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THE INTERACTION OF SECRETIN WITH PANCREATIC MEMBRANES

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SUMMARY

1. ^{125}I-labelled secretin bound rapidly and specifically to membranes from cat pancreas. Binding of labelled hormone was competitively inhibited by unlabelled secretin in the same range of concentrations that stimulated pancreatic adenylate cyclase in these membranes. The dissociation constant of the membrane binding sites for unlabelled secretin as evaluated by these displacement experiments was $4.1 \cdot 10^{-9}$ M and the number of binding sites 1.0 pmol per mg of membrane protein.

2. Studies using different concentrations of [^{125}I]secretin (at a constant ratio of labelled to unlabelled hormone) revealed a similar value of $4.4 \cdot 10^{-9}$ M for the dissociation constant.

3. Both the association and dissociation rate constants of [^{125}I]secretin binding were temperature sensitive; the dissociation rate constant increased more rapidly with increase in temperature. The ratio k_{-1}/k_{+1} (at 22 °C) gave a dissociation constant of $3.7 \cdot 10^{-9}$ M which agrees closely with the figure obtained from equilibrium data.

These data indicate that ^{125}I-labelled secretin and unlabelled secretin bind to the same binding site on pancreatic membranes, with high affinity.

4. Unlabelled secretin stimulated pancreatic adenylate cyclase with an apparent K_m of $8.4 \cdot 10^{-9}$ M, while [^{125}I]secretin apparently did not stimulate the adenylate cyclase. Together with the binding data this might suggest that different portions of the secretin molecule are responsible for binding and adenylate cyclase activation.

5. Studies on the specificity of [^{125}I]secretin binding carried out with various peptide hormones (glucagon, human gastrin, pancreozymin and caerulein) which are all inefficient in stimulating pancreatic fluid secretion, showed that these hormones have no influence on the binding of [^{125}I]secretin. In contrast, vasoactive intestinal polypeptide, which stimulates pancreatic fluid and bicarbonate secretion, showed a competitive inhibition of secretin binding to the plasma membrane preparation.

INTRODUCTION

The first step in the induction of a cellular process by peptide hormones appears to be a reversible binding of the hormone with a specific receptor located on the cell

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surface [1]. Although some indirect information about hormone-receptor affinity can be obtained by measuring the physiological response at various hormone concentrations, direct measurements of hormone binding to specific receptors are essential since the final response and the degree of receptor occupancy need not be simply related [2]. Consequently, during the past few years considerable progress has been made in the study and identification of a variety of peptide hormone receptors by means of direct measurement of hormone binding [3–6].

Electrolyte and water secretion of the pancreas is regulated by the peptide hormone secretin [7]. Previous studies have established that secretin increases cyclic AMP formation in pancreatic tissue in vivo [8] and stimulates an adenylate cyclase system present in membrane fractions of the pancreas [9, 10]. A direct interaction of secretin with a target site on pancreatic membranes, however, has not yet been demonstrated.

In the present study, the characteristics of the interaction of secretin with pancreatic membranes have been examined by the use of iodinated synthetic secretin. It has been shown that secretin binding exhibits certain characteristics of peptide hormone-receptor interaction such as specificity, reversibility and high affinity, which are consistent with the biological activity of the ligand.

MATERIALS

Synthetic secretin was a gift from Professor Dr. E. Wünsch (Max-Planck-Institut für Biochemie, Martinsried b. München, G.F.R.). The synthetic secretin was iodinated with ^{125}I or ^{127}I by a method described elsewhere [11]. Briefly, chloramine T was used to start the iodination of secretin in the presence of $Na^{125}I$. The reaction was terminated 30 s later by the addition of $Na_2S_2O_5$. Free iodine and degradation products were separated from [^{125}I]secretin by a cellulose powder column. To check if the biological activity of secretin was influenced by the procedure it was submitted to the same procedure in the absence of iodine. The specific activity of the iodinated secretin as determined from the absorbance at 230 nm and the radioactivity of the same batch was 100–200 Ci/g. This value was corrected for each batch by an iterative procedure described by Smigel and Fleischer [12]. It represents an approximate average of 0.3 iodine atom per molecule of secretin.

Porcine vasoactive intestinal polypeptide was a gift from Professor Dr. V. Mutt (Karolinska Institutet, Stockholm, Sweden). Pancreozymin was obtained from the Karolinska Institutet (G.I.H. Research Unit, Stockholm, Sweden), glucagon from Calbiochem (R. Paesel, Frankfurt, G.F.R.), human 2-17-gastrin from Imperial Chemical Industries (Cheshire, U.K.) and caerulein from Farmentici Italia (Milan, Italy). Bacitracin and albumin fraction V were from Sigma (Louisville, U.S.A.), soybean trypsin inhibitor, pyruvate kinase (crystalline suspension 10 mg per ml) and phosphoenolpyruvate (trisodium salt) were from Boehringer Mannheim (Mannheim, G.F.R.). (α-^{32}P)-labelled adenosine 5′-triphosphate (2–10 Ci/mmol, sodium salt) and [8-^{3}H]adenosine 3′, 5′-cyclic phosphate (spec. act. 27 Ci/mmol, ammonium salt) were from The Radiochemical Centre Amersham (Amersham, U.K.). Dowex 50 WX (200–400 mesh, hydrogen form) was from Serva Feinbiochemica (Heidelberg, G.F.R.). Filters (EHWPO, cellulose acetate, pore size 0.5 μm) were obtained from Millipore Corporation (Bedford, Mass., U.S.A.).

METHODS

Preparations of tissue

A male or female cat was anaesthetized by Nembutal (60 mg/kg body weight intraperitoneal) and exsanguinated by ventricular incision. The pancreas was removed, freed of fat and connective tissue, washed and placed into ice cold solution containing Tris · Cl buffer 20 mM pH 7.4, $MgCl_2$ 2.5 mM, EDTA 2.5 mM, Bacitracin 0.1 mg/ml and soybean trypsin inhibitor 0.5 mg/ml. The tissue was then cut into small pieces with scissors, weighed and homogenized in 4 vol. of the same solution, using a loose-fitting, hand-operated, teflon glass homogenizer. The homogenate was filtered through one layer of gauze and spun at $600 \times g$ for 10 min to remove nuclei and large debris. The supernatant was spun at $4000 \times g$ for 10 min and the pellet washed three times with the same solution by alternate homogenization and centrifugation. The final pellet was resuspended to a protein concentration of about 10 mg/ml in 20 mM Tris · Cl pH 7.4 containing 0.5 mg per ml soybean trypsin inhibitor. This preparation is enriched for secretin-stimulated adenylate cyclase activity by a factor of 4. It is refered to as "pancreatic membranes".

Binding assay of ^{125}I-labelled secretin to pancreatic membranes

The standard binding assay consisted of incubating membranes (150–300 μg of protein) at 22 °C for 30 min in 0.5 ml of 20 mM Tris · Cl/1 % albumin buffer pH 7.4 containing 0.1 mg trypsin inhibitor, 0.05 mg Bacitracin, [^{125}I]secretin ($0.1–0.5 \cdot 10^{-10}$ M) and, when necessary, different amounts ($0.5 \cdot 10^{-9}$ M–10^{-6} M) of unlabelled secretin. Reaction was started by adding membranes to the preequilibrated assay mixture. After incubation, the mixture was filtered without dilution through a millipore filter (EHWPO, pore size 0.5 μm), the filter was washed within 15–20 s with 10 ml of ice-cold 20 mM Tris · Cl pH 7.4 and transferred to a plastic vial for counting. Various millipore filter types were checked (BD, NR, WH, HA, EH) and of these tested, only the EH type, consisting of pure cellulose acetate, was satisfactory in having an adequately low blank (2 % retention of the radioactivity present in the sample in the absence of membranes). Radioactivity was determined in a γ-counter (Packard). The term "unspecific" binding was introduced by assuming that the general equation for any binding process $B = (N \times f \times K)/(1 + K \times f)$ will approximate $B = K \times N \times f$ if K is small or N is large [13]. B is the concentration of bound ligand at free ligand concentration f. K is the association constant of the ligand-receptor complex and N is the concentration of the receptor. Sites which can be characterized by this equation appear to be unsaturable and therefore will not exhibit competition with analogues. Corrections were made for unspecific binding of [^{125}I]secretin to membranes and to the filters by performing parallel incubations in which excess native secretin ($3 \cdot 10^{-6}$ M) was added to the membranes together with iodinated secretin. Under our standard conditions the unspecific binding represented 10–20 % of the total binding and could not be displaced by further addition of unlabelled hormone. "Bound" and "free" secretin levels were calculated in the following way. The counts per min obtained by filtration of the incubation mixture were reduced by the value obtained for "unspecific" binding. This corrected value was used to calculate the "bound" secretin (B) per sample. "Free" (f) secretin was obtained with the expression $f = T - b$ (b = "bound" secretin uncorrected for non-specific binding), where T is the total amount

of secretin present in the sample [13]. The ^{125}I values given in the text and figures comprise one third ^{125}I-labelled and two thirds unlabelled native secretin. Other details of the incubation procedures are specified in the legends to the figures and tables.

Adenylate cyclase assay

The composition of the incubation mixture for measuring adenylate cyclase activity was as follows: 0.8 mM [α-^{32}P]ATP ($1-2 \cdot 10^6$ cpm per 100 μl), 5 mM $MgCl_2$, 42 mM Tris · Cl, pH 7.4, 10 mM theophylline, 80–120 μg of membrane protein, with an ATP-regenerating system consisting of 5 mM phosphoenolpyruvate and 0.2 mg pyruvate kinase per ml. The final volume was 0.1 ml. Incubations were carried out at 30 °C for 10 min in the presence and absence of secretin. The reaction was stopped by placing the tubes for 2 min in a boiling water bath, whereafter 400 μl of a solution containing cyclic[^{3}H]AMP (0.5 mg per ml, 50 000 cpm per mg) were added. The tubes were centrifuged and the supernatant fluid was chromatographed on Dowex 50 WX (200–400 mesh, hydrogen form) columns (0.5×3.0 cm) as described by Krishna et al. [14]. The radioactivity was determined in a liquid scintillation spectrometer (Packard tricarb) using Bray's scintillation solution. Incubations without enzyme or with heat-denaturated enzyme were run as blanks; these blanks represented only 0.003 % of the radioactivity initially present. Enzyme activity was expressed as pmol cyclic AMP formed per mg protein per min. The hormone-stimulated activity was calculated by deducting the basal activity (15 pmol per min per mg) from the total activity observed in the presence of hormone.

Determination of protein

Membrane protein was determined by the method of Lowry et al. [15] after precipitation of the protein by 10 % trichloroacetic acid in the cold and dissolution of the precipitate in 1 M NaOH.

RESULTS

Time course and temperature dependence of binding of [^{125}I]secretin

The binding reaction was rapid, attaining a constant level of bound secretin within 10 min of incubation at 22 °C (Fig. 1). Binding of [^{125}I]secretin to pancreatic membranes was temperature dependent. At 15 and 22 °C the binding followed a similar time course. However, at 0 °C the rate of binding was slower and the maximal level of the hormone bound was lower than at higher temperatures. The constant level of bound hormone was maintained for at least 45–50 min in all cases. For subsequent studies described below, incubation times were chosen so that the bound levels had reached a stable equilibrium value.

Binding of [^{125}I]secretin as a function of membrane protein concentration and pH of medium

Binding of [^{125}I]secretin to pancreas membranes was a linear function of the concentration of membrane protein up to a protein concentration of about 0.6 mg per ml in the hormone concentrations range from 0.1 to $1.1 \cdot 10^{-10}$ M. A maximum of about 30 % of the total labelled hormone present in an incubation vial bound at the

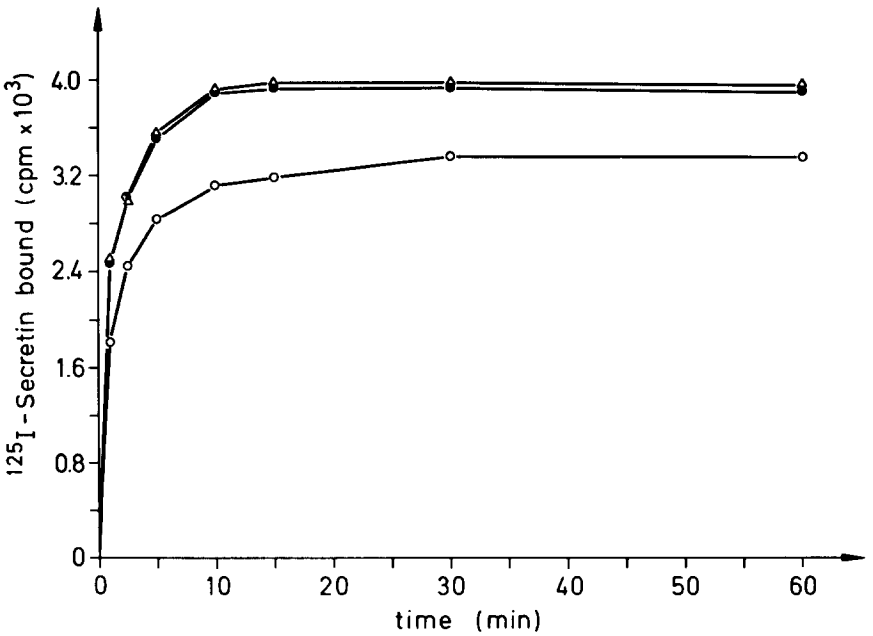

Fig. 1. Time dependence of specific binding of ^{125}I-labelled secretin to pancreas plasma membranes at various temperatures. ^{125}I-labelled secretin ($2.14 \cdot 10^{-10}$ M) was incubated with pancreas plasma membranes (480 μg of protein per ml) at 0 °C (○), 15 °C (●) or 22 °C (△) in 2.5 ml of 20 mM tris Cl buffer pH 7.4 containing 1 % (w/v) albumin, 0.1 mg/ml Bacitracin and 0.2 mg/ml soybean trypsin inhibitor. At indicated times specific binding was determined on 300 μl aliquots as described in the text. A duplicate tube containing excess cold secretin (10^{-6} M) was run at 3 temperatures and correction for nonspecific binding was made for each time. Points represent mean of 3 experiments.

highest concentration of membrane protein tested (0.8 mg/ml). Nonspecific binding, i.e. binding of the labelled secretin in the presence of excess of unlabelled secretin which occupies all specific binding sites was directly proportional to the membrane protein concentration throughout the whole range of protein concentrations used. Nonspecific binding represented 20 % of the total binding. All further binding studies

TABLE I

EFFECT OF pH OF THE MEDIUM ON SPECIFIC BINDING OF [^{125}I]SECRETIN

The results are expressed as percentage of specific binding obtained at pH 7.4. The buffers used were Tris · Cl 40 mM for pH 7.0–8.5 and phosphate buffer 40 mM for pH 6.0–7.0. Results are means of three experiments made in duplicate, with S.D.

pH	% bound [^{125}I]secretin
6.0	35 ± 5
6.5	72 ± 2
7.0	95 ± 3
7.4	100 ± 3
8.0	93 ± 4
8.5	34 ± 2

were performed at protein concentrations which were in the range of the linear part of the curve. The pH optimum for binding was between pH 7.0 and 8.0 with a decrease in binding on either side of this relatively broad range (Table I). Change in buffer composition, i.e. phosphate instead of Tris buffer, did not influence secretin binding.

Binding of [^{125}I]secretin as a function of its concentration

Binding of secretin to the pancreatic membranes performed, using different concentrations of hormone at constant ratio of labelled to unlabelled secretin is shown in Fig. 2. The dissociation constant derived from these data was $4.4 \cdot 10^{-9}$ M.

Competitive inhibition of [^{125}I]secretin binding by unlabelled secretin

Unlabelled secretin, when added simultaneously with ^{125}I-labelled secretin to the membranes, reduced tracer binding in a dose-dependent fashion, indicating that the same sites are involved in the binding of either species (Fig. 3). Small quantities of unlabelled hormone ($5 \cdot 10^{-10}$ M) readily inhibited tracer binding, and relatively little further displacement was obtained by increasing the concentration to above $6 \cdot 10^{-9}$ M. At secretin concentrations higher than $3 \cdot 10^{-8}$ M, further displacement could not be observed. The nonspecific binding represented 10–20 % of the specific

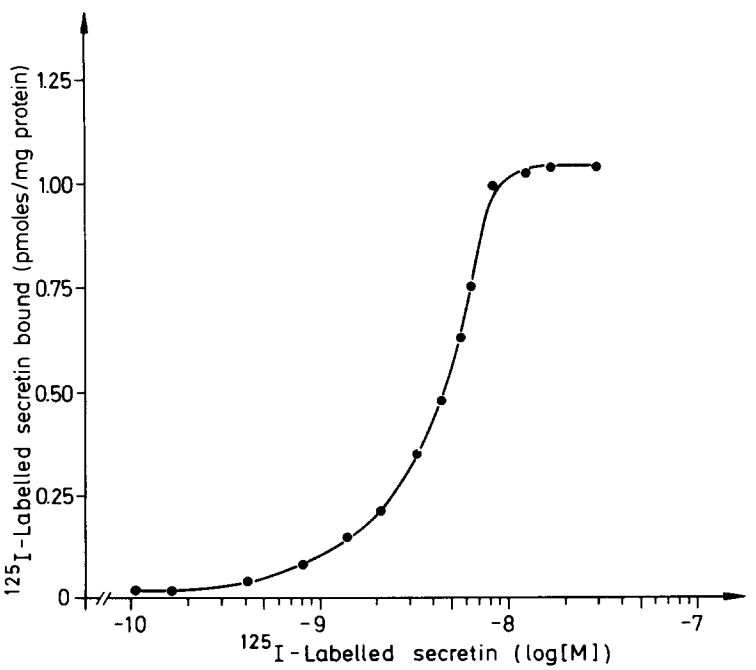

Fig. 2. Binding of ^{125}I-labelled secretin as a function of the concentration of labelled hormone. Incubations were carried out for 20 min at 22 °C in final volume 0.5 of medium containing 160 μg of membrane protein, 1 % albumin, 20 mM Tris · Cl pH 7.4, 0.1 mg soybean trypsin inhibitor, 0.05 mg Bacitracin and different concentrations of labelled secretin (10^5 cpm/pmol). Points represent mean of 3 experiments. The [^{125}I]secretin values on ordinate and abscissa represent labelled to unlabelled secretin at the ratio of 1:3990.

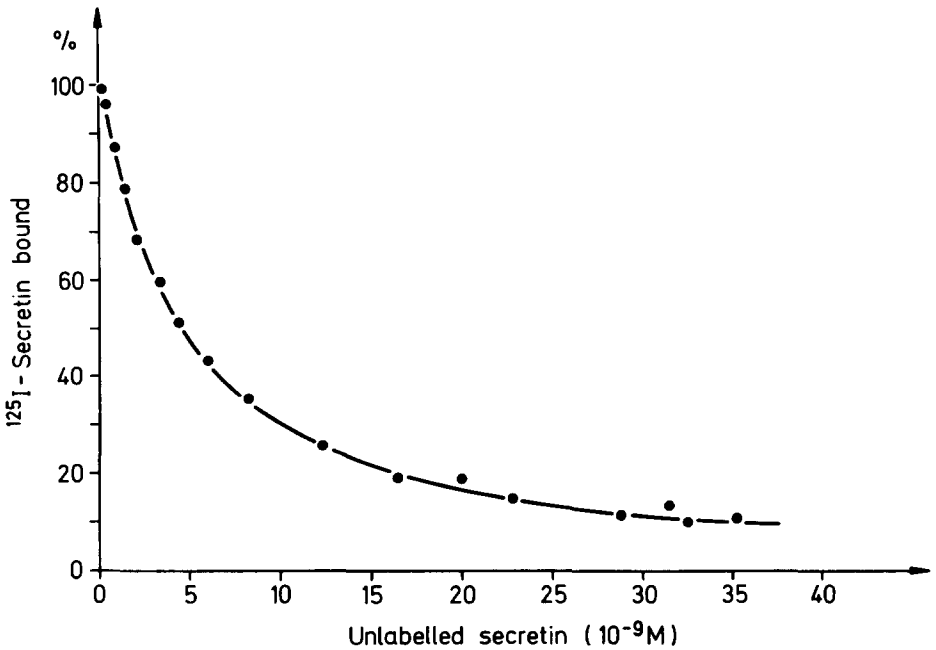

Fig. 3. Competitive inhibition of [^{125}I]secretin binding by unlabelled native secretin. Pancreas plasma membranes (300 μg of protein per ml) were incubated at 22 °C for 20 min in a final volume of 0.5 ml of the standard incubation medium containing labelled secretin ($11 \cdot 10^{-11}$ M) and the indicated concentrations of unlabelled secretin. Separation of membrane-bound from free secretin was carried out as described under "Methods". Points are mean of triplicate determination. In this and the next figure the term ^{125}I refers strictly to the labelled molecules.

binding depending on the batch of the tracer used. A discrimination between labelled and unlabelled secretin was made by plotting the data from Fig. 3 according to the equation (ref. 16).

$$\frac{1}{i} = 1 + \frac{K_i}{(I)}\left(1 + \frac{(f)}{K}\right).$$

The dissociation constant K_i derived from this plot was $4.8 \cdot 10^{-9}$ M, a value similar to $4.4 \cdot 10^{-9}$ M which was obtained by the experiment described in Fig. 2. A Scatchard plot derived from the same data (Fig. 3) corrected for non-specific binding is presented in Fig. 4. Between $5 \cdot 10^{-10}$ M and $3 \cdot 10^{-8}$ M of hormone concentration a linear relationship was obtained indicating the homogeneity of binding sites. From this plot the dissociation constant was estimated to be $4.1 \cdot 10^{-9}$ M which is comparable to those from Fig. 2 and from the above mentioned plot. The number of binding sites was calculated to be 1.0 pmol per mg membrane protein (Table II).

Specificity of secretin binding

Several peptide hormones were examined for their ability to compete with secretin for binding to pancreatic plasma membranes. Glucagon (10^{-6} M), pancreozymin ($0.5 \cdot 10^{-6}$ M), caerulein (10^{-5} M) and gastrin (10^{-5} M) caused virtually no

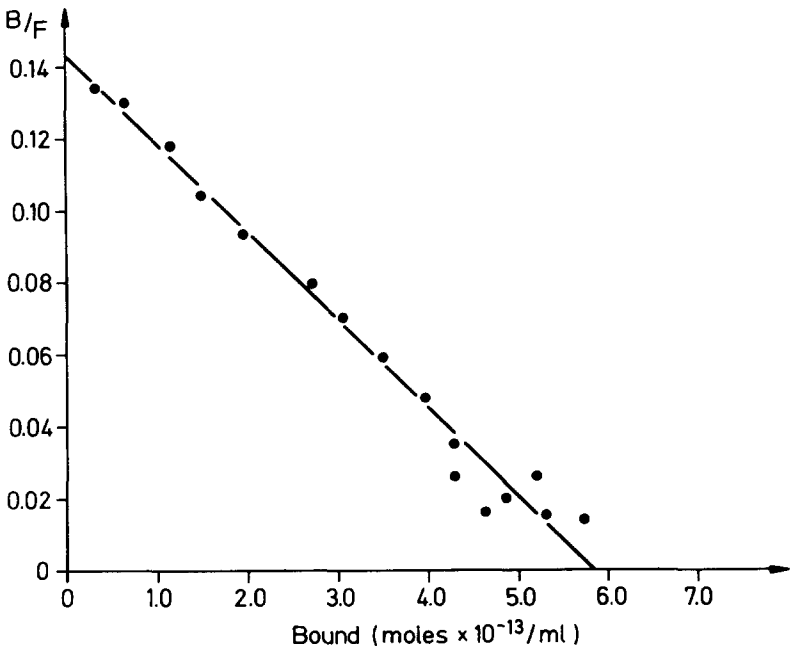

Fig. 4. Scatchard plot of the data of Fig. 3. The abscissa and ordinate value represent total (labelled + unlabelled) secretin. Since under equilibrium conditions the ratio labelled: total secretin is the same allover, the ratios of labelled secretin which could easily be evaluated at each concentration of unlabelled secretin correspond to the ratio of total secretin.

TABLE II

KINETIC CONSTANTS OF SECRETIN BINDING AND OF SECRETIN-STIMULATED ADENYLATE CYCLASE

The values are obtained as follows: K_d and number of binding sites from 8 experiments similar to that described in Fig. 3; ratio k_{-1}/k_{+1} from experiments presented in Figs. 1 and 6, respectively. Apparent K_m for adenylate cyclase stimulation was derived from a Lineweaver-Burk plot (plot not shown) of 10 experiments described under Fig. 7. The values presented are means ± S.D.

K_d		Number of binding sites (pmol/mg protein)
From equilibrium data:	$4.1 \pm 0.5 \cdot 10^{-9}$ M	1.0 ± 0.1
From k_{-1}/k_{+1} ratio:	$3.70 \cdot 10^{-9}$ M	
Apparent K_m for adenylate cyclase activation		V (pmol/mg protein per min)
Native secretin	$8.41 \pm 0.89 \cdot 10^{-9}$ M	31.81 ± 2.85
Partially iodinated secretin	$11.10 \pm 3.07 \cdot 10^{-9}$ M	32.05 ± 3.43

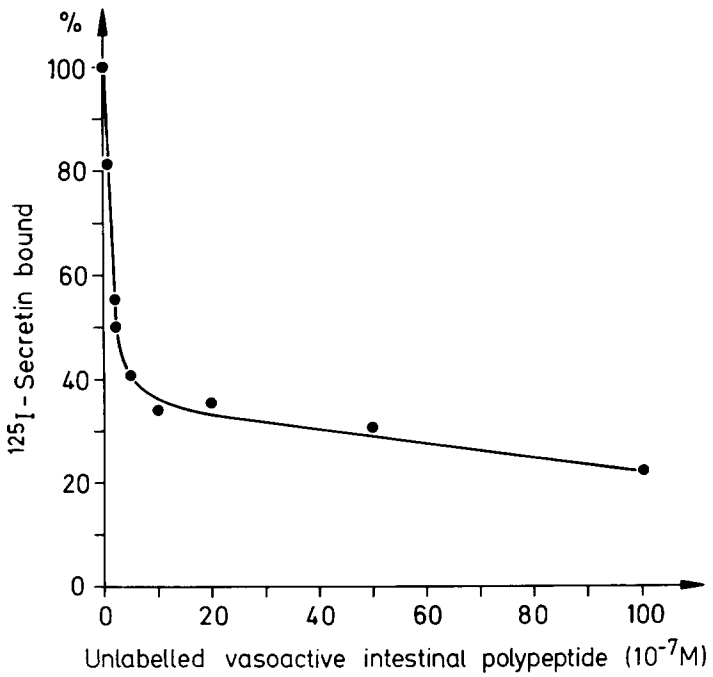

Fig. 5. Inhibition of binding of ^{125}I-labelled secretin by vasoactive intestinal polypeptide. ^{125}I-labelled secretin ($1.1 \cdot 10^{-10}$ M) which comprises two thirds of the unlabelled native secretin was incubated with pancreatic plasma membranes (300 μg of protein per ml) and varying concentrations of native vasoactive intestinal polypeptide in 0.5 ml 20 mM Tris · Cl pH 7.4, containing 1 % (w/v) albumin, 1 mg/ml Bacitracin and 0.2 mg/ml soybean trypsin inhibitor. After 20 min at 22 C, specific binding was determined by filtration procedures as described in the text. Mean values of triplicate determinations are shown.

inhibition of iodinated secretin binding even at high molar excess. It is of special interest that vasoactive intestinal polypeptide, a hormone closely similar to secretin in amino acid sequence [17] which, like secretin, stimulates pancreatic fluid and bicarbonate secretion, can compete with secretin for binding to the membranes (Fig. 5). The concentration of vasoactive intestinal polypeptide giving half-maximal inhibition of tracer binding is $4 \cdot 10^{-7}$ M, and the lowest concentration still giving measurable tracer displacement is 10^{-7} M. The affinity of vasoactive intestinal polypeptide for secretin binding sites is estimated as 100 times lower than that of secretin itself.

Dissociation of [^{125}I]secretin from pancreatic membranes

Binding of iodinated secretin to pancreatic membranes is reversible: once formed the [^{125}I]secretin membrane complex can dissociate upon addition of excess unlabelled hormone (Fig. 6). At 37 °C, dissociation is a first-order process with a half time of 1.5 min. Reducing the incubation temperature from 37 to 30, 22, 15 and 0 °C produces progressive slowing of the first-order dissociation process (Fig. 6, Table III). At these lower temperatures other changes in the kinetics of dissociation of the membrane-hormone complex became apparent. At 0 °C whilst the initial 60 % of

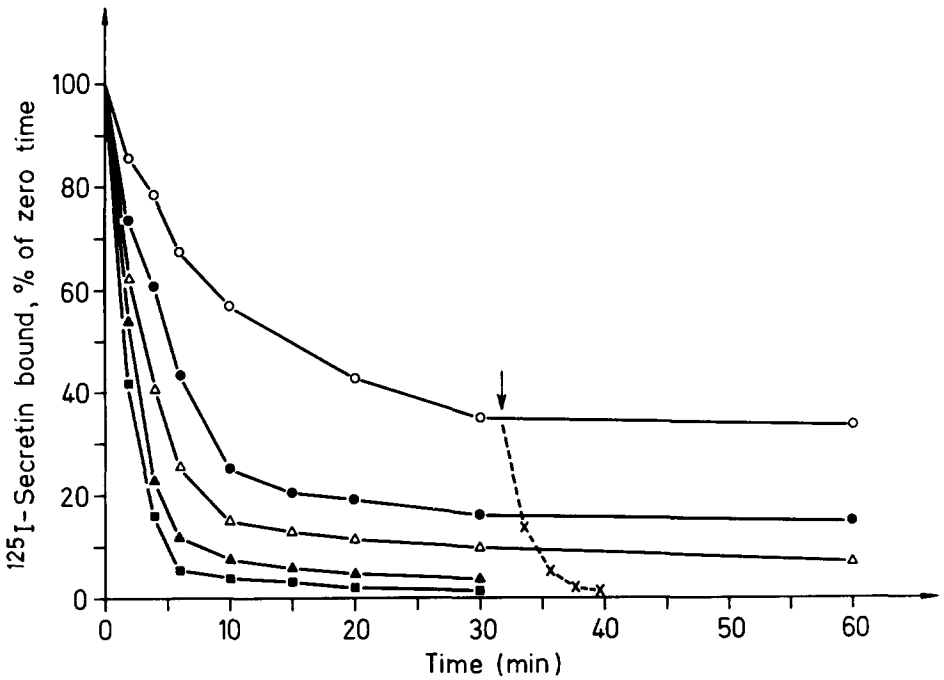

Fig. 6. Time dependence of the dissociation of ^{125}I-labelled secretin bound to pancreatic membranes at various temperatures. ^{125}I-labelled secretin ($5 \cdot 10^{-10}$ M) which comprises two thirds of the unlabelled native secretin was incubated with membranes (700 μg of protein per ml) at 22 °C in 4 ml of Tris · Cl buffer 20 mM pH 7.4, containing 1 % (w/v) albumin, 0.1 mg/ml Bacitracin and 0.4 mg/ml soybean trypsin inhibitor. After 20 min (zero time) 600 μl aliquots of the mixture were transferred to tubes pre-equilibrated at 0, 15, 22, 30 and 37 °C respectively, containing 20 mM Tris · Cl buffer pH 7.4, 1 % albumin, 2.4 mg trypsin inhibitor, 1.2 mg Bacitracin and excess of cold secretin (2 μM in 12 ml) and the samples were incubated further at 0 °C (○), 15 °C (●), 22 °C (△), 30 °C (▲) and 37 °C (■). At indicated times between immediately upon mixing (about 15 s) and 60 min the samples (300 μl) were rapidly filtered to determine specific binding of secretin to membranes. At the time indicated by the arrow an aliquot was taken from incubation at 0 °C and transferred to a 10 times greater volume of incubation mixture pre-equilibrated at 37 °C and incubation proceeded further on 37 °C. Results are expressed as percentage of secretin bound to membranes at zero time (0.15 pmol/mg protein). Each time point was corrected for nonspecific binding and represents mean of 5 determinations.

specifically bound hormone dissociates following first-order kinetics over a period of 6 min, the remaining 40 % dissociates very slowly with a half-time of about 8 h. At 22 °C the slowly dissociating fraction represents 10 % of the total binding (i.e. less than the nonspecific binding) and was not detectable at higher temperatures. If the slowly dissociating complex at 0 °C is rapidly brought to 37 °C (Fig. 6, arrow) there is an immediate dissociation of the membrane-hormone complex exhibiting the first-order kinetics expected for dissociation at 37 °C. This suggests that the slow dissociation of the membrane-hormone complex at low temperature cannot be explained by an irreversible damage of the system.

TABLE III

KINETIC CONSTANTS OF SECRETIN BINDING AT DIFFERENT TEMPERATURES

The rate of association k_{+1} was determined from the amount of ^{125}I-labelled secretin interacting with the membranes at varying times; the experimental values were substituted in a second-order equation as described previously [18]. Mean of 3 experiments ±S.D. is shown. At temperatures higher than 22 °C, the binding reaction was too quick to be accurately measured. The rate of dissociation k_{-1} was determined from a semi-logarithmic plot of the dissociation of labelled secretin bound to membranes as a function of time. The indicated values are means ±S.D. obtained from determinations made within the first 10 min ($n = 5$). The dissociation constant K_d was calculated from the ratio k_{-1}/k_{+1}.

Temp. (°C)	$k_{-1}(s^{-1}) \times 10^3$	$k_{+1}(M^{-1} \cdot s^{-1}) \times 10^6$	$K_d(M) \times 10^{-9}$
0	0.375 ± 0.023	0.261 ± 0.029	1.44
15	0.803 ± 0.055	0.361 ± 0.040	2.22
22	1.410 ± 0.170	0.381 ± 0.036	3.70
30	2.640 ± 0.190		
37	3.090 ± 0.280		

Relative dependence of binding and stimulation of adenylate cyclase on hormone concentration

The biological activity of partially iodinated secretin was compared with that of the unlabelled hormone in the adenylate cyclase assay (Fig. 7). As judged from

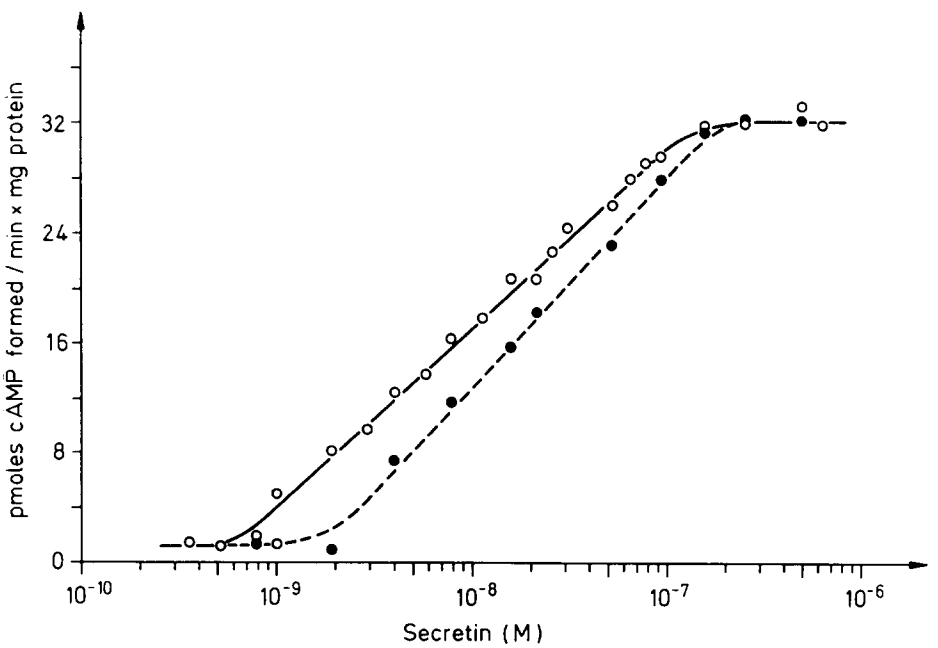

Fig. 7. Stimulation of adenylate cyclase activity at different concentrations of native and iodinated secretin. Pancreatic plasma membranes (80–140 μg) were incubated for 10 min at 30 °C in 0.1 ml of medium containing 0.8 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 42 mM Tris · Cl buffer pH 7.4, an ATP-regenerating system consisting of 5 mM phosphoenolpyruvate and 0.2 mg per ml pyruvate kinase, and native (○) or iodinated (●) secretin at the indicated concentrations. Mean values of 10 experiments are shown.

TABLE IV

THERMODYNAMIC PARAMETERS OF SECRETIN BINDING

$1H$ is obtained from the plot of equilibrium constant as a function of temperature on the base of the results presented in Table III. Mean values and for $1H$ and $1G$ S.D. values are given.

$1H$	8.05 ± 0.73	kcal/mol
$1G$ (295 °K)	−11.17 ± 1.40	kcal/mol
$1S$	10.57	cal/mol degree

results obtained in 10 experiments, partially iodinated secretin (about 0.33 atoms of iodine per molecule) shows an increase in the apparent K_m for adenylate cyclase stimulation by about 32 % compared with unlabelled hormone (Table II). It seems, therefore, that iodination of the N-terminal histidine does not preserve biological activity of the hormone.

Kinetic constants of secretin-membrane interaction

Kinetic constants and thermodynamic parameters of the secretin-membrane interaction derived from the data presented are summarized in Tables III and IV. Calculation of association and dissociation rate constants at different temperatures shows that the dissociation rate constant increases considerably with temperature, at a greater rate than the forward reaction rate constant (Table III). Hence the dissociation constant based on k_{-1}/k_{+1} ratio increases with increasing temperature. Dissociation constant obtained from equilibrium experiments at 22 °C ($4.1 \cdot 10^{-9}$ M) is in agreement with the k_{-1}/k_{+1} ratio for the same temperature ($3.7 \cdot 10^{-9}$ M).

From the dependence of the equilibrium constant on temperature the ΔH is about −8.1 kcal/mol and calculation of ΔF (295 °K) gives a value of −11.2 kcal/mol, so that the free energy term is predominantly controlled by changes in enthalpy.

DISCUSSION

An iodinated derivative of synthetic secretin has been used to study the interaction of this hormone with its target membranes. Lacking tyrosine residues the hormone can be iodinated only on the histidine residue in position 1 [18]. It has been established [19] that the N-terminal regions of the molecule are indeed critical for the biological properties of secretin since it was shown that peptide lacking histidine in position 1 (secretin 2-27) is practically inactive. In this respect it could be expected that incorporation of iodine into the biologically important histidine 1 may affect biological activity of the hormone. The apparent K_m for adenyl cyclase activation by iodinated secretin is 32 % higher than that of the native hormone (Table II). Since only 1 in 3 molecules were iodinated and since Chloramine T treatment alone under these conditions does not affect the biological activity of secretin (unpublished observation), we may conclude that iodination of histidine 1 destroys the biological activity of the hormone. In spite of this loss of biological activity, iodinated secretin exhibits a number of characteristics suggesting that its binding retains some features relevant to native hormone action. To estimate the effect of iodination on binding properties of secretin, we tested 100 % labelled ^{127}I-iodinated secretin (Fahrenholz

et al., manuscript in preparation) for displacement of [^{125}I]secretin tracer binding and for stimulation of adenylate cyclase in the cat pancreas. Completely labelled [^{127}I]secretin did not stimulate adenylate cyclase but showed similar displacement of tracer binding as unlabelled secretin. This, together with the binding data presented in this paper, suggests that the loss in biological activity of secretin is not followed by the loss in properties relevant for receptor binding. This discrepancy between receptor binding and adenyl cyclase stimulation found in our system is not unique. A similar finding was described in a recent study on the efficiency of different secretin fragments on cyclic AMP accumulation and vasoactive intestinal peptide binding in pancreatic tissue [20, 21], as discussed later.

Data obtained from competitive binding studies using a fixed low amount of ^{125}I-labelled secretin and increasing amounts of native hormone clearly indicate that competition for hormone binding sites between the two forms of the hormone occurs throughout the concentration range (Fig. 3). The dose-dependent inhibition of binding of iodinated secretin by unlabelled secretin extends to the low concentrations of unlabelled secretin which are effective to induce bicarbonate and fluid secretion in the intact organ. A reanalysis of these data in the form of a Scatchard plot suggests that the hormone binding sites in this membrane preparation are a homogeneous population. The slope of the Scatchard plot yields a dissociation constant of $4 \cdot 10^{-9}$ M. This value agrees quite well with the apparent K_m ($8 \cdot 10^{-9}$ M) for adenyl cyclase stimulation by this hormone in the same tissue preparation. This agreement is compatible with the assumption that secretin stimulation of adenylate cyclase proceeds through the binding of the hormone to the same set of sites. Furthermore, the high affinity of secretin binding is in the same range as that of other gastrointestinal peptide hormones, such as glucagon [4] or vasoactive intestinal polypeptide [20].

Our finding that only secretin and vasointestinal polypeptide, among the various peptide hormones tested such as glucagon, gastrin, pancreozymin and caerulein, reduced the binding of labelled secretin indicates that the binding sites studied are specific for this hormone.

Studies on the kinetics of the binding reaction showed that binding of ^{125}I-labelled secretin was rapid and reversible. The ratio of the rate constants for dissociation and association of the hormone binding gave a dissociation constant of $3.70 \cdot 10^{-9}$ M which agrees closely with the figure, quoted above, obtained from equilibrium data. Further examination of the kinetics of dissociation and its temperature dependence (Fig. 6) reveals less clear cut behaviour. It is observed that the extent of dissociation of ^{125}I-labelled secretin in the presence of a vast excess of native hormone is highly temperature dependent. While at temperatures higher than 22 °C, the dissociation process follows first-order kinetics, at 0 °C up to 40 % of the labelled secretin dissociates very slowly from the membranes. We have no definite explanation for this phenomenon but assume that it is connected with phase transition, which in mammalian cell membranes takes place at around 22 °C [22], the temperature at which the deviation of binding in our system also occurs. This modification of dissociation does not involve an irreversible change on the membrane since instantaneous and complete release of labelled hormone is obtained on warming. Similar observations on the reduced dissociation of bound peptide hormones at low temperatures have previously been made [18].

The same biological effects as with secretin in the intact pancreas can be ob-

tained by vasoactive intestinal peptide. The primary structure of this peptide hormone is very similar to that of secretin, nine amino acid residues appear in both hormones in the same position [17]. In addition to these similarities in structure and in biological action in the pancreas, remarkable similarities in binding properties have previously been reported [11, 18, 20]. Specific binding of ^{125}I-labelled vasoactive intestinal polypeptide to liver plasma membranes [18] and to adipose tissue [11] is competitively inhibited by secretin and recently a similar competition for ^{125}I-labelled vasoactive intestinal polypeptide binding sites was observed in isolated pancreatic cells [20]. In these latter studies the binding affinity for secretin of vasoactive intestinal polypeptide binding sites was at least two orders of magnitude lower than that of vasoactive intestinal hormone itself. A similar picture emerges from the present studies. It was shown (Fig. 5) that vasoactive intestinal polypeptide competes for specific secretin binding sites in our membrane preparation and that the affinity of native vasoactive intestinal polypeptide for these sites is lower by about two orders of magnitude than the affinity for secretin itself. A comparison between secretin and vasoactive intestinal polypeptide binding to the secretin binding sites and the binding of these two hormones to vasoactive intestinal polypeptide sites shows a strong parallelism. These observations, together with the similarity in structure of the two hormones suggest that there are distinct high affinity binding sites for each of these hormones which might have topographical similarities so that each hormone could also bind with lower affinity to the binding site of the other hormone. In binding studies performed by the use of an iodinated hormone the question of biological activity is obviously relevant. The possibility of a dichotomy existing between structural requirements for secretin receptor binding and adenylate cyclase stimulation is suggested by the results of two recent works. In the first [20], different secretin fragments were tested for their ability to compete with labelled vasoactive intestinal polypeptide in pancreatic tissue. In this study secretin fragment 5-27 was found to have a potency comparable with the entire secretin molecule (secretin 1-27), while a fragment with shorter chain length (secretin 14-27) and the N-terminal portion of secretin were practically ineffective. In the second study [21, 23], different secretin fragments were tested for cyclic AMP accumulation in guinea pig pancreatic cells. The secretin fragment 5-27 was found to lack agonist activity but demonstrated antagonistic properties when tested against native secretin. Since biologically inactive fragment 5-27 retained properties relevant to receptor binding, it was suggested that different portions of the secretin molecule are responsible for adenylate cyclase activation and receptor binding. Our observation that secretin iodinated on the biologically important histidine residue, although inactive, binds to the high affinity, secretin-specific single class of binding sites parallels the above mentioned findings.

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