Molecular architecture of secretin receptors: the specific covalent labelling of a 51 kDa peptide after cross-linking of [125 I]iodo-secretin to intact rat pancreatic acini

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p-Azidophenylglyoxal (APG), a heterobifunctional reagent with one group reacting selectively with arginine residues and another group photoactivable, was used to cross-link [125 I]secretin prebound to intact rat pancreatic acini. The best yield was obtained when the [125 I]secretin-acini complex was incubated under dim light with 2 mM APG at 37°C and pH 8.0, followed by photolysis at 312 nm. The main secretin binding peptide cross-linked under reducing conditions, when tested by SDS-PAGE and autoradiography: (i) had a molecular mass of 51 kDa and was not a subunit of a larger disulfide-linked structure, and (ii) was distinct from the main VIP binding peptide coexisting in the same preparation.

Secretin receptor; Azidophenylglyoxal, *p*-; (Rat pancreas)

1. INTRODUCTION

Secretin-preferring and VIP-preferring receptors coexist in intact rat pancreatic acini. They are endowed with overlapping affinities for agonists so that competition binding curves extend over more than 3 logarithms [1,2]. The same holds true in rat pancreatic membranes [3]. We recently showed that the main VIP binding site in intact rat pancreatic acini has a molecular mass of 77 kDa by acylating amino group(s) in both 125 I-VIP and its receptor with bifunctional cross-linking agents acting through *N*-hydroxysuccinimide ester groups [4]. The present study gives the first information about the molecular architecture of secretin receptors in intact pancreatic acini using a photoaffinity labelling approach. Porcine secretin has no lysine residues and its N-terminal histidine is directly in-

involved in receptor recognition ([5,6] and unpublished observations). Such an absence of free amino groups readily available in secretin prevents the testing of the cross-linkers we used for VIP receptors. On the other hand secretin contains four arginine residues so that we were persuaded to test *p*-azidophenylglyoxal (APG). This arylglyoxal is an ultraviolet-sensitive heterobifunctional reagent highly specific for arginine in its dione (α -dicarbonyl) moiety [7,8]. We reasoned that, after reversible [125 I]secretin binding to intact rat pancreatic acini, the glyoxal function of APG would react, under mild conditions, with the bifunctional nitrogen nucleophile of the guanidinium group in those arginine residues of the radioligand that were not directly involved in receptor recognition. The adduct formed would be a double carbinolamine with vicinal *cis*-hydroxyl groups. In the next step the still reversibly bound secretin derivative(s) could be irreversibly cross-linked to secretin receptors, after UV photoactivation of the azidophenyl group of APG, the reactive nitrene generated forming linkage with virtually any part of nearby binding sites. A similar approach had already been

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successfully undertaken for VIP receptors in liver plasma membranes [9].

2. MATERIALS AND METHODS

[¹²⁵I]Secretin was prepared with synthetic porcine secretin by minor modifications of the method of Jensen et al. [10] as described in [1,3] (labelling of the N-terminal histidine residue was initiated chloramine T). [¹²⁵I]Secretin corresponded to a single homogeneous peak after reverse phase HPLC on a μ Bondapak C-18 column with a 25–40% acetonitrile gradient containing 0.1% trifluoroacetic acid. It was diluted in 10 mM phosphate buffer (pH 7.4)–150 mM NaCl enriched with 0.1% NaN₃ and 0.5% Tween 20, was stored frozen at –20°C, and could be used over a 2 month period.

Dispersed acini from male Wistar rats (150–200 g) were prepared as in [1] and incubated for 45 min at 37°C with 0.5 nM [¹²⁵I]secretin (35 TBq/mmol) in 25 mM Dulbecco-Hepes medium supplemented with 2% bovine serum albumin, 0.1% bacitracin and 0.1% soybean trypsin inhibitor (STI). After binding equilibrium [1] the acini were washed four times by centrifugation/resuspension in cold PBS (10 mM phosphate buffer (pH 7.4)–145 mM NaCl) and resuspended in 25 mM borate–8 mM phosphate buffer (pH 8.0) at a 40 mg wet wt/ml concentration. 1 vol. APG solution was added to 99 vols of the acini suspension. APG was dissolved immediately before use in dry dimethylsulfoxide. After 15 min of incubation under dim light at the indicated temperature the acini were washed twice by centrifugation/resuspension in cold PBS, resuspended in PBS as above and exposed for 15 min at 4°C to a 6 W UV lamp (Lab Center, Ankersmit) wavelength 312 nm, at 10 cm.

Illuminated acini were centrifuged and the pellet was disrupted by sonification in a lysis buffer made of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.25 M sucrose, 0.1% bacitracin and 0.01% STI. Following centrifugation at 1000 × g for 10 min, the resulting supernatant was recentrifuged for 20 min at 50000 × g (in a J2-21 Beckman centrifuge) to obtain a crude membrane fraction. This second pellet, less overloaded with nuclear and zymogen granule proteins than the first one, was solubilized in an electrophoresis sample buffer made of 0.125 M Tris-HCl (pH 6.8) containing 5% SDS, 10% sucrose, 0.02% bromophenol blue and enriched with 1% dithiothreitol and 4% 2-mercaptoethanol.

SDS-PAGE under reducing conditions was performed as in [4] with a 5–20% gradient polyacrylamide separating gel (180 × 200 × 1.5 mm). Autoradiography (conducted for 6 weeks at –80°C), protein determination, and the origin of chemicals were as in [4]. Synthetic porcine secretin was a generous gift from Dr W. König (Hoechst Aktiengesellschaft, Frankfurt/Main, FRG).

3. RESULTS

Pretreating at 37°C the [¹²⁵I]secretin-intact acini complex with 2 mM APG at pH 8.0 or 9.0 (fig.1, lanes 5 and 11, respectively), provoked the incorporation of radioactivity into a major 54 kDa peptide. When provision is made for the molecular

Fig.1. Cross-linking of [¹²⁵I]secretin to rat pancreatic acini. Acini were incubated with [¹²⁵I]secretin in 1 ml aliquots, in the absence or presence of 1 μM unlabelled secretin. Washed acini were incubated for 15 min at 4, 20 or 37°C and at pH 8 or 9 in the presence of 1 mM APG. Further treatment was as described in section 2.

mass of secretin this suggests that the 51 kDa peptide was a part of the binding site. A faint 180–220 kDa secretin binding smear was also detectable under our reducing conditions (fig.1–3). Labelling was fully inhibited when the radioligand was incubated in the presence of 1 μM secretin (fig.1, lanes 6 and 12) and suppressed in the absence of APG or UV, while being unaffected after incubation in the presence of 1 μM vasoactive intestinal peptide (VIP) and 1 μM peptide histidine-isoleucinamide (PHI) (not shown). Fig.1 also shows that the optimal temperature was 37°C, secretin cross-linking being feeble at 20°C (lanes 3 and 9), and nonexistent at 4°C (lanes 1 and 7): this temperature of 37°C was a middle ground for optimal adduct formation of APG with arginine residues, in the face of the dissociation of secretin not yet cross-linked to its receptor.

When comparing 3 APG concentrations (5, 2, and 0.5 mM), the cross-linking of the main 54 kDa peptide was most specific with 2 mM APG (fig.2). We next tested the effects of pH on cross-linking. Theoretically, the reaction with a glyoxal is

Fig.2. Effects of APG concentration on the cross-linking of [125 I]secretin to rat pancreatic acini. Acini were incubated with [125 I]secretin in the absence or presence of 1 μ M unlabelled secretin. Washed acini were incubated for 15 min at 37°C in the presence of 0.5, 2 or 5 mM APG. Further treatment was as described in section 2.

facilitated when arginine is deprotonated i.e. at an unphysiologically high alkaline pH [8]. Under our conditions, the optimal pH was around 8.5 (fig.3, lane 7), an obvious compromise between optimal APG reaction with arginine residues in secretin and increasing dissociation of the hormone from intact receptors.

Finally (fig.4) secretin in the incubation medium competitively inhibited [125 I]secretin cross-linking to intact acini with an IC_{50} of 31 nM for both the main 51 kDa band and the smearing 200 kDa material. This IC_{50} value was comparable to the IC_{50} value of reversible [125 I]secretin binding to dispersed rat acini [1] but could differ from the true K_i by, e.g. an unusually high affinity of the tracer and/or the relatively high concentration of tracer (0.5 nM) we used to obtain enough labelling.

Fig.3. Effects of pH on the cross-linking of [125 I]secretin to rat pancreatic acini. Preincubated acini were suspended in 1 ml aliquots of 25 mM borate buffer adjusted to the indicated pH with phosphoric acid. Further treatment was as described in section 2.

Fig.4. Inhibition of [125 I]secretin cross-linking to rat pancreatic acini by increasing secretin concentrations in the preincubation medium. The general procedure is described in section 2.

4. DISCUSSION

Our photoaffinity probe of secretin binding sites was prepared by cross-linking prebound [125 I]secretin with APG, at 37°C and at pH 8.0. The conditions used maintained probably intact the plasma membranes of dispersed acini. However, the yield was very low and more than one month exposures were necessary to visualize the cross-linked secretin. We were unable to cross-link [125 I]secretin to semi-purified rat pancreatic plasma membranes probably as a result of receptor inactivation during the long cross-linking procedure.

The 51 kDa peptide we observed appeared distinct from the 62 kDa binding site observed [11] in plasma membrane preparations from rat antrum (in addition to a 33 kDa peptide) when using a cleavable chemical homobifunctional cross-linker (dithiobis(succinimidyl)propionate). The molecular mass of the present secretin binding peptide was also definitely lower than the molecular mass of 60–80 kDa of VIP binding peptides in rat pancreatic acini [4], rat tumoral pancreatic acinar cells AR 4-2J [12], rat and human intestinal epithelium, the human colonic HT-29 cell line, the main binding site of rat liver membranes, and membranes from human, rabbit and guinea pig lung. This molecular mass was more similar to that of other also unrelated VIP binding peptides characterized in rat liver and lung membranes, rat GH 3 pituitary cells and in human lymphoblasts (review [4]).

A variable proportion of a minor smearing material in the 180–220 kDa range could represent covalent linkage, at 37°C, and under conditions excluding interchain disulfide bridges between [125 I]secretin, its receptor, and neighboring proteins including those of the adenylate cyclase complex.

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REFERENCES

- [1] Dehaye, J.P., Winand, J., Damien, C., Gomez, F., Poloczek, P., Robberecht, P., Vandermeers, A., Vandermeers-Piret, M.C., Stiévenart, M. and Christophe, J. (1986) *Am. J. Physiol.* 251, G602–G610.
- [2] Bissonnette, B.M., Collen, M.J., Adachi, H., Jensen, R.T. and Gardner, J.D. (1984) *Am. J. Physiol.* 246, G710–G717.
- [3] Robberecht, P., Coy, D.H., De Neef, P., Camus, J.-C., Cauvin, A., Waelbroeck, M. and Christophe, J. (1987) *Eur. J. Biochem.* 165, 243–249.
- [4] Svoboda, M., Poloczek, P., Winand, J., Robberecht, P. and Christophe, J. (1988) *Eur. J. Biochem.* 174, 59–66.
- [5] Robberecht, P., Waelbroeck, M., Noyer, M., Chatelain, P., De Neef, P., König, W. and Christophe, J. (1982) *Digestion* 23, 201–210.
- [6] König, W., Bickel, M., Wissmann, H., Uhmman, R. and Geiger, R. (1979) in: *Hormone Receptors in Digestion and Nutrition* (Rosselin, G., Fromageot, P. and Bonfils, S. eds) pp.137–143, Elsevier/North-Holland Biomedical Press, Amsterdam.
- [7] Ngo, T.T., Yam, C.F., Lenhoff, H.M. and Ivy, J. (1981) *J. Biol. Chem.* 256, 11313–11318.
- [8] Patthy, L. and Thész, J. (1980) *Eur. J. Biochem.* 105, 387–393.
- [9] Robichon, A., Kuks, P.F.M. and Besson, J. (1987) *J. Biol. Chem.* 262, 11539–11545.
- [10] Jensen, R.T., Charlton, C.G., Adachi, H., Jones, S.W., O'Donohue, T.L. and Gardner, J.D. (1983) *Am. J. Physiol.* 245, G186–G195.
- [11] Bawab, W., Gespach, C., Marie, J.-C., Chastre, E. and Rosselin, G. (1988) *Life Sci.* 42, 791–798.
- [12] Svoboda, M., De Neef, P., Tastenoy, M. and Christophe, J. (1988) *Eur. J. Biochem.*, in press.