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# Solubilization of active GLP-1 (7–36)amide receptors from RINm5F plasma membranes

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Glucagon-like peptide-1 (7–36)amide (GLP-1 (7–36)amide) represents a physiologically important incretin in mammals including man. Receptors for GLP-1 (7–36)amide have been described in RINm5F cells. We have solubilized active GLP-1(7–36)amide receptors from RINm5F cell membranes utilizing the detergents octyl-β-glucoside and CHAPS; Triton X-100 and Lubrol PX were ineffective. Binding of radiolabeled GLP-1(7–36)amide to the solubilized receptor was inhibited concentration-dependently by addition of unlabeled peptide. Scatchard analysis of binding data revealed a single class of binding sites with $K_d$ = 0.26 ± 0.03 nM and $B_{max}$ = 65.4 ± 21.24 fmol/mg of protein for the membrane-bound receptor and $K_d$ = 22.54 ± 4.42 μM and $B_{max}$ = 3.9 ± 0.79 pmol/mg of protein for the solubilized receptor. The binding of the radiolabel to the solubilized receptor was dependent both on the concentrations of mono- and divalent cations and the protein/detergent ratio in the incubation buffer. The membrane bound receptor is sensitive to guanine-nucleotides, however neither GTP-γ-S nor GDP-β-S affected binding of labeled peptide to solubilized receptor. These data indicate that the solubilized receptor may have lost association with its G-protein. In conclusion, the here presented protocol allows solubilization of the GLP-1(7–36)amide receptor in a functional state, and opens up the possibility for further molecular characterization of the receptor protein.

GLP-1(7–36)amide; Receptor; Solubilization; RINm5F

## 1. INTRODUCTION

It is well established that postprandially released intestinal hormones positively modulate insulin secretory function [1]. Recent findings suggest glucagon-like peptide (GLP-1(7–36)amide) represents an important physiological incretin in mammals including man [2].

GLP-1(7–36)amide is a post-translational product of preproglucagon processing in the mammalian intestine [3,4]. It is released into the circulation in response to oral glucose [5] and has a potent stimulating effect on the glucose-induced insulin secretion [4,6–8]. Furthermore, it was demonstrated that GLP-1(7–36)amide strongly inhibits the pentagastrin-induced gastric acid output [9,10]. Thus, GLP-1(7–36)amide is, in addition to a proposed role as an incretin, also under consideration as an 'enterogastrone' candidate.

Previously, we identified and characterized GLP-1(7–36)amide receptors on rat insulinoma-derived RINm5F cells [11] and rat lung plasma membranes [12]. We demonstrated that, after binding to RINm5F cells, GLP-1(7–36)amide is internalized and further processed intracellularly [13]. Furthermore, binding of GLP-1(7–36)amide to RINm5F cells results in a cyclic AMP increase [11]. Moreover, binding of GLP-1(7–36)amide to RINm5F and rat lung plasma membranes is decreased in the presence of guanine nucleotides [14,15] indicating that GLP-1(7–36)amide receptors in RINm5F cells and rat lung are coupled to G-proteins.

In the present study we present a protocol allowing the solubilization of GLP-1(7–36)amide receptors in an active form. This is of particular interest since up to now the solubilized receptor for GLP-1(7–36)amide has not been available to allow further detailed biochemical characterization.

## 2. EXPERIMENTAL

### 2.1. *Materials*

GLP-1(7–36)amide was purchased from Peninsula Laboratories Europe Ltd., St. Helens, Merseyside, UK. Disuccinimidyl suberate, octyl-β-glucoside, Lubrol PX and Triton X-100 were from Pierce Europe B.V., Oud Beijerland, The Netherlands. Human serum albumin was from Behring, Marburg, Germany. Phenylmethylsulfonyl fluoride (PMSF) and CHAPS were obtained from Sigma, Deisenhofen, Germany. Bacitracin was purchased from Serva, Heidelberg, Germany. $^{125}$I-labelled GLP-1(7–36)amide (specific activity approximately 74 TBq/mmol) was prepared as described previously [11].

### 2.2. *Cells*

RINm5F cells were grown under conditions as described by Praz et al. [16]. Cells were detached from the surface of plastic culture bottles using phosphate-buffered saline (NaCl 135 mM, KCl 2.7 mM, $Na_2HPO_4$ 8.1 mM, $KH_2PO_4$ 1.5 mM, pH 7.3) containing 0.7 mM EDTA. Cell concentrations were determined using a Neubauer haemocytometer.

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2.3. *Preparation of RINm5F plasma membranes*

RINm5F plasma membranes were prepared as described previously [17]. Briefly, approximately $6 \times 10^8$ cells were resuspended in ice-cold buffer (Tris–HCl 10 mM, NaCl 30 mM, dithiothreitol 1 mM, PMSF 5 μM, pH 7.5) and disrupted using a glass-glass homogenizer. The homogenate was layered over a 41% (w/v) sucrose solution and centrifuged at 95,000 × *g* for 60 min at 4°C. The band of membranes at the interface of the layers was collected, diluted fourfold with homogenisation buffer and centrifuged for 30 min at 40,000 × *g* and 4°C. The pellets were resuspended in solubilization buffer (HEPES 10 mM, PMSF 1 mM, NaCl 118 mM, KCl 4.7 mM, $MgCl_2$ 5 mM, EGTA 1 mM, pH 7.4) frozen in liquid nitrogen and stored at −80°C. Protein concentrations were measured as described by Bradford [18].

2.4. *Solubilization of the GLP-1(7–36)amide receptor*

RINm5F plasma membranes were thawed and diluted with solubilization buffer containing different detergents (octyl-β-glucoside, CHAPS, Lubrol PX, Triton X-100; final concentration 1%). Only the solubilized proteins with octyl-β-glucoside and CHAPS retained sufficient quantities of GLP-1 (7–36)amide binding activity. For subsequent studies octyl-β-glucoside was used since it gave the highest yields. For solubilization of GLP-1(7–36)amide receptors RINm5F plasma membranes (1 mg/ml) were incubated in solubilization buffer containing octyl-β-glucoside (final concentration 1%, w/v) for 1 h at 4°C in a gently rotating plastic centrifugation tube. After centrifugation for 60 min at 100,000 × *g* at 4°C the supernatant was frozen in liquid nitrogen and stored at −80°C until use.

2.5. *Receptor binding studies*

Binding studies with [$^{125}$I]GLP-1(7–36)amide were carried out for 60 min at 20°C in a total volume of 250 μl/tube. The incubation buffer, unless otherwise indicated, contained HEPES 10 mM, $MgCl_2$ 5 mM, NaCl 118 mM, KCl 4.7 mM, EGTA 1 mM, PMSF 40 μM, bacitracin 0.01% (w/v) and human serum albumin 0.01% (w/v), pH 7.4. The final concentration of octyl-β-glucoside was 0.2%. Binding assay was terminated by addition of human γ-globulin (final concentration 0.25 mg/ml) and polyethylene glycol (PEG; final concentration 10%) followed by a centrifugation for 30 min at 2,000 × *g* at 4°C. Pellets were counted in a γ-counter.

Both radioactivity associated to the PEG pellet in the absence of solubilized receptors (blanks), and non-specific binding, defined as binding in the presence of an excess of unlabelled GLP-1(7–36)amide (1 μM), were substracted in each experiment.

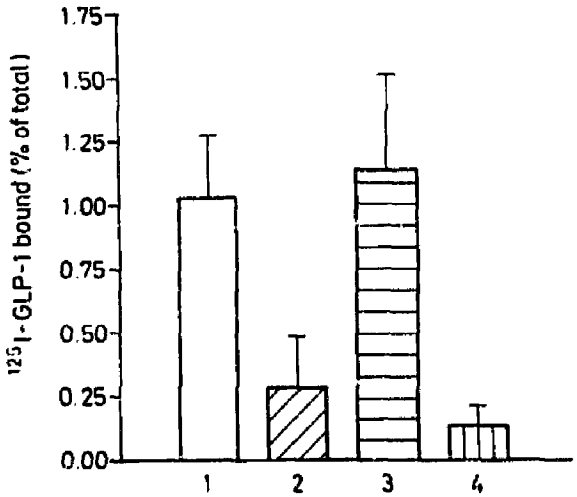


Fig. 1. Effect of different detergents on the solubilization of [$^{125}$I]GLP-1(7–36)amide binding activity. RINm5F plasma membranes (1 mg protein/ml) were incubated for 60 min at 4°C with octyl-β-glucoside (1% w/v) (1), Triton X-100 (1% w/v) (2), CHAPS (1% w/v) (3) or Lubrol PX (1% w/v) (4). After centrifugation for 60 min at 100,000 × *g* at 4°C aliquots of the supernatant were incubated with [$^{125}$I]GLP-1(7–36)amide (approx. 40,000 cpm) for 60 min at 20°C. Data show means ± S.E.M. of 10 experiments.

## 3. RESULTS

### 3.1 *Solubilization of GLP-1(7–36)amide receptors*

Four detergents (octyl-β-glucoside, Triton X-100, CHAPS and Lubrol PX) were investigated for their ability to solubilize active GLP-1(7–36)amide binding protein from RINm5F plasma membranes. The highest yields of GLP-1(7–36)amide binding activity were obtained with octyl-β-glucoside and CHAPS (Fig. 1). For the further experiments octyl-β-glucoside was used.

Solubilization of GLP-1(7–36)amide binding activity was optimal at an octyl-β-glucoside concentration of 1% (Fig. 2a). The use of a higher detergent concentration resulted in a decrease of the obtained binding activity. The optimal protein–detergent ratio was 1:15 (Fig. 2b).

Binding of [$^{125}$I]GLP-1(7–36)amide to solubilized receptors was dependent on the amount of solubilized protein used (Fig. 3a).

### 3.2. *Effect of cations on the binding of [$^{125}$I]GLP-1(7–36)amide to solubilized receptors*

In previous studies investigating the binding of [$^{125}$I]GLP-1(7–36)amide to RINm5F cells and RINm5F plasma membranes we used a modified Krebs-Ringer-HEPES buffer [11]. To optimize the conditions we now studied the effect of monovalent and divalent cations on the binding of tracer to solubilized receptors. Using a 10 mM HEPES-buffer (pH 7.4) containing 1 mM EGTA, human serum albumin 0.01% (w/v), bacitracin 0.01% (w/v) and 1 mM PMSF without cations no specific binding of tracer to solubilized receptors was detectable. Addition of $MgCl_2$ (5 mM) caused a significant increase of tracer binding which was stronger than that observed after the addition of $CaCl_2$ (5 mM) (Fig. 3b). The presence of monovalent cations was necessary for optimal binding of [$^{125}$I]GLP-1(7–36)amide to solubilized receptors. The optimal buffer had the following composition: HEPES 10 mM, $MgCl_2$ 5 mM, NaCl 118 mM, KCl 4.7 mM, EGTA 1 mM, PMSF 1 mM, bacitracin 0.01% (w/v) and human serum albumin 0.01% (w/v) pH 7.4.

### 3.3. *Effect of guanine nucleotides on binding of [$^{125}$I]GLP-1(7–36)amide to solubilized receptors*

To study the effect of guanine nucleotides on tracer binding, solubilized receptor proteins were incubated with [$^{125}$I]GLP-1(7–36)amide in the absence and presence of the non-hydrolyzable analogs GTP-γ-S (0.1 mM) and GDP-β-S (0.1 mM). Both guanine nucleotide analogs had no effect on the binding of [$^{125}$I]GLP-1(7–36)amide to solubilized GLP-1(7–36)amide receptors (Fig. 4).

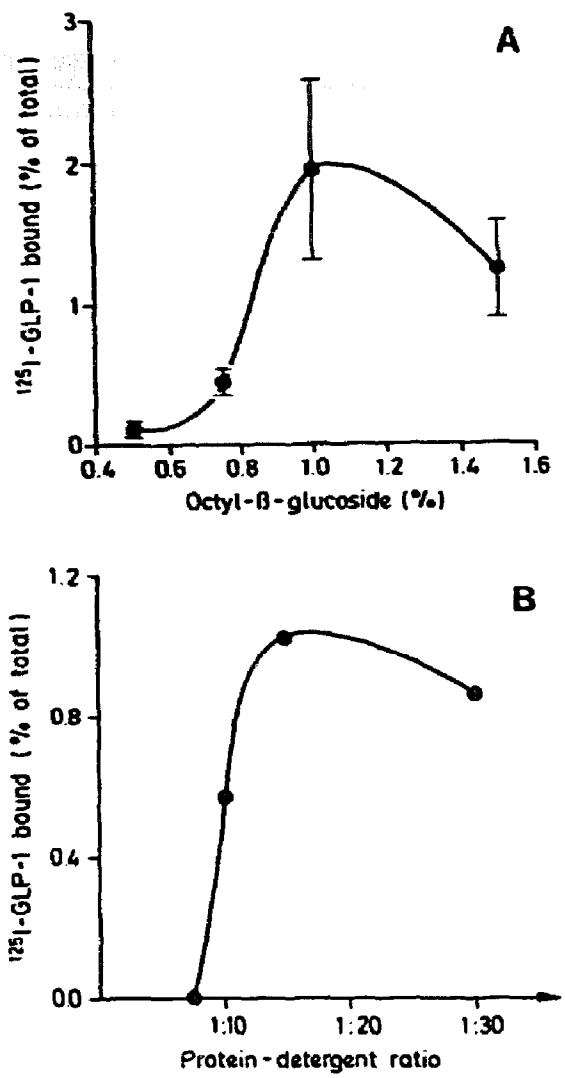


Fig. 2. (a) Effect of octyl-β-glucoside concentration on the solubilization of [$^{125}$I]GLP-1(7-36)amide binding activity. RINm5F plasma membranes (1 mg protein/ml) were incubated with the indicated octyl-β-glucoside concentrations for 60 min at 4°C. After centrifugation for 60 min at 100,000 × g at 4°C aliquots of the supernatant were incubated with [$^{125}$I]GLP-1(7-36)amide (approx. 40,000 cpm) for 60 min at 20°C. Data points show means ± S.E.M. of 10 experiments. (b) Binding of [$^{125}$I]GLP-1(7-36)amide as a function of octyl-β-glucoside-to-protein ratio. Solubilized receptors were incubated with [$^{125}$I]GLP-1(7-36)amide (approx. 40,000 cpm) in the presence of an increasing amount of octyl-β-glucoside/mg protein for 60 min at 20°C. Data points show means of triplicates of a single representative of three experiments.

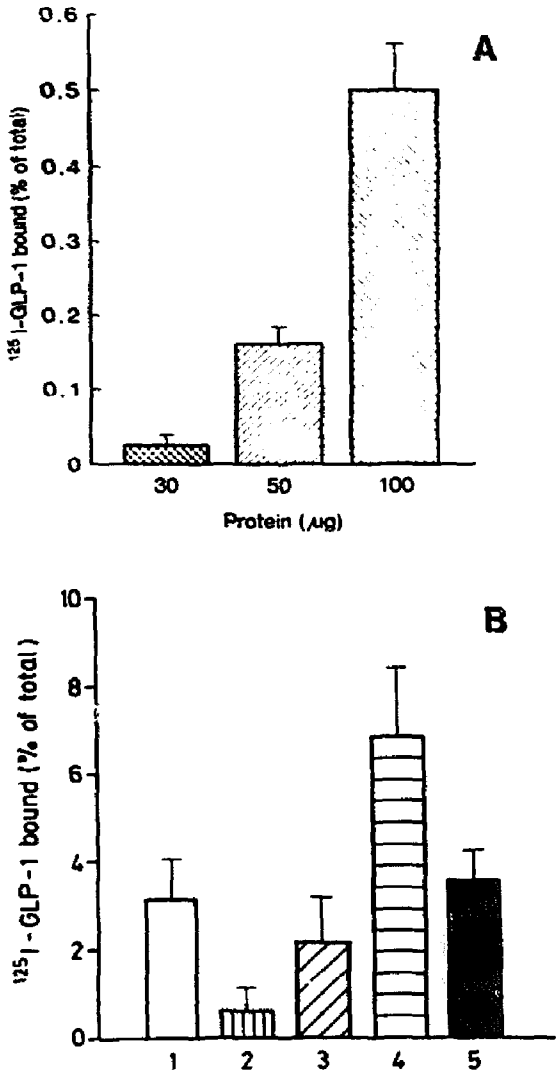


Fig. 3. Effect of protein concentration on binding of [$^{125}$I]GLP-1(7-36)amide. After solubilization of RINm5F plasma membranes (1 mg protein/ml) increasing protein concentrations were incubated with [$^{125}$I]GLP-1(7-36)amide (approx. 40,000 cpm) for 60 min at 4°C. Data points show means ± S.E.M. of 4 experiments. (b) Effect of mono- and divalent cations on [$^{125}$I]GLP-1(7-36)amide binding to solubilized receptors. Solubilized receptors were incubated with [$^{125}$I]GLP-1(7-36)amide (approx. 40,000 cpm) in HEPES (10 mM) buffer (pH 7.4) containing PMSF (1 mM) in the presence of (1) EGTA (1 mM + $MgCl_2$ (5 mM), (2) $CaCl_2$ (5 mM), (3) EGTA (1 mM) + NaCl (118 mM) + KCl (4.7 mM), (4) EGTA (1 mM) + $MgCl_2$ (5 mM) + NaCl (118 mM) + KCl (4.7 mM), or (5) $CaCl_2$ (5 mM) + NaCl (118 mM) + KCl (4.7 mM), for 60 min at 20°C. Data show means ± S.E.M. of 5 experiments.

### 3.4. *Effect of GLP-1(7-36)amide on binding of [$^{125}$I]GLP-1(7-36)amide to membrane-bound and solubilized receptors*

Binding of [$^{125}$I]GLP-1(7-36)amide was inhibited in a concentration-dependent manner by GLP-1(7-36)amide (Fig. 5). Scatchard analysis of the binding data revealed a single class of binding sites with $K_d$ and $B_{max}$ values of 0.26 ± 0.03 nM and 65.4 ± 21.24 fmol/mg of protein for membrane-bound receptors and 22.54 ± 4.42 μM and 3.9 ± 0.79 pmol/mg of protein for solubilized receptors, respectively.

## 4. DISCUSSION

In previous studies, we identified and characterized receptors for GLP-1(7-36)amide on RINm5F cells [11,17]. RINm5F cells possess a single class of GLP-1(7-36)amide binding sites which are coupled to the

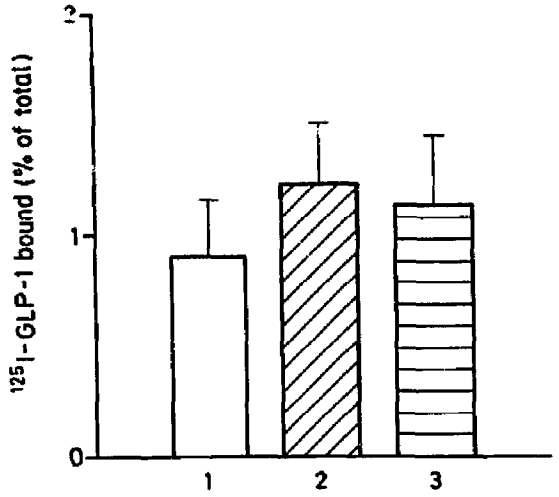


Fig. 4. Effect of guanine nucleotides on [$^{125}$I]GLP-1(7–36)amide binding. Solubilized receptors were incubated with [$^{125}$I]GLP-1(7–36)amide (approx. 40,000 cpm) in the absence (1) and presence of GTP-$\gamma$-S (0.1 mM) (2) or GDP-$\beta$-S (0.1 mM) (3) for 60 min at 20°C. Data show means ± S.E.M. of 16 experiments.

adenylate cyclase system [11,14,17]. Investigations using reducing agents showed that the binding protein of the receptor is not attached to other subunits via disulphide bonds [17]. In additional studies, radiolabelled GLP-1(7–36)amide was cross-linked to GLP-1(7–36)amide receptors in RINm5F plasma membranes. Further analysis by SDS-PAGE and autoradiography revealed that the molecular mass of the ligand-binding protein complex is 63,000 [17].

The present study was carried out to solubilize active GLP-1(7–36)amide receptors from RINm5F membranes thereby employing various detergents. Detergents are widely used to solubilize proteins from cellular membranes. Receptors have been solubilized using Triton insulin [20], IGF-II [21] and EGF [22]; Lubrol was used to solubilize benzodiazepin receptor [23], CHAPS for solubilization of opiate [24] and prolactin [25] receptors, and octyl-$\beta$-glucoside for solubilization of VIP receptor [26]. We investigated Triton X-100, Lubrol PX, CHAPS and octyl-$\beta$-glucoside for their ability to solubilize active GLP-1(7–36)amide receptors from RINm5F plasma membranes. Solubilization with octyl-$\beta$-glucoside and CHAPS gave highest yields of active GLP-1(7–36)amide receptors. There was no significant difference of the ability of these both detergents in solubilizing active GLP-1(7–36)amide receptors.

For further experiments octyl-$\beta$-glucoside was used. The optimal final concentration of the detergent was 1% (w/v) and the optimal protein–detergent ratio was 1:15. Binding of [$^{125}$I]GLP-1(7–36)amide was proportional to the amount of solubilized protein. Investigating the role of mono- and divalent cations we found that the presence of $Na^+$, $K^+$ and $Mg^{2+}$ in the incubation buffer was important for maximal specific ligand binding. Similar results have been found in studies with solubilized CCK receptors from mouse pancreas [27].

Receptors for GLP-1(7–36)amide are coupled to the adenylate cyclase system [14]. Therefore it is easy to understand that binding of [$^{125}$I]GLP-1(7–36)amide to RINm5F plasma membranes is decreased when guanine nucleotides are added to the test system [14]. However, binding of [$^{125}$I]GLP-1(7–36)amide to solubilized receptors was not affected by guanine nucleotides which indicates that the GLP-1-receptor protein may be solubilized as a single protein without a G-protein attached. Another finding argues in the same direction: tracer binding to RINm5F plasma membranes is inhibited by GLP-1(7–36)amide in a concentration-dependent manner with a $K_d$ of 0.26 nM. The same is true for binding of radiolabelled GLP-1(7–36)amide to solubilized receptors but the $K_d$ is 22.54 $\mu$M which indicated a significantly reduced affinity of the receptor against the ligand. Support for these results comes from previous observations that $\alpha_2$-adrenergic, $\beta$-adrenergic, CCK and $D_2$-dopaminergic receptors lose their sensitivity to guanine nucleotides and their high-affinity binding after solubilization [28–32]. Another interesting feature of solubilized GLP-1-receptors is that they exhibited a much higher binding capacity than membrane-bound receptors. This could be due to an exposition of cryptic GLP-1(7–36)amide-specific binding sites as has been suggested similarly for CCK receptors [27]. On the other hand, solubilization and separation from other membrane proteins could mean a relative enrichment of specific binding sites.

Our results show that active GLP-1(7–36)amide receptors can be solubilized from RINm5F membranes. This opens up the possibility to further purify these

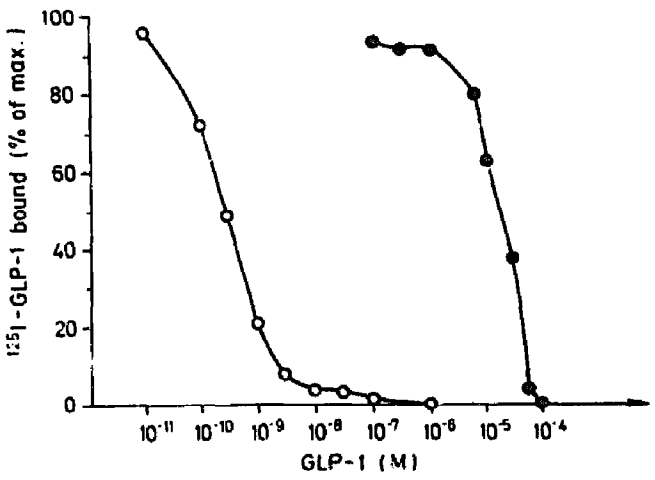


Fig. 5. Inhibition of [$^{125}$I]GLP-1(7–36)amide binding by unlabelled GLP-1(7–36)amide. RINm5F plasma membranes (○) were incubated with [$^{125}$I]GLP-1(7–36)amide (approx. 40,000 cpm) for 30 min, solubilized receptors (●) for 60 min at 20°C in the presence of the indicated concentrations of unlabelled GLP-1(7–36)amide. Data show means ± S.E.M. of 6 and 7 experiments, respectively.

receptors which is mandatory in order to characterize their molecular properties in more detail.

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