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IMPORTANCE OF DISULFIDE BONDS IN RECEPTORS FOR VASOACTIVE INTESTINAL PEPTIDE AND SECRETIN IN RAT PANCREATIC PLASMA MEMBRANES

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(1) Vasoactive intestinal peptide (VIP), secretin, and C-terminal octapeptide of cholecystokinin (CCK-8) receptors were identified in rat pancreatic plasma membranes by the ability of these peptides to stimulate adenylate cyclase activity. The membrane preparation procedure was conducted through a series of steps including discontinuous sucrose density gradient fractionation. 5 mM β-mercaptoethanol was added stepwise. Membrane preparations obtained stepwise were preincubated for 10 min at 25°C in the presence of various concentrations of β-mercaptoethanol or dithiothreitol before assaying adenylate cyclase. The use of the reducing agents exerted no effect on p[NH]ppG-, NaF-, and CCK-8- stimulated activities. By contrast, stimulation of adenylate cyclase by low VIP concentrations was specifically altered when β-mercaptoethanol was used during tissue homogeneization at 5°C. (2) In addition, both VIP and secretin responses were highly sensitive towards a preincubation of 10 min at 25°C in the presence of dithiothreitol. (3) These results were likely to reflect alterations at the receptor level. ^{125}I-VIP binding was, indeed, reduced after dithiothreitol preincubation, low concentrations of the thiol reagent decreasing the apparent number of high-affinity VIP receptors and higher dithiothreitol concentrations reducing the affinity of VIP receptors.

Introduction

We described previously the presence, in purified plasma membranes from rat pancreas [1], of specific receptors for VIP, secretin [2], and CCK-like peptides [3]. Based of the capacity of these peptides to stimulate adenylate cyclase activity in the same preparation, we further demonstrated the existence of high- and low-affinity receptors for secretin and of only one class of VIP receptors with relatively low affinity [2]. Recently, however, the presence of high-affinity VIP receptors was documented in intact rat pancreatic acini, whose occupancy leads to increased cyclic AMP levels [4,5].

The present study was undertaken in order to evaluate the possible disappearance of such high-affinity VIP receptors, coupled to an adenylate cyclase system, during the preparation of rat pancreatic plasma membranes.

We first observed that the presence of β-mercaptoethanol in the homogenizing buffer selectively reduced the apparent K_{act} of VIP-stimulated adenylate cyclase. We then compared the effects of β-mercaptoethanol and dithiothreitol on hormone-stimulated adenylate cyclase activities in rat pan-

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Abbreviations: VIP, vasoactive intestinal peptide; CCK-8, C-terminal octapeptide of cholecystokinin-pancreozymin (CCK_{32-39}); p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

creatic plasma membranes prepared in the absence of any reducing agent. These effects were finally interpreted in terms of alterations of hormonal receptors based on ^{125}I-VIP binding data.

Materials and Methods

Preparation of rat pancreatic plasma membranes. Rat pancreatic plasma membranes were prepared at 5°C, as previously described [1,2]. Pancreases from 30 rats were minced and homogenized with a glass-teflon pestle in 5 vol. of ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 500 kallikrein inhibitor units/ml Trasylol, 1 mg/ml liver phospholipids, without (control) or with 5 mM β-mercaptoethanol. This homogenate was diluted to 10% (v/v) with the same buffer, filtered on medical gauze and centrifuged for 10 min at 180 × *g*. The supernatant was centrifuged for 15 min at 15000 × *g* and the resulting pellet resuspended in 30 ml of the same buffer (without or with 5 mM β-mercaptoethanol). Further purification was achieved by discontinuous sucrose density gradient fractionation, using a SW 27 swinging-out bucket rotor in the L 2-65 B Beckman ultracentrifuge. The gradient layers were prepared as previously described [1], in the same buffer containing or not 5 mM β-mercaptoethanol. The plasma membrane-rich fraction was collected and stored as described in Ref. 1 in the absence or presence of 5 mM β-mercaptoethanol.

Preincubation of rat pancreatic membranes with β-mercaptoethanol or dithiothreitol. Pancreatic membranes (±3 mg membrane protein/ml) were thawed and incubated for 10 min at 25°C in absence of presence of various concentrations of β-mercaptoethanol or dithiothreitol. They were then diluted 2-fold with the ice-cold homogenizing buffer, without β-mercaptoethanol and immediately assayed for adenylate cyclase activity (20 µl of the diluted membranes were incubated in a final volume of 60 µl, so that the final concentration of the reducing agent, during the assay, was one-sixth of that used during the preincubation period.

Adenylate cyclase assay. Adenylate cyclase activity was determined by the procedure of Salomon et al. [6] with minor modifications. About 30 µg membrane protein were incubated for 8 min at 37°C in a total volume of 60 µl containing 0.5 mM [α-^{32}P]ATP ($1 \cdot 10^6$ cpm), 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cyclic AMP, 1 mM theophylline, 10 mM phospho*enol*pyruvate, 30 µg/ml pyruvate kinase, and 30 mM Tris-HCl (pH 7.6). When hormonal stimuli were tested, 10 µM GTP was added to the buffer. The reaction was stopped by adding 0.5 ml of a 0.5% sodium dodecylsulfate solution containing 0.5 mM ATP, 0.3 mM cyclic AMP and 20000 cpm of cyclic [8-^{3}H]AMP (for determination of cyclic nucleotide recovery). Cyclic AMP was separated from ATP by two successive chromatographies on Dowex 50W-X8 and neutral alumina.

Radioiodination of VIP and ^{125}I-VIP binding studies

VIP was iodinated with a chloramine-T technique and purified as described in Refs. 7 and 8. ^{125}I-labelled VIP samples with specific radioactivity 200 to 250 µCi/µg were stored at 25°C.

Studies of ^{125}I-labelled VIP binding to rat pancreatic membranes were carried out in a 50 mM Tris-maleate (pH 7.4) buffer containing 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 100 KIU (kallikrein inhibitor unit) kallikrein inhibitor/ml, 1% bovine serum albumin, 20–30 pM ^{125}I-labelled VIP, increasing concentrations of unlabelled peptide and 100 µg membrane protein in a final volume of 240 µl. Incubations were conducted at 37°C for 15 min in order to obtain binding equilibrium as previously described [2]. At the end of the incubation period, 180 µl of the medium were pipetted, mixed in a centrifuge tube with 90 µl of ice-cold buffer (without peptide) and centrifuged in a 42-2Ti rotor, at 25000 rpm for 5 min at 2°C, with the L8-55 Beckman ultracentrifuge. The supernatant was discarded and the tip of the centrifuge tube was counted in a Packard spectrometer.

Non specific binding was determined in the presence of 1 µM unlabelled VIP and accounted for approx. 25% of total binding. Specific binding was defined as total binding minus non specific binding and always represented less than 10% of the total number of counts offered.

Origin of the main chemicals used

Synthetic secretin, VIP and CCK-8 were gener-

ous gifts from, respectively, Dr. W. König, (Hoechst Aktiengesellschaft, Frankfurt/Main, F.R.G.), Dr. J.P. Durieux (UCB-Bioproducts, Brussels, Belgium) and Dr. S.J. Lucania (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.). β-Mercaptoethanol and dithiothreitol were from Merck (Darmstadt, F.R.G.) and Aldrich-Europe (Beerse, Belgium), respectively. ATP, cyclic AMP, GTP, p[NH]ppG, pyruvate kinase and phospho*enol*pyruvate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [α-^{32}P]ATP (15 Ci/mmol) and cyclic [8-^{3}H]AMP (20-30 Ci/mmol) were purchased from New England Nuclear Corporation (Dreieich, F.R.G.) and the Radiochemical Centre (Amersham, Bucks, U.K.), respectively.

Results

I. Comparison of adenylate cyclase activity in rat pancreatic plasma membranes prepared in absence or presence of 5 mM β-mercaptoethanol

Specific activities of adenylate cyclase, measured in the absence or presence of maximal concentrations of p[NH]ppG, NaF, and CCK-8, were the same, whether the membranes were prepared in the absence or presence of 5 mM β-mercaptoethanol (Table I). The concentrations of p[NH]ppG, NaF, and CCK-8 giving half-maximal activation were also identical (data not shown). By contrast, enzyme activities, tested in the presence of maximal concentrations of VIP and secretin, were reduced by 37% and 54%, respectively, in membranes prepared with 5 mM β-mercaptoethanol (Table I). The VIP and secretin concentrations required for half-maximal enzyme activation also increased (Fig. 1), and more markedly so with VIP (30-fold) than with secretin (3-fold) (Fig. 1). The relatively flat curve of VIP stimulation of adenylate cyclase, obtained with control mem-

TABLE I

COMPARISON OF ADENYLATE CYCLASE ACTIVITY OF RAT PANCREATIC PLASMA MEMBRANES PREPARED IN THE ABSENCE OF PRESENCE OF 5 mM β-MERCAPTOETHANOL

Figures are represented as means ± S.E. of four preparations.

Agent tested	Adenylate cyclase activity (pmol cyclic AMP produced per min per mg protein)	
	no β-mercaptoethanol	5 mM β-mercaptoethanol
None	34 ± 4	27 ± 3
100 μM p[NH]ppG	461 ± 32	385 ± 43
10 mM NaF	295 ± 28	364 ± 24
10 μM CCK-8	866 ± 53	926 ± 48
10 μM secretin	686 ± 48	430 ± 25 [a]
10 μM VIP	595 ± 33	271 ± 18 [a]

[a] These values, obtained with membranes prepared in the presence of 5 mM β-mercaptoethanol, were significantly lower ($P < 0.02$) than those observed with control membranes.

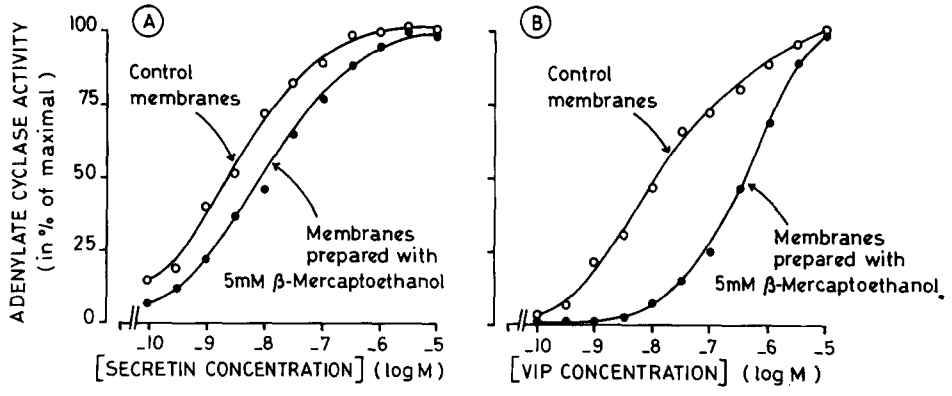

Fig. 1. Dose-response curves of adenylate cyclase activation by secretin (A) and VIP (B) in rat pancreatic plasma membranes prepared in the absence (open circles) or presence (closed circles) of 5 mM β-mercaptoethanol. The results were expressed in % of activity observed in the presence of 10 μM secretin or 10 μM VIP and were the means of four preparations (absolute values are reported in Table I).

branes, was replaced by a steeper curve when membranes were prepared in the presence of 5 mM β-mercaptoethanol. By contrast, secretin dose-effect curves for adenylate cyclase activation remained parallel in membranes prepared without and with β-mercaptoethanol.

2. Effects of β-mercaptoethanol on adenylate cyclase activity in rat pancreatic plasma membranes prepared without β-mercaptoethanol

Membranes prepared without β-mercaptoethanol were preincubated, for 10 min at 25 °C or for 30 min at 4 °C, in the presence of 5 mM β-mercaptoethanol. This pretreatment exerted no significant effect on adenylate cyclase stimulations by VIP (Fig. 2A) and secretin (data not shown). Further experiments were conducted to specify at which step the presence of β-mercaptoethanol, during the membrane preparation procedure, led to an alteration of VIP- and secretin-stimulations of adenylate cyclase.

When pancreases were homogenized in the absence of β-mercaptoethanol, and the first pellet was further processed in the presence of 5 mM β-mercaptoethanol, the response to VIP of the final preparation was comparable to that of membranes prepared in the absence of the reducing agent (Fig. 2B). When the tissue was homogenized in the presence of 5 mM β-mercaptoethanol and the first pellet further purified in the absence of the reducing agent, the characteristics of the resulting membranes were comparable to those of membranes prepared in the constant presence of 5 mM β-mercaptoethanol (Fig. 2B).

3. Effects of dithiothreitol on adenylate cyclase activity in rat pancreatic plasma membranes prepared with or without β-mercaptoethanol

Membranes prepared in the absence (control membranes: Fig. 3) or presence (Fig. 4) of 5 mM β-mercaptoethanol were preincubated for 10 min with various dithiothreitol concentrations, as described in Materials and Methods. On control membranes (Fig. 3), a pretreatment with 1 or 2 mM dithiothreitol induced a slight decrease in maximal adenylate cyclase activities and 3- to 10-fold shifts to the right of secretin and VIP dose-effect curves. Dithiothreitol when used at 5 and 10 mM concentrations, provoked further decreases in the efficacy and potency of both peptides so that the general shape of dose-effect curves was markedly altered.

On membranes prepared in the presence of 5 mM β-mercaptoethanol, dithiothreitol, when used at 1 or 2 mM concentrations, shifted the dose-effect curve of secretin, but not that of VIP, to the right (Fig. 4). At 5 and 10 mM concentrations, dithiothreitol increased the threshold dose of

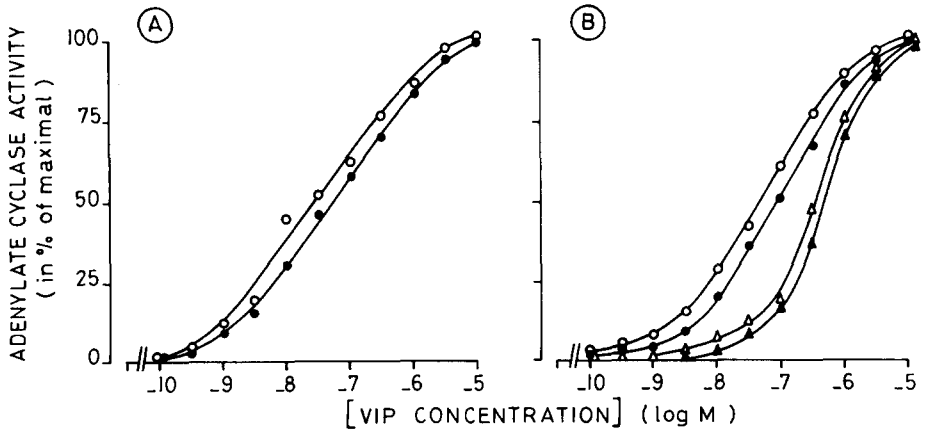

Fig. 2. Dose-response curves of adenylate cyclase activition by VIP in rat pancreatic plasma membranes. (A) Membrane were prepared in absence (○) or presence (●) of 5 mM β-mercaptoethanol. (B) Membranes were prepared without β-mercaptoethanol (○) or with 5 mM β-mercaptoethanol during the whole preparation procedure (▲), during the homogenization procedure only (△), or after collecting the first pellet (●). The results, expressed in % of activity in the presence of 10 μM VIP, were representative of three experiments.

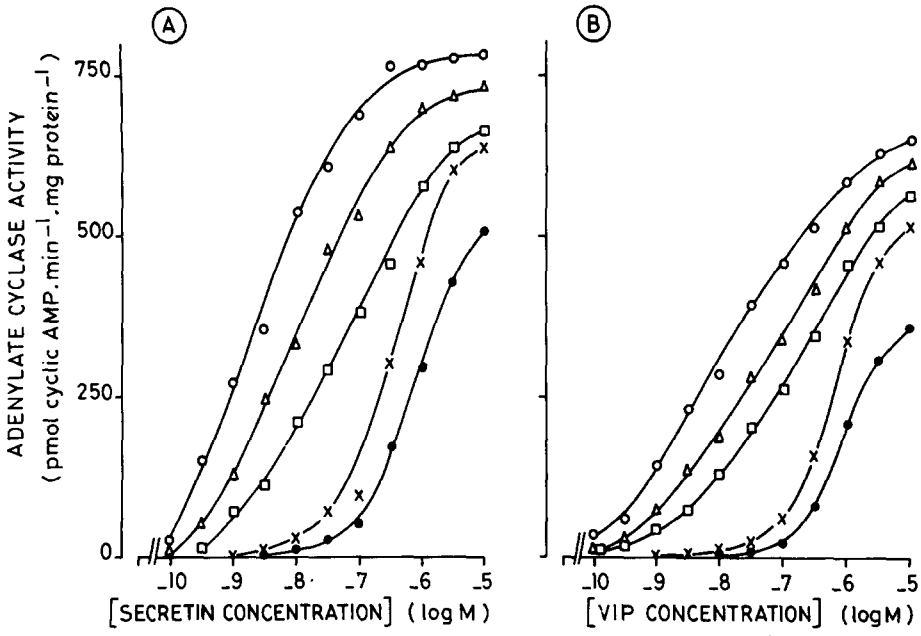

Fig. 3. Dose-response curves of adenylate cyclase activation by secretin (A) and VIP (B) of rat pancreatic plasma membranes prepared in the absence of β-mercaptoethanol and preincubated in the absence (○) or presence of 1 mM (△), 2 mM (□), 5 mM (×), and 10 mM (●) dithiothreitol. The results are presented in pmol cyclic AMP produced per min per mg protein over basal value. Mean of three experiments performed in duplicate.

secretin 300- to 1000- fold (from 0.3 nM to 100 and 300 nM, respectively), dithiothreitol also reduced the efficacy of VIP (without modifying the VIP concentration required for half-maximal enzyme stimulation).

In both control membranes and membranes

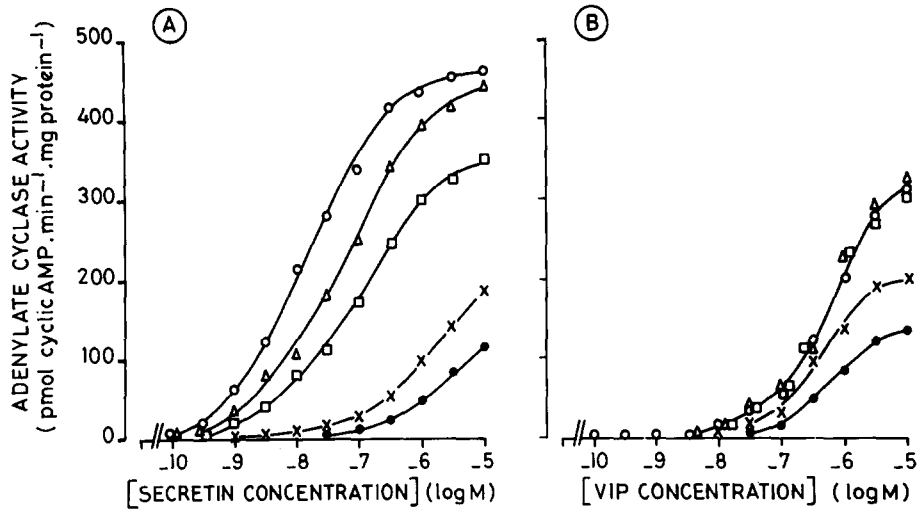

Fig. 4. Dose-response curves of adenylate cyclase activation by secretin (A) and VIP (B) in rat pancreatic plasma membranes prepared in the presence of 5 mM β-mercaptoethanol and preincubated in the absence (○) or presence of 1 mM (△), 2 mM (□), 5 mM (×), and 10 mM (●) dithiothreitol. The results are presented in pmol cyclic AMP produced per min per mg protein over the basal value. Mean of three experiments performed in duplicate.

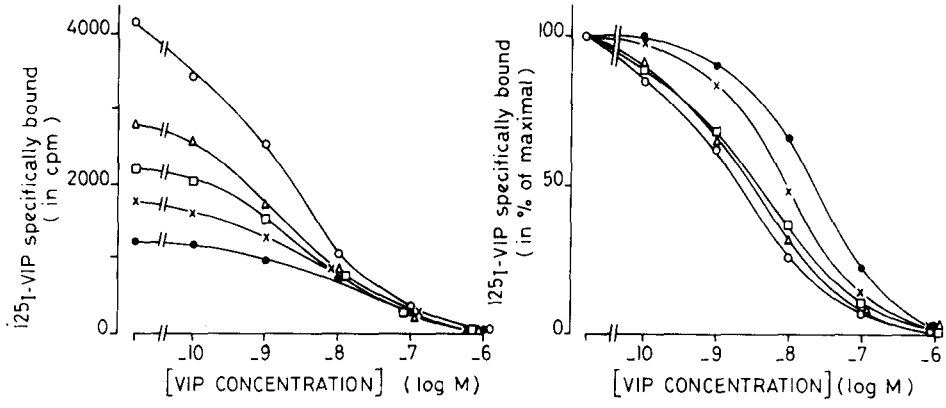

Fig. 5. Inhibition, by increasing concentrations of unlabelled VIP, of ^{125}I-labelled VIP (^{125}I-VIP) binding to rat pancreatic membranes prepared in absence of β-mercaptoethanol and pretreated without (○) or with 1 mM (△), 2 mM (□), 5 mM (×) and 10 mM (●) DTT. The data were expressed as the amount of tracer specifically bound (left panel) and in percent of tracer bound in absence of unlabelled VIP (right panel). Mean of four experiments performed in duplicate.

prepared with β-mercaptoethanol, the responses of adenylate cyclase to p[NH]ppG, NaF and CCK-8 were unaffected by a pretreatment with as much as 10 mM dithiothreitol (data not shown).

4. Effects of dithiothreitol on ^{125}I-labelled VIP binding on rat pancreatic plasma membranes prepared without β-mercaptoethanol

Membranes prepared in the absence of β-mercaptoethanol were preincubated for 10 min with various dithiothreitol concentrations. Specific tracer binding was reduced by 30%, 45%, 55%, and 70% with, respectively, 1.0, 2.0, 5.0 and 10 mM dithiothreitol (Fig. 5, left panel). These pretreatments were without effect on non specific binding, and on the tracer stability (the trichloroacetic acid precipitability being 85% after 15 min incubation at 37°C under all experimental conditions). The ability of unlabelled VIP to compete with ^{125}I-labelled VIP was not significantly modified after preincubation with 1.0 and 2.0 mM dithiothreitol but decreased after exposure to 5.0 and 10.0 mM dithiothreitol. When 10.0 mM dithiothreitol was used, a 10-fold higher concentration of unlabelled VIP was required for competition with ^{125}I-labelled VIP binding (Fig. 5, right panel).

Discussion

Our original technique for preparing rat pancreatic plasma membranes was basically developed to study the properties of adenylate cyclase [1]. The addition of a reducing agent was justified by the fact that all types of membranous adenylate cyclase are extremely sensitive to the oxidation of -SH groups [9]. The choice of β-mercaptoethanol as a reducing agent was determined by its efficacy in protecting adenylate cyclase, and its limited deleterious effects on β-adrenergic receptors (despite the high sensitivity of these receptors to the reduction of essential S-S bonds by dithiothreitol: Ref. 10).

The present results demonstrated, however, that β-mercaptoethanol and dithiothreitol, two reagents commonly used for the reductive cleavage of disulfide bounds [11,12], selectively altered VIP- and secretin stimulations of adenylate cyclase in rat pancreatic plasma membranes, without affecting basal-, as well as p[NH]ppG-, NaF-, and CCK-8- stimulated activities. The preservation of the responses to the hormone CCK-8 and to agents acting on the guanine nucleotide regulatory site strongly suggests that the decreased responses to VIP and secretin were related to a change in the number and/or affinity of VIP and secretin binding sites, and neither to a general membrane perturbation, nor to an alteration of the catalytical subunit, and/or to the inactivation of the ATP-regenerating system.

This hypothesis is further supported by our data on the effects of dithiothreitol preincubation on ^{125}I-labelled VIP binding. Used at low con-

centration (1 and 2 mM), dithiothreitol reduced the apparent number of VIP receptors as the amount of tracer bound decreased with no alteration in the apparent affinity of VIP receptors for the ligand. When used at higher concentrations (5 and 10 mM), dithiothreitol reduced the apparent affinity of VIP receptors. A comparison of binding data with our results on adenylate cyclase activation remains, however, difficult since: (a) ^{125}I-labelled VIP binding and VIP-stimulated adenylate cyclase could not be studied under the same experimental conditions as the GTP concentration required for adenylate cyclase activation dramatically reduces the binding of the tracer to pancreatic membranes, an observation similar to that previously made on membranes from liver [13], lung [14], and intestinal epithelial cells [15], and (b) only the occupancy of high-affinity VIP receptors was explored accurately by ^{125}I-labelled VIP binding. The present data demonstrated, nevertheless, that S-S bonds play an essential role in the recognition site of VIP receptors and, although secretin receptors were not directly studied, it is reasonable to interpret similarly our data concerning the secretin stimulation of adenylate cyclase.

VIP and secretin receptors showed distinct susceptibilities towards dithiothreitol and β-mercaptoethanol: both classes of receptors were extremely sensitive towards dithiothreitol whereas only VIP receptors were altered when β-mercaptoethanol was brought into the homogeneization medium, i.e., when solely the external surface of plasma membranes was exposed during the brief period preceding cell disruption. It is not clear where these disulfide bonds are located but it is tempting to suggest that readily accessible S-S bounds play an essential role in high-affinity VIP receptors, their notable sensitivity further supporting the view that VIP and secretin receptors represent distinct entities in rat pancreatic acini [3,16].

It also appears that the same S-S bonds are accessible to a mono- and a bifunctional reagent, as the VIP response of adenylate cyclase was no longer affected by low dithiothreitol concentrations in membranes prepared in the presence of β-mercaptoethanol. Dithiothreitol was, however, more efficient than β-mercaptoethanol in altering receptors, in line with the respective redox potentials of the two agents [11,12], and in agreement with a similar observation made on β-adrenergic receptors [10].

The present data are the first demonstration of the capacity of reducing agents to alter essential disulfide bonds in VIP and secretin receptors. Similar data on α-adrenergic, β-adrenergic and dopaminergic [10.17,18,19], acetylcholine [20], thyrotropin [21], gonadotropin [22], growth hormone [23] and insulin [24,25] receptors have previously been obtained.

The mechanism of action of S-S bonds in or near VIP and secretin receptors is not evident. Schultz and Milutinović [26] have documented, on cat pancreas, the effects of the alkylating agent *p*-chloromercuribenzoate on secretin receptors, secretin-stimulated adenylate cyclase, and secretin-induced fluid secretion: *p*-chloromercuribenzoate stimulates the secretin-activated parameters at low concentration while inhibiting secretin binding as well as secretin stimulation of adenylate cyclase at higher concentration. The model proposed by these authors opposes superficial (readily accessible) SH-groups and buried SH-groups, all in close contact with secretin receptors. S-S groups, between buried and superficiel protein segments, may then play a considerable role: when *p*-chloromercuribenzoate titrates superficial SH-groups at low concentration, deeper SH-groups may participate in sulfhydryl-disulfide exchange mechanisms thereby enhancing the biological response to secretin. This interpretation, if valid for the effects of an alkylating agent, may be transposed to our results showing reduced hormone-responsiveness after disruption of disulfide bonds. In line with this hypothesis, reducing agents impair the insulin-stimulated transport of amino acids and glucose [27] while the alkylating agent *p*-chloromercuribenzoate stimulates these transports at low concentration and exerts opposite effects at higher concentration [28].

Acknowledgements

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