Biochemical Pharmacology, Vol. 32, No. 18, pp. 2689–2695, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

SPECIFIC SECRETIN BINDING SITES IN RAT PANCREAS

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(*Received* 1 *February* 1983; *accepted* 8 *April* 1983)

Abstract—The dynamic nature of specific secretin binding sites in the pancreas was studied to simulate the interaction of secretin with its receptors in living organisms using isolated perfused rat pancreas. [^{125}I]Secretin or [^{125}I]-[Tyr^{1}] secretin bound to certain binding sites in the perfused pancreas was displaced only by secretin in a dose-dependent manner, not by glucagon or vasoactive intestinal peptide, and the majority of the displaced radioactivity was demonstrated to be undegraded. On the basis of a secretin binding study with subcellular components, it was considered that the displacement would occur on the plasma membranes. These findings suggest that secretin which is distributed in the pancreas may rapidly and reversibly bind to specific binding sites or receptors on the plasma membranes with high affinity.

Secretin plays an important role in pancreatic exocrine secretion, especially water and bicarbonate secretion [1]. The interaction of secretin with its receptors in the pancreas has been investigated by the stimulation of adenylate cyclase and by the binding of [^{125}I]secretin or [^{125}I]vasoactive intestinal peptide to plasma membrane preparation or pancreatic acinar cells [2–4]. However, specific binding sites responsible for the action of secretin have not been demonstrated in the intact pancreas. We reported previously that isolated perfused rat pancreas maintained in physiological state rapidly decomposed secretin [5], although the relationship between receptor binding and degradation has not been confirmed. The rat pancreas is less sensitive to secretin than the pancreas of other species, such as dog and man [6]. Vasoactive intestinal peptide (VIP), a structurally related peptide to secretin, also stimulates the pancreatic exocrine secretion, although secretin and VIP receptors were recently indicated to be distinct entities in rat pancreatic plasma membranes [3]. Therefore, the present study was undertaken to examine the characteristics of specific secretin binding sites in the isolated perfused rat pancreas, and to compare the results with findings on the binding of secretin to rat pancreatic plasma membranes.

MATERIALS AND METHODS

Perfusion of pancreas. Wistar male rats, weighing 240–300 g, were anaesthetized with pentobarbital and the pancreas was isolated and perfused by the modified method of Penhos *et al.* [7] as reported earlier [5]. All blood vessels between the pancreas and duodenum were ligated. The pancreas–duodenum was isolated and perfused at a constant temperature (37°) with Krebs–Ringer bicarbonate solution supplemented with glucose (5.8 mM), dextran T-70 (4.6%) and 0.5% bovine albumin which prevents adsorption of secretin to cannula and tubings [8]. The perfusate was continuously oxygenated by carbogen gas (95% O_2, 5% CO_2) through a fibre-type oxygenator (Microporous Hollow Fiber, Mitsubishi Rayon Co.) and maintained at pH 7.4. Perfusion was performed at the rate of 2 ml/min in a non-recirculating system. The pancreas was initially perfused without secretin for 15 min for conditioning.

Preparation of pancreatic plasma membranes. Isolation of plasma membranes from rat pancreas was carried out by differential and Percoll gradient centrifugation according to the isolation method of basolateral plasma membranes from rat kidney cortex established in this laboratory [9]. The pancreas was removed from Wistar male rats (190–230 g) under pentobabital anaesthesia and placed in ice-cold buffer containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 U/ml Trasylol (buffer A). The pancreas was freed of fat and connective tissue, weighed and minced with a razor blade. Homogenization was carried out in 5 vol. of buffer A with 10 strokes of a Dounce homogenizer (homogenate). This was centrifuged briefly and stopped as soon as 2400 *g* was attained. Centrifugation of the supernatant was repeated twice in the same manner. A pellet (pellet 1) was obtained and the supernatant was centrifuged again at 2400 *g* for 15 min, after which a second pellet (pellet 2) was obtained. The supernatant was further centrifuged at 20,500 *g* for 20 min and the fluffy layer, separated from a tightly packed pellet (pellet 3), was resuspended in buffer A and homogenized with a glass–Teflon Potter homogenizer with 10 strokes at 1000 rpm (crude plasma membranes). The supernatant was centrifuged again at 100,000 *g* for 60 min in a Hitachi RPS27 rotor. Finally, the fourth pellet (pellet 4) and the supernatant were obtained. Next, the suspension of crude plasma membranes was mixed with Percoll (10%, v/v) in buffer A, after which this mixture (total volume, 30 ml) was centrifuged in a Hitachi RP50T rotor at 48,000 *g* for 30 min. On the basis of the distribution of 5′-nucleotidase activity, an 8–12 ml aliquot from the top was

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Table 1. Distribution of marker enzymes and protein in the fractions obtained during purification of plasma membranes*

	5'-Nucleotidase		Cytochrome c oxidase		Amylase		Protein
	sp. act	%	sp. act.	%	sp. act.	%	%
Homogenate	5.3 ± 0.5	100	3.7 ± 0.4	100	1.1 ± 0.1	100	100
Pellet 1	4.0 ± 0.7	25.6	2.9 ± 0.6	27.5	1.0 ± 0.1	33.5	35.3
Pellet 2	2.9 ± 0.4	12.1	6.6 ± 2.3	38.1	1.3 ± 0.1	25.3	21.3
Pellet 3	4.7 ± 0.2	2.6	7.1 ± 1.4	6.5	0.9 ± 0.2	2.4	2.8
Crude plasma membrane	25.4 ± 1.7	16.9	2.9 ± 0.7	2.6	0.1 ± 0.1	0.3	3.4
Pellet 4	11.8 ± 2.6	23.6	0.9 ± 0.1	2.7	0.6 ± 0.1	6.4	10.7
Supernatant	6.6 ± 1.5	29.6	0.2 ± 0.1	1.2	1.0 ± 0.1	23.2	24.7
Plasma membrane	52.0 ± 7.4	3.1	0.3 ± 0.1	0.1	N.D.	0.0	0.3

* Fractions were obtained as described in Materials and Methods. The specific activity of 5'-nucleotidase is expressed in nmole/min per mg protein; that of cytochrome *c* oxidase in ΔO.D./min per mg protein; that of amylase in kilo units/30 min per mg protein. N.D., not detectable. Each value represents the mean ± S.E. of three preparations. % represents the percentage of the enzyme activity found initially in the homogenate.

used as the plasma membrane fraction. This fraction was diluted with buffer A and centrifuged at 100,000 *g* for 60 min to remove the Percoll particles. The obtained pellets were resuspended in the buffer containing 100 mM Tris–HCl (pH 7.5), 0.1 mM PMSF and 100 U/ml Trasylol (buffer B). All these procedures were performed at 0–4°. Table 1 shows the distribution of 5'-nucleotidase, cytochrome *c* oxidase and amylase in the fractions obtained during the isolation of plasma membranes. In the plasma membrane fraction the specific activity of 5'-nucleotidase, a marker enzyme of plasma membranes, was enriched ten-fold, while little activity of cytochrome *c* oxidase, a mitochondria marker, and amylase, a marker of zymogen granules, was observed. 5'-Nucleotidase was determined by the method of Widnell and Unkeless [10]. The activity of cytochrome *c* oxidase was assayed according to the method of Peters *et al.* [11], amylase by the method of Caraway [12], and protein by the procedure of Lowry *et al.* [13] using bovine serum albumin as a standard.

Binding of secretin to subcellular fractions. The subcellular fraction (30–100 μg of protein) was incubated at 25° for 1 min in 0.2 ml of buffer B supplemented with 60 pM [^{125}I]-[Tyr^1] secretin (or 300 pM [^{125}I]secretin) and 2% bovine serum albumin. Binding was initiated by adding the subcellular fraction to the assay mixture, and terminated by the addition of 1 ml of ice-cold buffer B supplemented with 2% bovine serum albumin and filtration through a Millipore cellulose acetate filter. The filter was washed with 7 ml of ice-cold buffer B after which radioactivity in the filter was measured. To determine the specific binding, 2.5 μM of secretin was added to the parallel incubations. Non-specific binding was 1–2% of the total radioactive secretin added.

Materials. Highly purified secretin (16,000 Crick, Haper and Raper units/mg), synthetic secretin (20,000 Crick, Haper and Raper units/mg) and purified secretin (3110 Crick, Haper and Raper units/mg) free from other gastrointestinal hormones were supplied by Eisai Co. (Tokyo, Japan). [^{125}I]-[Tyr^1]Secretin was donated by Daiichi Radioisotope Lab. (Tokyo, Japan) and purified by gel filtration. Synthetic secretin or highly purified secretin was radioiodinated by the chloramine T method [14] and purified by gel filtration using Sephadex G-50. The specific activity of [^{125}I]secretin or [^{125}I]-[Tyr^1] secretin was about 20–40 mCi/mg or 100–200 mCi/mg, respectively. Carrier-free Na^{125}I was purchased from the Radiochemical Centre (Amersham, U.K.); glucagon from Novo Industri A/S. (Copenhagen, Denmark); vasoactive intestinal peptide (VIP) from Calbiochem–Behring Corp. (San Diego, CA); Percoll, dextran T-70 and Sephadex G-50 from Pharmacia Fine Chemicals (Uppsala, Sweden); bovine serum albumin from Povite Producten B.V. (Amsterdam, Holland), and Toyopearl HW-55 from Toyo Soda Mfg. Co. (Tokyo, Japan).

RESULTS

Secretin binding sites in isolated perfused rat pancreas

When [^{125}I]secretin or [^{125}I]-[Tyr^1] secretin was perfused in the pancreas, radioactivity in the portal effluent attained an equilibrium within 5 min (Fig. 1). For the next 5 min, the pancreas was perfused without secretin, and the radioactivity in the effluent decreased rapidly. Then, unlabelled secretin (20 nM) was perfused for 5 min, and the radioactivity in the effluent rose immediately, peaking within the first minute. The displacement phenomenon was similar in both the [^{125}I]secretin and [^{125}I]-[Tyr^1] secretin experiments. When [^{125}I]tyrosine, a metabolic product of [^{125}I]-[Tyr^1] secretin, was perfused, no displacement of [^{125}I]tyrosine by secretin was noted. To study the nature of the displaced radioactivity, the effluents before and after the addition of unlabelled secretin were subjected to gel filtration. Chromatography revealed that most of the displaced radioactivity had co-migrated with authentic secretin (Fig. 2), while a low-molecular-weight component, the fraction of degraded products released from the pancreas [5], was also observed. These results suggested that specifically bound secretin would be released in an undegraded form. To confirm that secretin binds competitively to specific binding sites in the perfused pancreas, the displacement of labelled secretin by various concentrations of unlabelled secretin was examined. Figure 3 shows that labelled secretin was displaced in proportion to the concentration of unlabelled secretin. This dose-dependent character-

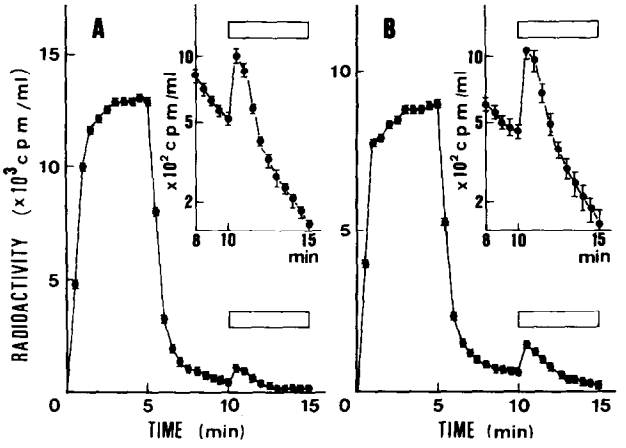

Fig. 1. Effect of unlabelled secretin on the concentration of [^{125}I]secretin (A) or [^{125}I]-[Tyr^1] secretin (B) in effluent from isolated perfused rat pancreas. Labelled secretin was perfused for 5 min, followed by 5 min perfusion without secretin. During the last 5 min, perfusion medium supplemented with unlabelled secretin (20 nM) was perfused (as indicated by open column). The displacement phenomenon is clearly demonstrated semilogarithmically in the inset. Each point represents the mean ± S.E. of three (A) or four (B) experiments.

istic of secretin displacement indicates that there is an occurrence of competitive binding with high affinity in the isolated perfused pancreas. Furthermore, the observation that the displaced amount was small as compared to the initial concentration in the perfusate suggests that the binding site for secretin in the pancreas is located within a limited area.

In order to clarify the specificity of the secretin binding sites in the the pancreas, glucagon (0.6 μM) or VIP (1.5 μM), structurally related peptide, was examined for its ability to compete with secretin for binding. Significant changes in radioactivity of the effluent caused by these peptides were not observed, but it continued to decrease. Therefore, we posit that secretin binding sites in the perfused pancreas have high structural specificity.

Secretin binding sites in rat pancreatic plasma membranes

The binding of secretin with its receptors in the

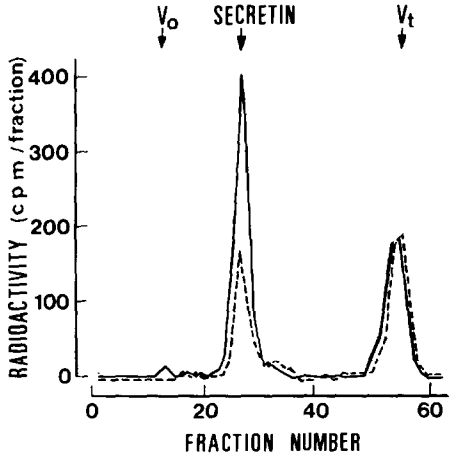

Fig. 2. Gel filtration profiles of effluents from [^{125}I]-[Tyr^1] secretin perfusion. The effluents, which were collected 1 min before (– – –) and 1 min after the beginning of perfusion with unlabelled secretion (2 μM) (——), were subjected to gel filgration on a 1.5 × 90 cm Toyopearl HW-55 column eluted with 3 M guanidine–HCl and 2.4 M formic acid at 4°. The column was calibrated with blue dextran as a void volume marker (V_0), native secretin (SECRETIN) and [^{125}I]tyrosine to indicate the internal volume (V_t).

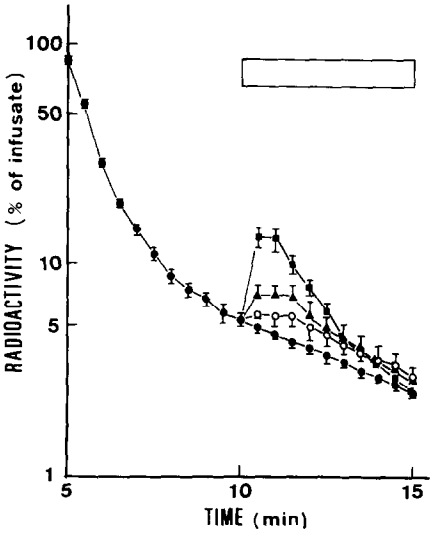

Fig. 3. Effect of unlabelled secretin on the concentration of [^{125}I]-[Tyr^1] secretin in the effluent from isolated perfused rat pancreas. A dose-dependent displacement was observed by the perfusion with unlabelled secretin [15 nM (■), 3 nM (▲). 0.6 nM (○)] and without unlabelled secretin (●). Each point represents the mean ± S.E. of at least three experiments.

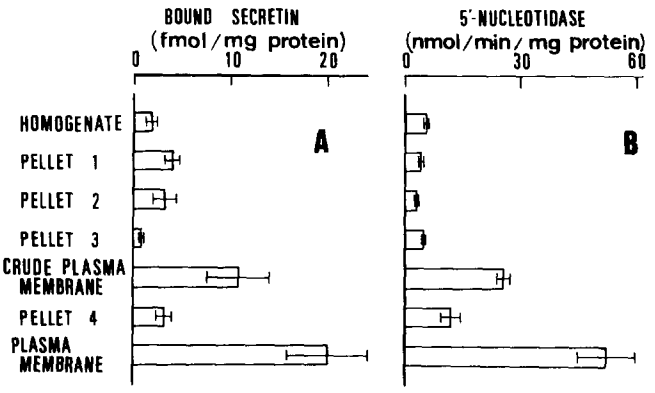

Fig. 4. Distribution of specific binding (A) and 5′-nucleotidase (B) in various fractions. Fractionation was performed as described in Materials and Methods. Each fraction (30–100 μg of protein) was incubated with [^{125}I]-[Tyr^1] secretin (60 pM) at 25° for 1 min. To determine the specific binding, 2.5 μM secretin was added to parallel incubations. Each column represents the mean ± S.E. of three to four experiments.

pancreas using ^{125}I-labelled secretin as the tracer was only reported by Milutinovic *et al.* [2] in cat pancreatic membranes. The characterization of the binding sites or receptors is considered important to investigate further the secretin binding sites in rat pancreas. Thus, fractionation of the pancreas was performed to determine the subcellular localization of secretin binding sites observed in the perfused pancreas. By Percoll density gradient centrifugation, we obtained a plasma membrane fraction in which 5′-nucleotidase was enriched ten-fold as compared with that found in the homogenate.

Figure 4 shows the subcellular distribution of the specific binding sites of secretin in the pancreas. The maximum specific binding was observed in the plasma membrane fraction, and the distribution pattern of secretin binding activity was similar to that of 5′-nucleotidase among the fractions obtained during the isolation of plasma membrane fraction. After centrifugation of the incubation mixture, the radioactivity bound to plasma membranes was extracted and analysed by gel filtration. The majority of the radioactivity appeared at the position of intact secretin (Fig. 5). These results suggest that the main binding sites of secretin are located on the plasma membranes, and that secretin binds to its receptor in an intact form. The time course of secretin binding to plasma membranes indicated a fast association rate with a binding equilibrium attained within 1 min, as shown in Fig. 6. This binding decreased rapidly on adding 1 μM of unlabelled secretin.

The binding of [^{125}I]secretin to pancreatic plasma

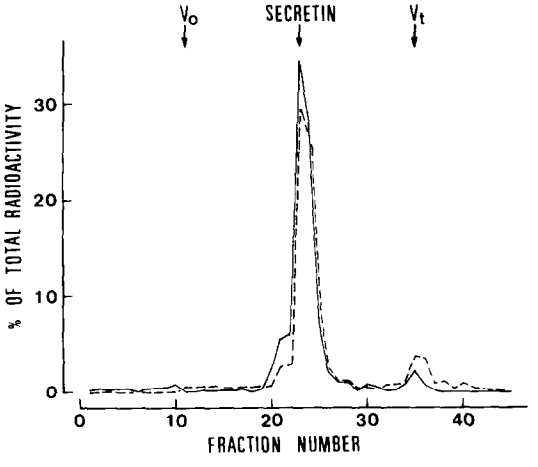

Fig. 5. Gel filgration profile of membrane-associated radioactivity. Pancreatic plasma membranes (100 μg of protein) were incubated with [^{125}I]-[Tyr^1] secretin (0.3 nM) at 4° for 10 min. Bound radioactivity, separated by centrifugation at 20,500 *g* for 20 min at 4°, was extracted with 1 ml of 5.4 M guanidine–HCl and 2.4 M formic acid. The extract was subjected to gel filtration on a 0.9 × 62 cm Toyopearl HW-55 column eluted with 0.1 M acetic acid at 4°. The broken line represents the gel filtration profile of the standard tracer of secretin.

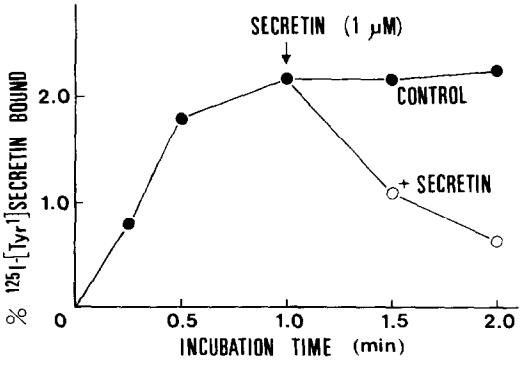

Fig. 6. Time course of [^{125}I]-[Tyr^1] secretin binding to plasma membranes. The plasma membrane fraction (51 μg of protein) was incubated with [^{125}I]-[Tyr^1] secretin (60 pM) at 25°. Dissociation of tracer from plasma membranes was observed after the addition of 1 μM unlabelled secretin at an equilibrium (○). Each point is the mean of three to six determinations.

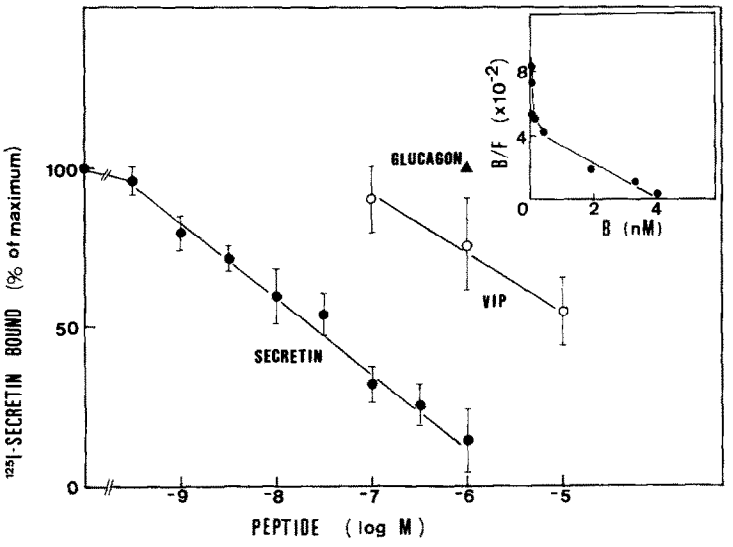

Fig. 7. Effect of secretin, VIP or glucagon on binding of [^{125}I]secretin to pancreatic plasma membranes. Plasma membrane fraction (200 ± 10 μg/ml of protein) was incubated with [^{125}I]secretin (0.3 nM) plus increasing concentrations of unlabelled secretion (●), VIP (○), and 1 μM glucagon (▲). Each point is the mean ± S.E. of four separate experiments. Inset: Scatchard plot of a representative experiment.

membranes was competitively inhibited by increasing the concentration of unlabelled secretin (Fig. 7). VIP was effective only at a high concentration, as observed in cat pancreatic membranes [2]. The discrepancy between the effect of VIP on secretin binding in the perfused pancreas and that in the membranes was interpreted as the difference in experimental conditions. Glucagon was found to be ineffective at a concentration of 1 μM. Scatchard plots of the binding data from four separate experiments were compatible with the presence of two classes of binding sites: high affinity sites with a K_d of 1.05 ± 0.22 nM (mean ± S.E.) having a binding capacity of 0.67 ± 0.19 pmoles/mg of protein; and low affinity sites with a K_d of 80 ± 11 nM having a binding capacity of 16.8 ± 3.8 pmoles/mg (Fig. 7, inset).

DISCUSSION

There have been few reports concerning the dynamic state of hormonal receptors in living target organs under a physiological environment. In the case of insulin, Petersen *et al.* [15] have recently suggested the presence of insulin-specific peritubular receptors by examining the fate of iodoinsulin removed from peritubular circulation of isolated perfused rat kidney. In the present study, we have demonstrated the dynamic nature of secretin binding in the isolated perfused rat pancreas. The adsorptive effect of secretin to perfusion tubings and cannula was almost negligible. Radioiodinated aprotinin, a similar basic peptide as secretin, was similarly tested, but no displacement of [^{125}I]aprotinin was observed by the addition of excessive unlabelled aprotinin (unpublished data). Therefore, it was concluded that a limited amount of entering secretin binds to specific binding sites in the perfused pancrease in a dose-dependent manner, although a considerable amount of this secretin would be degraded by the perfused pancreas [5]. Under this experimental condition, the half-maximal concentration of secretin for the displacement of ^{125}I-labelled [Try^1]secretin was approximately 4 nM (Fig. 3) and that for the stimulation of pancreatic juice secretion was about 1 nM [5]. These two comparable parameters suggest that the binding sites for secretin in the perfused pancreas may reflect the location of receptors on duct and centroacinar cells serving in the secretion of bicarbonate and water [16].

However, as there is a limitation to analysis of secretin binding in the perfused pancreas in a quantitative investigation of the relationship between the receptor occupancy and hormonal action, we studied the binding of secretin to purified plasma membranes from rat pancreas. The plasma membrane fraction showed maximal binding of secretin and there was a correlation between the distribution of secretin binding and that of 5'-nucleotidase among the subcellular fractions (Fig. 4). These findings indicate that the displacement of iodinated secretin by excessive secretin in the perfused pancreas is a consequence of the binding property of secretin to the specific binding sites on plasma membranes (Fig. 6).

Scatchard plots of secretin binding to plasma membranes demonstrated the presence of two classes of binding sites: high affinity sites (K_d of 1 nM) and low affinity sites (K_d of 80 nM). The inability to detect low affinity sites in the perfused pancreas is probably due to the release of [^{125}I]secretin bound to low affinity sites during the washing period (Fig. 1, 5–10 min). From the stimulation of adenylate cyclase and the binding of [^{125}I]VIP, Robberecht *et al.* [3] have recently suggested that secretin and VIP receptors are distinct entities in rat pancreatic plasma membranes, and have classified them into three sub-

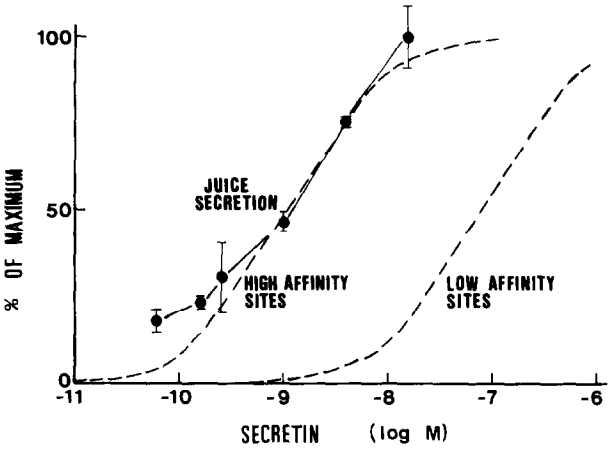

Fig. 8. Relationship between the concentration of secretin to stimulate juice secretion in perfused pancreas and to occupy the high affinity binding sites on pancreatic plasma membranes. ——, Juice secretion as per cent maximum; – – –, theoretical occupancy curves (as per cent of maximum) for the high and low affinity binding sites.

types: (1) high affinity secretin receptors (apparent K_d of 0.3 nM); (2) low affinity secretin receptors (apparent K_d of 300 nM); and (3) VIP-preferring receptors. Schuz *et al.* [17] have isolated duct cells from rat pancreas by the counterflow technique, and reported that secretin activates adenylate cyclase in duct cells more effectively than acinar cells, with an approximate half-maximal concentration of 1 nM. The high affinity binding sites demonstrated in the present study, which are compatible with their reports [3, 17], could reflect a contribution of membranes from centroacinar and duct cells. Figure 8 shows the occupation of high and low affinity binding sites in plasma membranes and stimulation of juice flow in the perfused pancreas [5]. Although there are several differences in the conditions of the experiments, the stimulation of juice flow correlated closely with the occupancy of the high affinity sites.

VIP is considered to be a functional neurotransmitter at neuroacinar synapse controlling enzyme secretion [18]. On the contrary, secretin is a hormone acting on centroacinar and duct cells to secrete a bicarbonate-rich fluid [1]. The lack of the ability of VIP to displace bound secretin does not indicate the competition of VIP with secretin for binding (Fig. 7), but suggests that VIP does not interfere with secretin binding in the pancreas under a physiological environment.

The dynamic characteristics of secretin binding in the perfused pancreas may represent a reasonable explanation for the rapid response of pancreatic secretion induced by secretin, and the combined study using the plasma membrane preparation provided valuable knowledge on the interaction of secretin with its receptors in the pancreas as related to hormonal action.

We have also observed the presence of secretin binding sites in the basolateral membranes of rat gastric mucosa [19] and the perfused stomach (unpublished data). Comparison of pancreatic receptors with gastric receptors will provide important information for elucidating the mechanism of secretin action on exocrine secretion.

Acknowledgements—We are indebted to Eisai Co. Ltd., Tokyo, for the donation of secretin, and also to Daiichi Radioisotope Lab., Tokyo, for the supply of radioiodinated [Try^1]secretin.

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