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Inactivation of cholecystokinin receptors in rat pancreatic membranes by sulfhydryl reagents: protection by guanosine 5′-triphosphate (GTP) but not N^2, O^2-dibutyryl guanosine 3′,5′-cyclic monophosphate (dibutyryl cGMP)

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Dithiothreitol (DTT*) and EGTA have been reported to increase specific [^{125}I]CCK binding in pancreatic tissue [1, 2]. The mode of action of DTT and EGTA on [^{125}I]CCK receptor binding is unknown. A possible common mechanism for both agents may be their ability to protect essential sulfhydryl groups associated with [^{125}I]CCK binding, DTT by preventing their oxidation and EGTA by chelating SH reactive heavy metals. To investigate this possibility, and the potential importance of functional sulfhydryl groups for CCK receptor binding, the effects of various sulfhydryl reagents upon [^{125}I]CCK binding to pancreatic membranes were examined.

Pancreatic membranes of Sprague–Dawley rats (250–350 g) were prepared as described previously [1]. The resulting membrane suspensions were reacted with NEM (with or without GTP or dibutyryl cGMP), DTNB, or PCMB at the concentrations, time periods, and temperatures indicated in the text. The reaction was stopped either by adding DTT to give a final concentration of 5 mM (after treatment with NEM), or by diluting the reaction mixture ten times with cold Tris buffer (after treatment with DTNB or PCMB) and immediately centrifuging at 50,000 *g* for 10 min. The resulting pellets were washed two more times by resuspending and centrifuging. The pellets were resuspended in 200 vol. of Tris buffer (pH 7.4 at 37°), 5 mM $MgCl_2$, 0.2% BSA and 0.1 mM bacitracin with (for NEM-treated) or without (for DTNB- and PCMB-treated) 5 mM DTT and used for [^{125}I]CCK receptor binding as described previously [1, 3].

Pretreatment of pancreatic membranes with NEM for 20 min at 25° caused a concentration-dependent reduction in specific [^{125}I]CCK receptor binding (Table 1). Scatchard analysis indicated that the reduction in [^{125}I]CCK receptor binding was due to a reduction in receptor number (B_{max}) without change in receptor affinity (K_D) (Fig. 1). Pretreatment with other sulfhydryl reagents, DTNB (0.1 mM) and PCMB (0.1 mM), under the same conditions also significantly decreased [^{125}I]CCK receptor binding (67 ± 7 and 89 ± 5% respectively). The data indicate that intact sulfhydryl groups are essential for [^{125}I]CCK binding to pancreatic membranes. The results are also consistent with the proposal that the previously reported ability of DTT and EGTA to increase [^{125}I]CCK binding to pancreatic membranes [1, 2] may be due to protection of SH groups essential for CCK receptor binding from oxidation or inactivation by heavy metals respectively.

The guanine nucleotide GTP has been shown previously to modulate binding of agonists to pancreatic CCK receptors [1]. However, using the present incubation and washing protocol, GTP (0.1 mM) did not significantly affect [^{125}I]CCK receptor binding and significantly prevented the reduction of [^{125}I]CCK binding produced by NEM (Table 2). As shown in Fig. 1, the mean B_{max} values of three triplicate determinations were 36.1 ± 5.6, 15.9 ± 1.1 and 24.6 ± 2.1 fmoles per assay for control, NEM-treated and NEM + GTP-treated respectively. The corresponding K_D values for the three treatments were 0.98 ± 0.19, 0.90 ± 0.08 and 0.93 ± 0.15 nM respectively. The present finding that NEM inactivation of [^{125}I]CCK binding is prevented by GTP suggests that essential sulfhydryl groups associated with the GTP binding protein regulating CCK receptors may be the site of action of NEM. However, the possibility must also be considered that GTP binding changes membrane configuration in such a way as to make the essential sulfhydryl groups less accessible to NEM.

Table 1. Effect of various concentrations of NEM on [^{125}I]CCK receptor binding in rat pancreatic membranes*

NEM conc (mM)	Specific [^{125}I]CCK receptor binding	
	fmoles/assay	% of Control
0	6.19 ± 0.23	100 ± 3.8
0.025	5.81 ± 0.20	94 ± 3.2
0.05	5.77 ± 0.09	93 ± 1.3
0.125	3.27 ± 0.41	53 ± 6.7
0.25	2.44 ± 0.11	40 ± 1.9
0.5	2.26 ± 0.06	37 ± 0.9
1	0.94 ± 0.07	15 ± 1.1
5	0.10 ± 0.04	1.6 ± 0.8

*To 9 ml of pancreatic membranes in 50 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 ml of NEM solution was added to give the indicated final concentration. The reaction was allowed to proceed at 25° for 20 min stopped by adding 1.1 ml of 50 mM DTT, and centrifuged. Pellets were further washed twice by resuspension and centrifugation. The resulting pellets were then resuspended in assay buffer and used for [^{125}I]CCK binding. The values are the mean ± S.E. from three experiments each run in triplicate.

Similar experiments were conducted using the competitive antagonist of CCK, dibutyryl cGMP [4]. The complete inhibition of specific [^{125}I]CCK binding produced by dibutyryl cGMP (1 mM) using unwashed pellets (data not shown) was not evident using the present protocol, demonstrating that dibutyryl cGMP could be removed from the membranes by washing. Preincubation with 1 mM dibutyryl cGMP did not significantly ($P > 0.1$) prevent the decrease in [^{125}I]CCK binding produced by NEM. In three triplicate experiments specific [^{125}I]CCK binding in control membranes was 6.23 ± 0.37 fmoles compared to 2.40 ± 0.21 (−39 ± 3%) and 2.94 ± 0.18 (−47 ± 3%) fmoles in NEM- and NEM+ dibutyryl cGMP-treated membranes respectively. Similar experiments that were attempted using CCK-8 as a ligand to protect the CCK receptor were not successful due to difficulty in washing CCK-8 from the membrane. The inability of dibutyryl cGMP to protect NEM inactivation of [^{125}I]CCK binding suggests that the sulfhydryl groups with which NEM react are not closely associated with active sites on the CCK receptor, although other explanations are also possible.

* Abbreviations: DTT, dithiothreitol; EGTA, ethyleneglycolbis (amino-ethylether) tetra-acetate; BSA, bovine serum albumin; GTP, guanosine 5′-triphosphate; CCK, cholecystokinin; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; and dibutyryl, cGMP, N^2, O^2-dibutyryl guanosine-3′,5′-cyclic monophosphate.

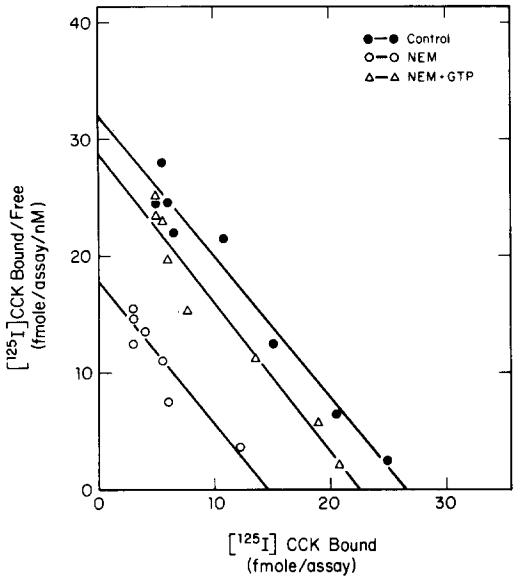

Fig. 1. Scatchard analysis of specific [^{125}I]CCK receptor binding in pancreatic membranes. Pancreatic membranes in 50 mM Tris buffer were incubated with or without GTP (0.1 mM final concentration) at 25° for 30 min. NEM was then added to give a final concentration of 0.25 mM and incubated for an additional 20 min. The reaction was stopped by adding DTT to give a final concentration of 5 mM and centrifuged. The pellets were washed twice by resuspending and centrifuging. Pellets were then resuspended in the binding assay buffer and used for [^{125}I]CCK receptor binding. The experiments, which were performed in triplicate, were replicated three times. The mean ± S.E. of the B_{max} and the K_D values from these determinations are indicated in the text.

The data could alternatively indicate that the sites of receptor attachment for CCK and dibutyryl cGMP may not overlap. The inaccessibility of a suitable CCK agonist which could be washed from the receptor precluded further study of these possibilities.

In summary, the present data provide evidence that functional sulfhydryl groups are located on the GTP binding protein regulating pancreatic CCK receptors but not on active sites of the CCK receptor itself. The previously reported increase in specific [^{125}I]CCK binding in pancreatic tissue produced by DTT and EGTA might thus be explained by the ability of these agents to protect sulfhydryl groups essential for receptor binding.

Table 2. Protection from NEM inhibition of [^{125}I]CCK binding by preincubation with GTP*

	Specific [^{125}I]CCK receptor binding	
	fmoles/assay	% of Control
Control	5.00 ± 0.18	100 ± 3.6
NEM	2.09 ± 0.08	41.8 ± 1.6†
GTP	5.68 ± 0.22	113.8 ± 4.5‡
NEM + GTP	3.96 ± 0.48	79.2 ± 9.6§,‖

* Pancreatic membranes in 50 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.2% BSA and 0.1 mM bacitracin were incubated with or without GTP (0.1 mM) for 30 min at 37°. NEM, when used, was then added to give a concentration of 0.25 mM and the mixture was incubated for an additional 2 min. The reaction was stopped by adding DTT to give a final concentration of 5 mM and then centrifuged. Pellets were washed twice by resuspending and centrifuging with Tris buffer. Final pellets were resuspended in assay buffer and used for [^{125}I]CCK binding. Values are the mean ± S.E from three determinations each run in triplicate.

† $P < 0.001$ (vs control).
‡ $P > 0.05$ (vs control).
§ $P < 0.025$ (vs NEM-treated).
‖ $P > 0.1$ (vs control).

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