Communications to the Editor

Chem. Pharm. Bull. 30(8)3050—3053(1982)

INTERACTION OF SECRETIN WITH BASOLATERAL MEMBRANE FRACTION FROM RAT GASTRIC MUCOSA: MEMBRANE PREPARATION AND SECRETIN BINDING

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Studies on secretin receptors of the stomach need an appropriate methodology. Plasma membrane fraction enriched in basolateral membranes was obtained from rat gastric mucosa using differential centrifugation and Percoll density gradient centrifugation. $(Na^+ + K^+)$-ATPase, a marker enzyme for basolateral membranes, was enriched 10.5-fold compared with that found in the homogenate. The binding of ^{125}I-[Tyr^1]secretin to the basolateral membrane fraction was rapid, reversible and saturable with an equilibrium K_d of 6.4 nM and the binding capacity of 0.6 pmol/mg of protein.

KEYWORDS——basolateral membranes; secretin; specific binding; membrane isolation; rat gastric mucosa

Secretin, a well-known enterogastrone, suppresses gastric acid and gastrin secretion,[1] and stimulates pancreatic exocrine secretion.[2] Although its interaction with pancreatic plasma membranes has been studied,[3] the specific binding sites in the stomach have not been characterized. In addition, an enriched preparation of basolateral plasma membranes from gastric mucosa has only recently reported by Culp and Forte[4] using density gradient centrifugation of dextran. However, they did not examine the usefulness of their membrane preparation for the membrane-bound hormonal receptor analysis. Thus, in the present study, a modified Percoll gradient method for the isolation of basolateral membrane fraction from rat gastric mucosa is presented, and the interaction of secretin with this membrane fraction is analyzed.

Basolateral membrane fraction from rat gastric mucosa was prepared by density gradient centrifugation with Percoll, as previously described for the isolation of basolateral membrane vesicles from rat kidney cortex.[5] The stomachs of Wistar male rats (190-230 g) were removed and rinsed with ice-cold buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml Trasylol and 0.1 μM pepstatin (buffer A). The gastric mucosa was scrapped and homogenized in ice-cold buffer A (1:7, w/v) with 10 strokes of a Dounce homogenizer (homogenate; H). Then, it was briefly centrifuged and stopped as soon as 2400×g was attained. The supernatant was recentrifuged two times in the same manner. The pellets

obtained were gathered and resuspended in buffer A (half the volume of the initial homogenate) and rehomogenized. This new suspension was centrifuged 3 times rapidly and briefly at 2400xg as described. A pellet (P1) was obtained. To improve the recovery, the supernatants were combined and recentrifuged at 2400xg for 15 min. A pellet (P2) was obtained. The supernatant was centrifuged at 20500xg for 20 min and the fluffy layer, separated from a tightly packed pellet (P3), was resuspended in buffer A, and homogenized with a glass/Teflon Potter homogenizer with 10 strokes at 1000 rev./min (crude plasma membrane; CPM). The supernatant was further centrifuged at 100000xg for 60 min in a Hitachi RPS27 rotor. Finally a pellet (P4) and the supernatant (S) were obtained. Now, the suspension of CPM was mixed with Percoll (Pharmacia Fine Chemicals; 10%, v/v) in buffer A. This membrane-Percoll mixture (total volume, 30 ml) was centrifuged in a Hitachi RP50T rotor at 48000xg for 30 min. Five fractions (Fr. I-V, 6 ml each) were collected from the top using a Hitachi DGF-U density gradient fractionator. In order to remove the Percoll particles, each fraction was diluted with buffer A and centrifuged at 100000xg for 60 min in a Hitachi RPS27 rotor. The membrane material was resuspended in the buffer containing 100 mM Tris-HCl (pH 7.5), 0.1 mM PMSF, 50 U/ml Trasylol and 0.1 μM pepstatin (buffer B) and homogenized. The suspension was centrifuged again in the same manner. The final pellet was resuspended in buffer B by sucking the suspension through a fine needle (0.4x20 mm). All procedures were performed at 0-4°C.

Fig. 1 shows the distribution of $(Na^+ + K^+)$-ATPase, a marker enzyme of the basolateral plasma membranes, and alkaline phosphatase, a marker enzyme of the apical membranes, after the centrifugation in the Percoll medium. Alkaline phosphatase migrated toward the bottom of the tube, whereas maximal $(Na^+ + K^+)$-ATPase activity was located at the upper part of the gradient. The distribution pattern of $(K^+ + H^+)$-ATPase, a marker enzyme for apical and tubulovesicular membranes, was similar to that of alkaline phoshatase. To reduce the contamination from apical membranes, only fractions 7-12 were taken as the basolateral membrane fraction (Fr. II).

Table I lists the distribution of marker enzyme activity and protein during the preparation. The basolateral membrane fraction, Fr. II, had 10.5-fold and 8.3-fold higher specific activities relative to those in the homogenate for $(Na^+ + K^+)$-ATPase and 5'-nucleotidase (a marker enzyme for plasma membranes), respectively. In contrast, the specific activity of $(K^+ + H^+)$-ATPase was lower than that in the crude plasma membrane fraction. Contamination of acid phosphatase derived from lysosome and cytochrome c oxidase from mitochondria was small.

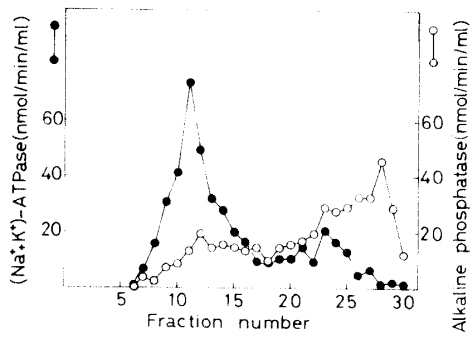


Fig. 1. Distribution of Marker Enzymes for Basolateral and Apical Membranes on Percoll Gradient
The Percoll gradient was collected from the top into 30 fractions of 1 ml.
● ; $(Na^+ + K^+)$-ATPase
○ ; alkaline phosphatase

This enriched preparation of basolateral plasma membranes from rat gastric mucosa had a purification factor for $(Na^+ + K^+)$-ATPase comparable to that

TABLE I. Distribution of Marker Enzymes, Protein and Secretin Binding Sites during Preparation of Basolateral Membrane Fraction

The following marker enzymes were assayed: $(Na^+ + K^+)$-ATPase[6]; 5'-nucleotidase[7]; alkaline phosphatase[8]; $(K^+ + H^+)$-ATPase[4]; acid phosphatase[9]; cytochrome c oxidase[10], and expressed as specific activity (nmol/min per mg protein)(Δ A550/min per mg protein for cytochrome c oxidase). Protein was determined, after precipitation with 10%(w/v) trichloroacetic acid, by the method of Lowry et al.,[11] and expressed as percent of homogenate. Secretin binding sites in subcellular fractions were examined as follows: the obtained fraction was incubated with ^{125}I-[Tyr^1]secretin (60 pM, Daiichi Radioisotope Laboratory) for 1 min at 25°C in 0.2 ml buffer B supplemented with 2% bovine albumin. The separation of bound radioactivity was performed by rapid filtration technique using a Millipore filter.[3] To determine the specific binding, 2.5 μM secretin (16000 Crick, Haper and Raper units/mg, Eisai Co.) was added to parallel incubation. The results were expressed in fmol/mg protein. Enzyme and secretin binding activities in each Percoll fraction were determined after the removal of Percoll. We could not determine these activities in Fr. I from the low recovery of membraneous material. Each value represents the mean ± S.E. for 3-4 experiments.

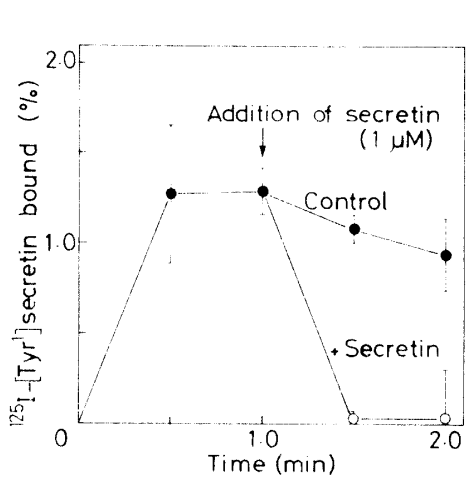
	$(Na^+ + K^+)$-ATPase	5'-nucleotidase	Alkaline phosphatase	$(K^+ + H^+)$-ATPase	Acid phosphatase	Cytochrome c oxidase	Protein	Secretin binding
H	7.8±0.9	1.2±0.1	34.8±5.2	17.2±2.8	279±25	13.9±2.7	100.0	6.2±0.5
P1	13.8±4.0	0.6±0.1	19.0±1.5	16.8±1.7	171±26	11.0±2.0	21.3±1.8	5.3±1.2
P2	2.9±0.4	1.1±0.1	14.2±4.8	24.3±4.7	316±81	27.8±9.4	31.7±0.5	8.6±1.2
P3	8.1±4.7	4.0±0.1	24.8±2.6	12.1±5.4	980±15	59.5±7.3	1.4±0.1	6.6±1.7
P4	10.2±5.9	6.0±0.2	55.2±14.2	29.2±8.5	434±13	3.2±0.7	3.5±0.8	5.6±2.3
S	5.9±1.7	0.7±0.1	46.8±2.4	2.4±1.0	275±7	0.2±0.03	37.6±1.4	——
CPM	42.9±8.3	5.0±0.7	82.3±26.1	157.0±29.1	541±60	17.4±2.7	4.4±0.7	8.6±3.1
Fr. II	81.7±2.1	10.4±1.7	72.5±8.8	100.3±25.3	392±76	1.6±0.4	0.3±0.03	29.1±4.3
Fr. III	90.1±8.8	5.2±1.0	112.8±17.5	170.4±31.2	374±23	2.5±0.2	0.4±0.04	8.0±5.7
Fr. IV	40.8±7.4	4.3±0.9	96.7±18.2	206.6±37.3	431±11	5.8±0.7	0.7±0.1	8.3±1.2
Fr. V	5.7±2.7	6.7±1.0	60.3±23.1	90.6±18.1	579±94	35.4±2.5	0.7±0.1	3.9±1.6

obtained from gastric glandular cells of rabbit using a dextran gradient method as reported by Culp and Forte.[4]

The subcellular distribution of the specific binding sites for secretin is also shown in Table I. Maximum specific binding of ^{125}I-[Tyr^1]secretin was seen in the basolateral membrane fraction, Fr. II (4.7±0.5 times binding of homogenate). This value was almost the same as the secretin interaction of pancreatic membrane fraction reported by Milutinovic et al..[3] The binding to other subcellular fractions was as small as that to the homogenate. The time course of secretin binding to the basolateral membrane fraction indicated a fast association rate with a binding equilibrium attained within one minute, as shown in Fig. 2. This binding was decreased rapidly by the addition of 1 μM unlabeled secretin. Fig. 3 shows the effect of unlabeled secretin on the binding of ^{125}I-[Tyr^1]secretin to basolateral membrane fraction. A dose dependent inhibition was detected. The Lineweaver-Burk plot of the binding data was linear, and the dissociation constant and maximum binding capacity were calculated as 6.4±4.5 nM and 0.6±0.2 pmol/mg protein (n=3), respectively. A Hill plot of the specific binding yielded a line with a slope of 0.98.

The collected data indicate the presence of secretin receptor on the basolateral membranes of gastric mucosa, having reversible and saturable characteristics, although one is left with the problem of cell specificity.

In conclusion, the use of the Percoll gradient method provides a simple and rapid method for the preparation of basolateral membrane fraction from rat gastric



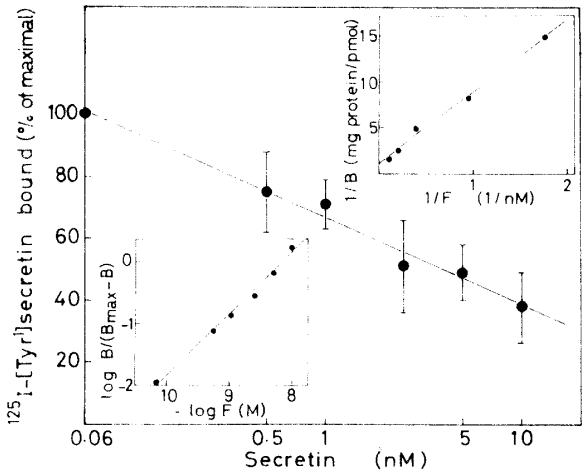


Fig. 2. Time Course of ^{125}I-[Tyr^1]secretin Binding to Basolateral Membrane Fraction
Basolateral membrane fraction (18.2 μg of protein) was incubated with ^{125}I-[Tyr^1]secretin at 25°C. Dissociation of tracer from basolateral membrane fraction was observed after the addition of 1 μM unlabeled secretin (○). Each point is a mean ± S.E. of 3 determinations. Specific binding was calculated as described in the legend of Table I.

Fig. 3. Effect of Unlabeled Secretin on the Binding of ^{125}I-[Tyr^1]secretin to Basolateral Membrane Fraction
Basolateral membrane fraction (27±3 μg of protein) was incubated with ^{125}I-[Tyr^1]secretin (60 pM) and unlabeled secretin (0.5-10 nM) at 25°C for one minute. Each point is a mean ± S.E. of 3 separate experiments performed in triplicate. For details, see the legend of Table I. Upper inset: Lineweaver-Burk plot. Lower inset: Hill plot.

mucosa, and offers valuable material for the analysis and characterization of secretin receptors.

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(Received June 21, 1982)