Partial purification and characterization of the glucagon receptor

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Received 21 March 1983

Specific labeling of liver plasma membrane glucagon receptors has been achieved by the photoincorporation of a ^{125}I-labeled photoderivative of glucagon, N^E-4-azidophenylamidinoglucagon. Identification of glucagon receptors was facilitated by irradiating membranes in the presence of excess unlabeled glucagon. Isoelectric focusing of radioiodinated membrane proteins revealed one major band of glucagon displaceable material which had an isoelectric point of 5.85. When this material was isolated and run on SDS–polyacrylamide gels a major labeled band of M_r 55000 was obtained which had properties consistent with those of the glucagon receptor. These studies indicate that a purification of the glucagon receptor of >700-fold can be attained through the use of isoelectric focusing and SDS–polyacrylamide electrophoresis.

Glucagon *Hormone receptor* N^E-*4-Azidophenylamidinoglucagon*

1. INTRODUCTION

Although the glucagon receptor has been studied extensively, it has not been purified. Attempts at purification have relied on detergent solubilization of liver plasma membrane-receptor binding sites prebound with saturating amounts of ^{125}I-glucagon [1,2]. Unfortunately, detergent treatment of the membranes disrupts the hormone–receptor complex and detergent micelles containing the radio-ligand are formed which further complicate the analysis. These limitations, together with the relatively low concentrations of receptors in the cell, have severely hampered progress in isolation and purification of the glucagon receptor.

Abbreviations: APA-glucagon, N^E-4-azidophenylamidinoglucagon; DTT, dithiothreitol; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Here, a new approach, based on photoaffinity labeling of the glucagon receptor, has been taken. We have synthesized a photoaffinity analogue of glucagon which when radiolabeled was able to covalently attach to a protein of M_r 55000 (D.E.W. et al., submitted). The incorporation into the receptor was reduced by the addition of native glucagon or GTP, providing evidence that the protein labeled is the glucagon receptor or some subunit thereof.

Using photoaffinity labeling in conjunction with isoelectric focusing and SDS–PAGE, we have been able to achieve purification of the glucagon receptor of >700-fold.

2. MATERIALS AND METHODS

The synthesis, purification and iodination of APA-glucagon was as submitted (D.E.W. et al.).

Liver plasma membranes were prepared as in [3]. Photoaffinity crosslinking of radiolabeled APA-glucagon to liver plasma membranes was

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00145793/83/$3.00

carried out as follows: membrane preparations (1 mg protein/ml) were suspended in 30 mM Tris buffer (pH 6.9) with or without unlabeled bovine glucagon (1 μM). ^{125}I-Labeled APA-glucagon (spec. act. 8×10^5 cpm/pmol) was then added to 0.2–1 nM final conc., the membrane suspension was incubated for 15 min and the resulting pellet was resuspended in 2 ml Tris buffer; 0.5 ml aliquots of the resuspended membranes were placed in wells on a porcelain plate which was cooled on ice. The membranes were photolyzed for 1 min with a Rayonet RMR-400 photochemical reactor (254 nm lamp) with the lamp placed 2 cm over the wells. Following photolysis, the membranes were separated from non-covalently bound label by centrifugation in Tris buffer containing 1% albumin, then by centrifugation with Tris buffer alone.

Samples for isoelectric focusing were either heated for 5 min at 100°C in 2% SDS 50 mM DTT or were treated at room temperature with 10 mM CHAPS. The samples were cooled to room temperature and buffer was added to give 8 M urea, 2% Nonidet P-40 and 2% carrier ampholytes (Bio-Rad) final conc.

Isoelectric focusing was performed in a similar manner to that in [4]. The gel composition was: 4% acrylamide, 0.25% *N,N'*-methylenebisacrylamide, 2% Nonidet P-40, 8 M urea, 2% carrier ampholytes pH 3/10 and 5/7 (Bio-Rad), 0.11% *N,N,N',N'*-tetramethylenediamine and 0.02% ammonium persulfate. Isoelectric focusing was done in 13 cm long glass tubes with 5 mm i.d. Sample solution (100 μl) containing from 50–200 μg protein was placed on the gel which was overlayered with 50 μl 8 M urea. The catholyte was 30 mM NaOH and the anolyte was 10 mM phosphoric acid. The samples were electrofocused at 2 mA constant current for 1 h and at 400 V for the remainder of the run.

After isoelectric focusing was complete, gels were either frozen at −80°C or were fixed in 50% methanol, 12% trichloroacetic acid, 0.5% $CuSO_4$ for ⩾6 h and stained with 0.1% Coomassie G250 (Sigma), in 27% isopropanol, 10% acetic acid, 0.5% $CuSO_4$ for 2 h. After staining, the gels were destained in 20% methanol, 7% acetic acid for ⩾6 h on a shaker until the protein bands were discernible from background. The frozen gels were serially sliced into 1 mm fragments with a Hoefer vibrating wire gel slicer and the slices were counted in a Packard gamma counter. After counting, gel slices were taken up in 1 ml water (4 slices/tube), left for 1 h and the pH was determined [5]. Protein was recovered from the pooled gel slices by adjusting the pH to 7 with 0.1 M NaOH and incubating them at room temperature for 24 h with vortexing at frequent intervals. With this procedure up to 80% of the radioactivity in the slices could be recovered. Protein isolated from the isoelectric focusing gels was dialyzed against water for 48 h, lyophilized and resolubilized in Laemmli buffer [6] for application to cylindrical 8% acrylamide resolving gels. The gels were sliced as above and counted.

3. RESULTS AND DISCUSSION

Isoelectric focusing of liver plasma membrane proteins covalently labeled with ^{125}I-labeled APA-glucagon revealed the presence of 4 minor radioactive components, at pH 6.4, 6.05, 5.25 and 4.84, and one major peak which focused at pH 5.85 (fig.1).

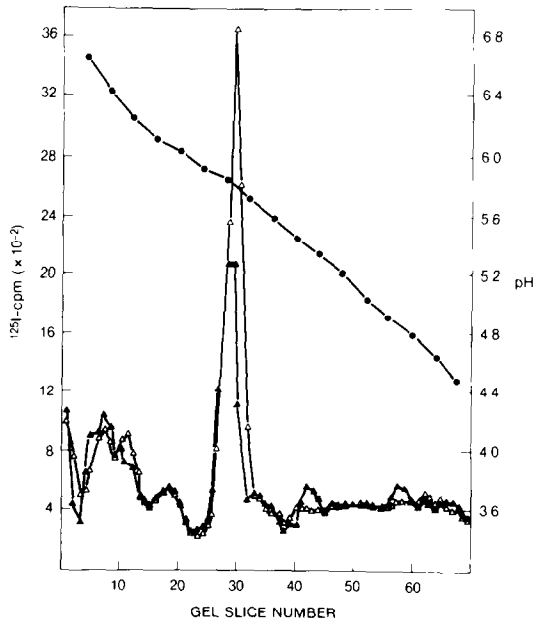

Fig.1. Gel isoelectric focusing of SDS–solubilized liver plasma membranes photolysed with ^{125}I-labeled APA-glucagon incubation in the presence (▲) or absence (△) of 1 μM unlabeled glucagon. Focusing was carried out for 7000 V.h at 25°C. Experimental conditions were as in section 2.

Photoaffinity labeling of the major band could be selectively inhibited by preincubation of membranes with excess unlabeled glucagon while the minor labeled bands were unaffected. The content of glucagon receptors in liver plasma membranes, based on ^{125}I-labeled glucagon binding data, was 1–2 pmol/mg membrane protein [7]. In fig.1, 290 fmol ^{125}I-labeled APA-glucagon/mg membrane protein were incorporated into the protein peak focusing at pH 5.85. This labeling was reduced by 35% in the presence of excess unlabeled glucagon.

The Coomassie blue staining pattern of the focused membrane proteins is shown in fig.2; at least 6 major bands and several minor components can be seen. The lack of any major staining protein band focusing at pH 5.85 is consistent with the idea that the glucagon receptor is present in only very small amounts in the cell membrane. An estimate of the amount of glucagon receptor protein present in liver plasma membranes as a percentage of total membrane protein, assuming a receptor M_r of 55000 [8,9], suggests that glucagon receptors comprise $<0.01\%$ of the total membrane protein.

Solubilization of samples for electrofocusing was routinely achieved by boiling membranes in 2% SDS [10]. Although classical methods of electrofocusing rely purely on non-ionic detergents, such as Nonidet P-40 to solubilize samples [11], we have found them to be inadequate for solubilizing liver plasma membranes for electrofocusing. Similar problems were encountered in [10] where Nonidet P-40 failed to solubilize certain protein components of bacterial membranes. Although ionic detergents have been used in gel isoelectric focusing [4,10] their presence can give rise to spurious isoelectric points [12]. We thus felt that it was important to establish an isoelectric point for the glucagon receptor solubilized in the absence of SDS. Mixtures of non-ionic and zwitter-ionic detergents have been successfully used in the past to solubilize liver microsomal membrane proteins for gel electrofocusing [13] and we have used a similar approach here; i.e., CHAPS and Nonidet P-40. A plot of pI for both the SDS and CHAPS solubilized glucagon receptors *vs* isoelectric focusing time is shown in fig.3. In both cases a steady state of focusing after about 5000 V.h was attained. The isoelectric points for the CHAPS-solubilized (6.00) and SDS-solubilized (5.92) glucagon receptor were almost identical.

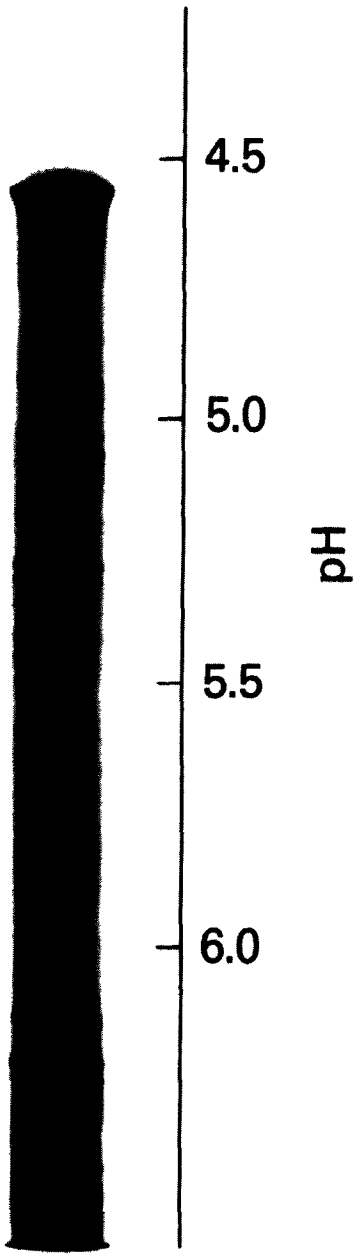

Fig.2. Isoelectric focusing pattern of liver plasma membranes: 200 μg liver plasma membranes were solubilized in SDS sample buffer and applied to isoelectric focusing gels (pI 7–4) as in section 2; proteins were stained with Coomassie brilliant blue.

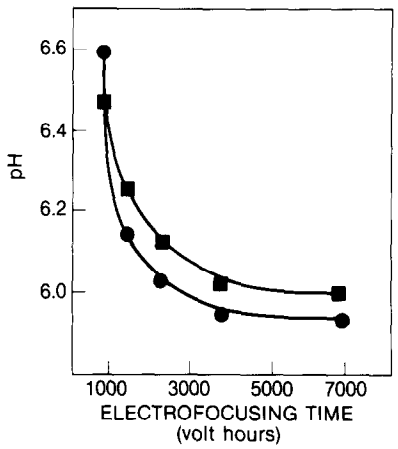

Fig.3. Effect of electrofocusing time on the apparent isoelectric points of SDS-solubilized (●) and CHAPS-solubilized (■) liver plasma membrane glucagon receptors photolysed with ^{125}I-labeled APA-glucagon. Liver plasma membranes, labeled as in section 2, were electrofocused for the indicated times and the apparent pI of the glucagon receptor was determined.

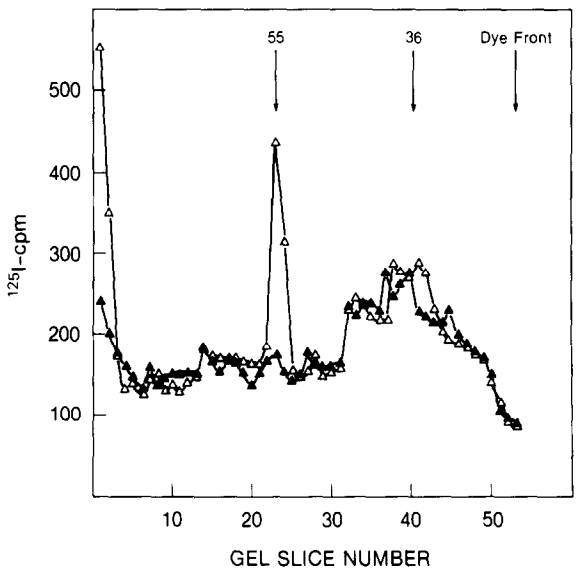

Fig.4. SDS–PAGE of isoelectric focused purified glucagon receptor. The major peak of ^{125}I-labeled protein migrating with a pI of 5.75–5.95 (fig.1) was eluted from isoelectric focusing gels as in section 2 and rerun on SDS–PAGE. Material was derived from labeled receptor incubated in the presence (▲) or absence (△) of 1 μM glucagon. The positions of M_r 55000 and 36000 markers are indicated.

The radioactive protein peak identified as the glucagon receptor from the isoelectric focusing experiments was eluted from the gel slices, resolubilized in SDS sample buffer and subjected to SDS–PAGE (fig.4). SDS–PAGE of the protein from the pI 5.85 peak revealed two minor bands of radioactivity with M_r 36000 and 45000 and one major peak migrating with M_r 55000. Only the M_r 55000 protein was sensitive to preincubation with excess unlabeled glucagon. The identification of the SDS-denatured glucagon receptor as an M_r 55000 protein is fully consistent with that reported [8,9]. In fig.4, about 30 fmol ^{125}I-labeled APA-glucagon/mg membrane protein were incorporated into the M_r 55000 protein (glucagon receptor). These data indicate that about 3% of the glucagon receptors are covalently labeled by the photoaffinity probe.

A summary of the partial purification of the glucagon receptor by isoelectric focusing is shown in table 1. Based on these data a 70-fold enrichment of the glucagon receptor has been achieved. A far greater enrichment of the receptor is possible if isoelectric focusing of membranes is followed by SDS–PAGE. When the material focusing at pH 5.85 was run on SDS–PAGE and stained for protein, only two bands, with M_r 70000 and 38000,

Table 1

Partial purification of the SDS-denatured rat liver plasma membrane glucagon receptor

Step	Protein (mg)	Spec. act. (fmol/mg)[b]	Purific. (-fold)
SDS-solubilized membranes	2	40	1
pH 5.85 peak from isoelectric focusing	0.01[a]	2500[c]	70

[a] Measured by Coomassie blue staining

[b] Specific activity refers to fmol APA-glucagon specifically incorporated into the glucagon receptor as determined by SDS–PAGE

[c] This figure was obtained by running the isolated material from the pH 5.85 peak on SDS–PAGE and determining the amount of radioactive APA-glucagon associated with the receptor peak

Labeled membranes (2 mg) were solubilized in SDS and subjected to isoelectric focusing; protein associated with the peak of radioactivity at pH 5.85 was eluted from the gel and run on SDS–PAGE as in section 2

were seen (not shown). The absence of any stainable protein of M_r 55000 (from the pH 5.85 peak) reflects the low concentration of glucagon receptors in the cell but also indicates that no other major protein contaminants with similar charge and molecular size were present. Since 10 μg protein were recovered from the pH 5.85 peak (table 1) and the receptor constituted <1 μg of this material, the minimum amount of protein we can detect with our Coomassie blue-staining protocol, then a minimum 10-fold purification of the receptor over and above that from isoelectric focusing was achieved by SDS–PAGE. Based on these data we estimate that the glucagon receptor can be enriched >700-fold using the purification scheme discussed above.

These results show that the glucagon receptor is an acidic protein with an isoelectric point ($\pm$ SEM, data from 3 expt) of 5.85 $\pm$ 0.15 at 25°C. This information should prove useful in future investigations aimed at purifying the receptor since separation of the receptor on the basis of charge properties appears to be highly effective in freeing it from other protein contaminants. Studies are underway in our laboratory to isolate and purify sufficient quantities of the glucagon receptor to permit a more extensive characterisation. Purified glucagon receptor might also be of great value clinically in testing for, and recognizing, autoimmune disease states of the receptor. In addition it might also be possible to raise monoclonal antibodies to the glucagon receptor. These would be of great value in studying hormone–receptor interaction, receptor biogenesis and, most importantly, identification of the glucagon receptor gene.

ACKNOWLEDGEMENTS

The authors thank Dr Andreas Chrambach for helpful discussions and Ms Bonnie Richards for typing the manuscript.

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