptor binding activity.** Membranes were pre-incubated with either 1 mM cysteamine or buffer for 90 min at 24°C and washed three times. Membranes were then incubated with [^{125}I]NPY for 90 min at 24°C in the presence of 1 mM cysteamine, 1 μM unlabeled NPY or buffer as indicated. Experimental methods were the same as described in Fig. 1.

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REFERENCES

1. Tatemoto, K., Carlquist, M., and Mutt, V. (1982) Nature 296, 659-660.
2. Adrian, T., Allen, J., Bloom, S., Ghatei, M., Rossor, M., Roberts, G., Crow, T., Tatemoto, K., and Polak, J. (1983) Nature 306, 584-586.
3. Allen, Y. S., Adrian, T. E., Allen, J. M., Tatemoto, K., Crow, T. J., Bloom, S. R., and Polak, J. M. (1983) Science 221, 877-879.
4. Sundler, F., Hakanson, R., Ekblad, E., Uddman, R., and Wahlestedt, C. (1986) Int. Rev. Cytol. 102, 243-269.

5. Hokfelt, T., J.M., L., Lagercrantz, H., Tatemoto, K., Mutt, V., Lindberg, J., Terenius, L., Everitt, B. J., Fuxe, K., Agnati, L., and Goldstein, M. (1983) Neurosci. Lett. 36, 217-222.
6. Potter, E. K. (1988) Pharmaco. Ther. 37, 21-273.
7. Tatemoto, K. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5485-5489.
8. Fuhlendorff, J., Johansen, N. L., Melberg, S. G., Thogersen, H., and Schwartz, T. W. (1990) J. Biol. Chem. 265, 11706-11712.
9. Unden, A., Tatemoto, K., Mutt, V., and Bartfai, T. (1984) Eur. J. Biochem. 145, 525-530.
10. Walker, M. W., and Miller, R. J. (1988) Mol. Pharmacol. 34, 779-792.
11. Cherdchu, C., Deupree, J. D., and Hexum, T. D. (1989) Eur. J. Pharmacol. 173, 115-119.
12. Wahlestedt, C., Yanaihara, N., and Hakanson, R. (1986) Reg. Pept. 13, 307-318.
13. Sheikh, S. P., Hakanson, R., and Schwartz, T. W. (1989) FEBS Lett. 245, 209-214.
14. Sheikh, S.P., O'Hare, M.M.T., Tortora, O., and Schwartz, T.W. (1989) J. Biol. Chem. 264, 6648-6654.
15. Li, W., and Hexum, T. D. (1991) Brain. Res. 553, 167-170.
16. Inui, A., Okita, M., Inoue, T., Sakatani, N., Oya, M., Morioka, H., Shii, K., Yokono, K., Mizuno, N., and Baba, S. (1989) Endocrinol. 124, 402-409.
17. Sheikh, S. P., and Williams, J. A. (1990) J. Biol. Chem. 265, 8304-8310.
18. Li, W., MacDonald, R. G., and Hexum, T. D. (1992) Life Sci., 50, 695-703.
19. Li, W., MacDonald, R. G., and Hexum, T. D. (1991) Eur. J. Pharmacol. 207, 89-91.
20. Li, W., MacDonald, R. G., and Hexum, T. D. (1992) J. Biol. Chem. in press.
21. Massague, J., and Czech, M. P. (1982) J. Biol. Chem. 257, 5038-5045.
22. Laemmli, U. K. (1970) Nature 227, 680-685.