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Purification and characterization of the head-activator receptor from a multi-headed mutant of *Chlorohydra viridissima*

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In hydra the neuropeptide head activator (HA) is responsible for head-specific growth and differentiation processes. The effects of HA are mediated by high and low affinity receptors on hydra cells. In the current study HA receptors from a multi-headed mutant of *Chlorohydra viridissima* were solubilized from the membrane fraction using 1% Triton X-100 and 2.5 M urea. Scatchard analysis showed that the solubilized receptor had a K_d of 1.55×10^{-9} M, indicating the low affinity subtype of the HA receptor. The solubilized receptor was purified by DMAE chromatography and subsequent affinity chromatography to homogeneity. SDS-PAGE revealed a single protein band with a molecular mass of 96 ± 4 kDa. The native receptor eluted during gel filtration as a 113 kDa protein, and focussed with an isoelectric point of 4.8.

Head activator; Neuropeptide receptor purification; *Chlorohydra viridissima*

1. INTRODUCTION

For head-specific growth and differentiation processes in hydra the neuropeptide head activator (HA) is necessary [1]. This is an undecapeptide with the sequence pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe [2]. Two effects of HA can be distinguished at the cellular level in hydra. At very low concentrations (> 10^{-13} M) it acts as a mitogen for all proliferating cell types, and at higher concentrations (> 10^{-11} M) it affects the determination of uncommitted stem cells into the head-specific direction [3,4]. In good agreement with these in vivo results Scatchard analyses and kinetic studies using isolated hydra membrane preparations revealed the presence of two types of HA binding sites on hydra cells [3]. The low-affinity binding site had a K_d value of 10^{-9} M, whereas the high-affinity binding site had a K_d value of 10^{-11} M. This suggested that the differential effects of HA at the cellular level may be mediated by two different types of HA receptors.

For the isolation of HA receptors we made use of a multi-headed mutant of *Chlorohydra viridissima* which over-expresses both types of HA receptors [3]. Only the low-affinity receptor, which is 100-times more abundant than the high-affinity receptor, survived the solubilization from the membrane fraction. The biochemical purification of this receptor is described in this paper.

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2. MATERIALS AND METHODS

2.1. *Animals*

A multi-headed mutant of *Chlorohydra viridissima*, originally provided by H. Lenhoff, Irvine, was used throughout this study. The animals were cultured in a medium containing 1 mM $CaCl_2$, 0.1 mM KCl and 0.1 mM $MgCl_2$ in 0.5 mM sodium phosphate buffer (pH 7.6) at 19 ± 2°C. They were fed daily with nauplii of *Artemia salina* and washed 6 h later.

2.2. *Membrane preparation and solubilization*

Membranes from *Chlorohydra viridissima* were prepared as described previously [3] and stored at − 70°C until use. For the solubilization, hydra membranes with a protein concentration of 1 mg/ml were mixed 1:1 with solubilization buffer (10 mM ammonium acetate, pH 6.0, 2% Triton X-100, 5 M urea) and incubated for 1 h on ice with vortexing every 5 min. PMSF was added for protease inhibition at a final concentration of 3 mM. The solubilized proteins were separated from the membrane by ultracentrifugation (100 000 × *g*, 4°C, 1 h).

2.3. *HA binding assay and protein determination*

Radioactive [^{125}I]YHA-HA (600 Ci/mmol) was used as a ligand for the HA receptor in binding studies. This biologically active analogue of HA was prepared as described in [3]. Varying amounts of sample were incubated in a total volume of 200 µl of assay buffer which contained 10 mM ammonium acetate, pH 6.0, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgCl_2$, 0.1% Triton X-100, and 10^{-9} M [^{125}I]YHA-HA. After 3 h of incubation at 4°C binding was monitored by separating bound from free ligand by filtration over GF/C filters (Whatman) presoaked over-night in 10% polyethylene glycoll (PEG 6000). The filters were washed 4 times with 4 ml of ice-cold assay buffer without ligand and counted in a γ-counter (Packard, Germany). For the determination of non-specifically bound tracer, the same analysis was performed in the presence of a 1 000-fold molar excess of non-radioactive [I]YHA-HA. All binding data represent the mean of triplicate determinations.

Scatchard analyses were performed in the same way except that varying concentrations of ligand were used. For these experiments [^{125}I]YHA-HA was HPLC purified, which resulted in a higher specific radioactivity of 3,000 Ci/mmol.

Protein concentrations were determined with the BCA protein assay (Pierce, Germany) using BSA as a standard.

2.4. *Chromatography*

All chromatographic steps were performed at 4°C on a FPLC system (Pharmacia, Germany). Fractogel EMD-DMAE-650(M) was used as the stationary phase for anion-exchange chromatography. The separation was done in a non-linear gradient from 0.0 to 1.0 M KCl in 50 mM ammonium acetate, pH 6.0, containing 0.05% Triton X-100. The stationary phase for affinity chromatography was prepared by coupling HA peptide to activated CH-Sepharose 4B according to the instruction manual from Pharmacia, Germany. In an aliquot of this gel, coupling efficiency and quality of the stationary phase was tested by RIA and by chromatography of a polyclonal α-HA serum. The final peptide concentration was 1.6–2.0 mg/ml gel and the matrix showed a high selectivity for α-HA antibodies. For control experiments, stationary phases were prepared by blocking the reactive groups of the CH-Sepharose 4B with an excess of ethanolamine. For the affinity chromatography of the HA receptor, the pooled fractions of the DMAE-chromatography, which contained HA binding activity, were applied to a HA–Sepharose column (10 mm i.d. × 20 mm) equilibrated with 10 mM ammonium acetate, pH 6.0, containing 0.1% Triton X-100 at a flow rate of 0.25 ml/min at 4°C. Non-specifically bound protein was washed from the column with 10 mM ammonium acetate (pH 6.0) containing 0.1% Triton X-100 and 0.1 M NaCl and then with 50 mM Na_2CO_3 buffer (pH 8.0) containing 0.1% Triton X-100 and 2 M NaCl. The bound receptor was eluted with 2 M acetic acid.

The molecular mass of the native receptor protein was determined by gel filtration on a Fractogel TSK-HW65(S) column (16 mm i.d. × 500 mm), equilibrated with ammonium acetate buffer (10 mM, pH 6.0, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgCl_2$, 0.1% Triton X-100) [5]. The elution volume of the native receptor was determined with the HA binding assay. Thyreoglobulin, apoferritin, β-amylase, alcohol dehydrogenase, BSA, and carbonic anhydrase were used as calibration standards.

2.5. *Electrophoresis*

For SDS-PAGE, samples were boiled for 5 min in denaturing buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 12% glycerol, 5% β-mercaptoethanol, 0.01% Bromphenol blue). The proteins were then separated on a 7% polyacrylamide gel according to Laemmli [6], fixed, and visualized by silver staining [7]. For liquid-phase isoelectric focusing, 15 ml of membrane containing 500 mg protein was solubilized as described. The supernatant fluid was diluted to a final volume of 55 ml containing 1% Triton X-100 and 550 μl Bio-Lyte ampholytes, pH range 3–10 (Bio-Rad, Germany). The whole mixture was loaded into a Rotofor electrophoresis cell (Bio-Rad, Germany). Focusing was carried out at 12 W constant power for 3 h at 4°C. The initial conditions were 500 V and 23 mA. At equilibrium the values were 2 000 V and 6 mA. 20 fractions were harvested and their pH values determined. After dialysis against binding-assay buffer, HA binding activity was measured in each fraction as described.

2.6. *Chemicals*

Radioactive ^{125}I (629 GBq/mg) was obtained from Du Pont (Germany), chromatographic materials from Merck (Germany) or Pharmacia (Germany), and all other chemicals were from Sigma, Pierce, or Merck (Germany).

3. RESULTS

3.1. *Alkaline treatment of the membrane and receptor solubilization*

In a previous study [3] we showed that, during cell fractionation, about 90% of the HA receptors were enriched in the membrane fraction of hydra cells. To test the stability of the protein membrane interaction, the membrane was treated with buffers of increasing alkalinity in the presence of 10 mM EDTA. After precipitating and re-dissolving the membrane at pH 6.0, the proportion of retained HA receptors was determined by binding assays. As shown in Fig. 1 nearly 80% of the receptor remained in the membrane even after treatment at pH 11. Most proteins which are only membrane attached were separated from the membrane under such conditions [8]. From this we deduced that the HA receptors are integral membrane proteins.

Solubilization of the active receptor proteins could be achieved by treating the membrane with 1% Triton X-100 in 2.5 M urea (Fig. 2). 60–80% of the receptors were released from the membrane into the supernatant of the subsequent 100 000 × *g* centrifugation. A Scatchard analysis of the solubilized proteins revealed that only the low-affinity type of HA binding sites with a K_d value of 1.55×10^{-9} M had survived the solubilization procedure (Fig. 3).

3.2. *HA receptor purification*

For the isolation of the low-affinity receptor protein, we solubilized a membrane fraction containing 10 mg of total protein. The supernatant of the ultracentrifugation was then used for a purification by means of FPLC-chromatography. The solubilization conditions (1% Triton X-100 and 2.5 M urea) interfered with HA receptor interaction and decreased the receptor stability. Thus a rapid reduction of the non-ionic detergent concentration was necessary. This was achieved during the first purification step by loading the solubilized sample

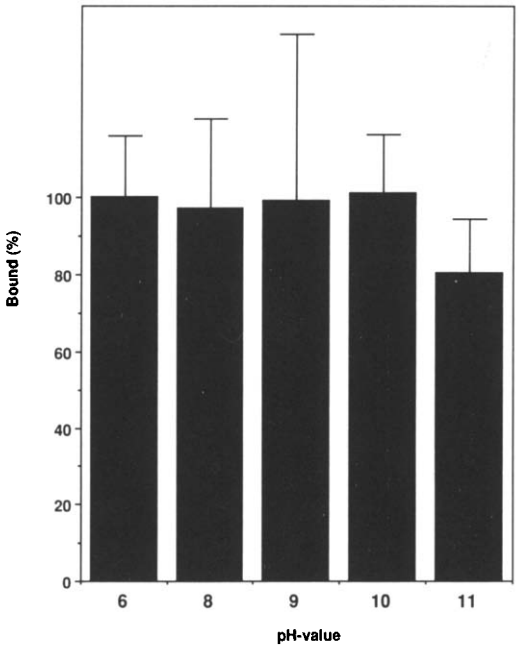

Fig. 1. Stability of the HA receptor membrane interaction. Aliquots of a membrane preparation were incubated in 10 mM Na_2CO_3 buffer (pH 8.0–11.0) containing 10 mM EDTA for 1 h at 4°C. After centrifugation at 100 000 × *g* the membrane was re-dissolved in binding assay buffer (pH 6.0) and binding activity was determined. As a control, the membrane was washed with 10 mM ammonium acetate buffer (pH 6.0) and its binding activity taken as 100%.

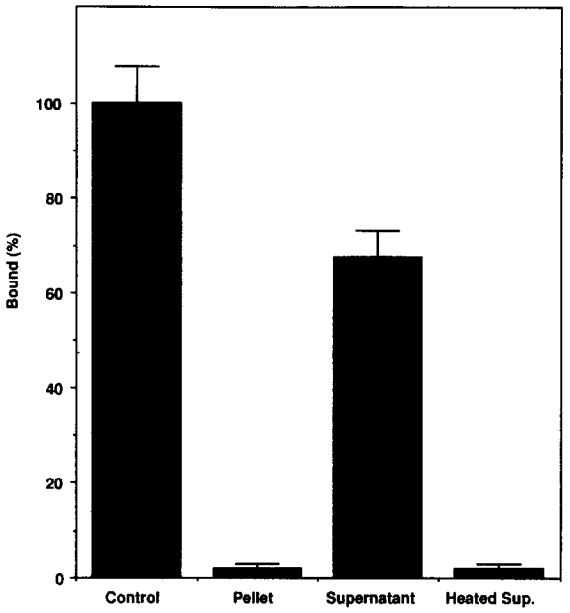

Fig. 2. Solubilization of the HA receptor. Hydra membrane containing 0.5 mg/ml protein was incubated with 1% Triton X-100 and 2.5 M urea for 1 h on ice. After centrifugation (100 000 × *g*, 1 h, 4°C) each fraction was assayed for binding activity. Untreated membrane was taken as a control. Solubilization of a heated membrane served as a negative control.

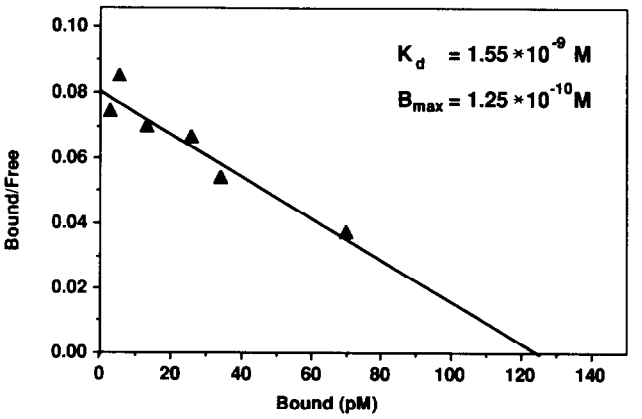

Fig. 3. Scatchard analysis of the solubilized HA receptor. After solubilization, binding assays were performed in the presence of increasing concentrations of radioactive ligand ([^{125}I]YHA-HA). The protein concentration was 0.1 mg/ml. K_d and B_{max} values were derived from a plot of bound/free against bound ligand.

onto a weak tentacle, anion-exchange column (Fractogel-EMD-DMAE) and washing with several vols. of buffer containing only 0.1% Triton X-100 and no urea. The receptor was then eluted with a non-linear gradient of KCl (Fig. 4). During this step the receptor was enriched by a factor of 4.2 with a yield of 71.8% (Table I).

The most active fractions were pooled, dialyzed, and submitted to affinity chromatography on a HA–Sepharose column. Non-specifically bound protein was washed from the column with increasing concentrations of NaCl. The bound receptor did not elute with NaCl but required 2 M acetic acid for the elution. Under these extreme conditions a rapid inactivation of the HA binding activity was observed. But, when dialyzed immediately against a large volume of binding assay buffer 7.15% of the HA binding activity was recovered (Table I). The most active fraction had a specific activity of 2,712.68 fmol/mg. It should be noted, however, that the protein content of these fractions were always close to the detection limit of the BCA protein assay (which was 10 μg/ml) even though they were concentrated prior to protein determination. Thus, the calculated specific activity and the resulting enrichment factor of 243 may be an underestimation. The purity of the final receptor preparation was tested by SDS-PAGE. After silver staining only one protein band was observed (Fig. 5). This band ran as a protein of 96±4 kDa according to molecular mass markers. In some preparations an additional band with a molecular mass of 45 kDa was observed. Since the HA binding activity was present in those purification runs where only the 96 kDa band was detected, we consider it to be the true receptor protein. None of these protein bands was observed when the affinity chromatography was performed using a control Sepharose without bound HA.

3.3. *Determination of the molecular mass and the isoelectric point of the native receptor protein*

Because of the rapid inactivation of the affinity purified receptor some of the biochemical properties were determined using partially purified receptor preparations. To determine the molecular mass of the native receptor the most active fraction from a DMAE-chrom-

Table I

Purification of the HA receptor from *Chlorohydra viridissima*

Purification step	Bound (fmol/ ml)	Yield (fmol)	Yield (%)	Protein (mg/ml)	Spec. bound (fmol/ mg)	Enrichment (factor)
Solubilisized membrane	5.57	11.15	100	0.5	11.15	1.0
DMAE-chromatography	5.34	47.22	80.04	0.113	47.22	4.2
Affinity chromatography	2.66	7.98	7.15	9.8×10^{-4}	2 712.68	234.4

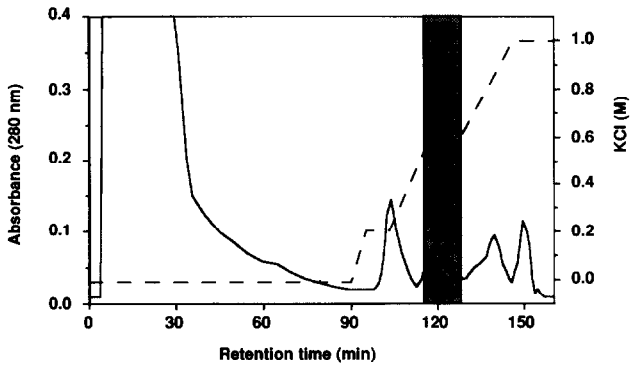

Fig. 4. DMAE-chromatography of the HA receptor. 63 mg of solubilized protein was loaded onto a Fractogel EMD-DMAE-650(M) column (16 mm i.d. × 100 mm) equilibrated with 50 mM ammonium acetate, pH 6.0, containing 0.05% Triton X-100, and eluted with a gradient of KCl at a flow rate of 1 ml/min at 4°C. Samples indicated with a shaded bar contained HA binding activity and were used for the affinity chromatography.

atography was applied to a gel filtration column (Fractogel TSK-HW65(S)). The receptor was detected in the eluate by HA binding assay. The resulting elution volume corresponded to a molecular mass of 113 kDa (Fig. 6). This is a difference of 17 kDa compared to the result of the SDS-PAGE.

We used liquid phase isoelectric focusing to determine the isoelectric point of the native HA receptor. In a first focusing step a solubilized receptor preparation was fractionated in a gradient from pH 3 to 10. The most active samples were pooled and submitted to a fractionation in the narrower gradient from pH 4 to 6. The receptor was focussed between pH 4.6 and 5.0 with a maximum at pH 4.8 (Fig. 7).

4. DISCUSSION

The fresh water coelenterate, hydra, is a suitable model system for developmental biology studies, because it consists of only a few basic cell types and because the pathways from stem cells to terminally differentiated cells are short, comprising a single cell cycle in the case of epithelial cells and nerve cells and 2–4 cycles in the case of nematocytes. An additional advantage is that the substances which influence these differentiations are known. HA, which is available as a synthetic molecule, is necessary for cell growth and for head-

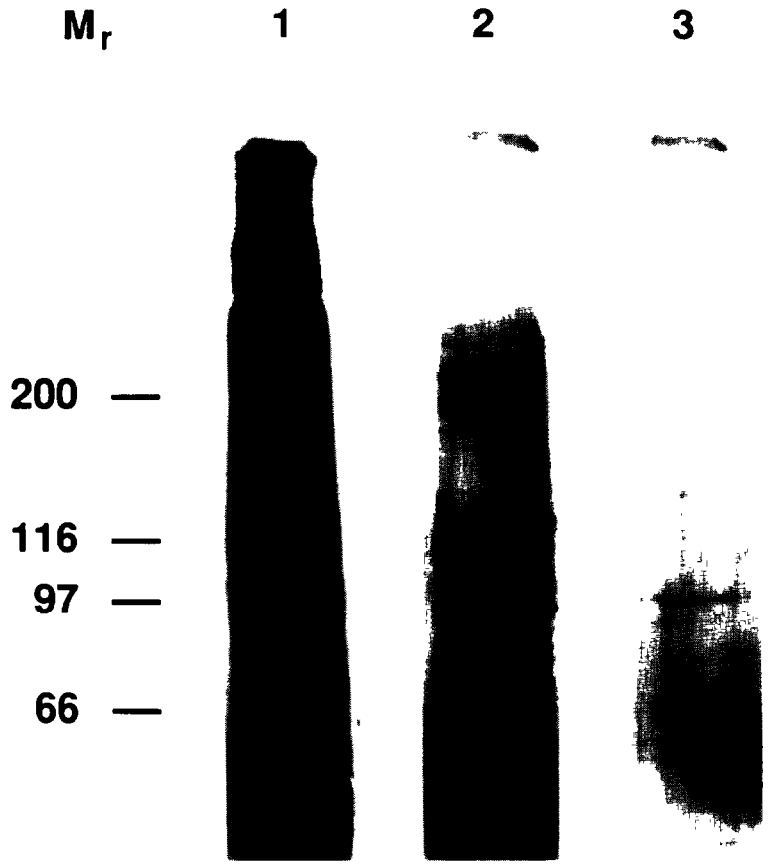

Fig. 5. Analysis of receptor purity by SDS-PAGE. Samples from the different purification steps were denatured and run on a 10% SDS-polyacrylamide gel as described. Lane 1, solubilized membrane; lane 2, pooled active fractions from the DMAE-chromatography; lane 3, affinity-purified receptor which was eluted with 2 M acetic acid from the HA–Sepharose and concentrated by acetone precipitation prior to denaturation. Protein bands were visualized by silver staining. Positions of marker proteins are indicated on the left

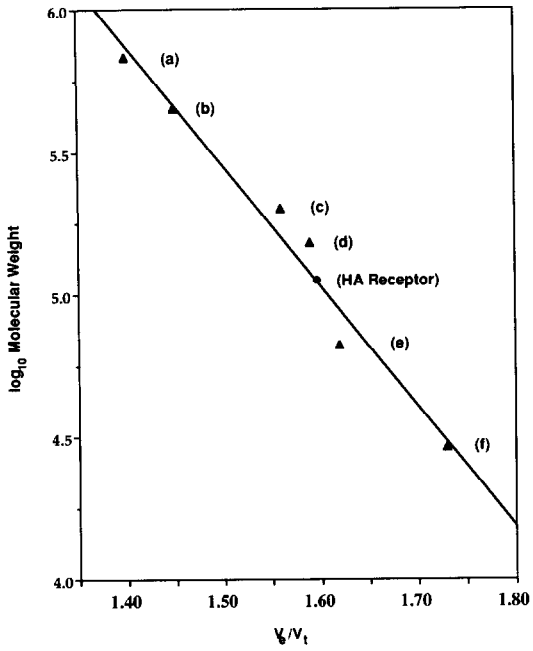

Fig. 6. Molecular mass determination by gel filtration of a partially purified HA receptor. The most active fraction from a DMAE-chromatography was subjected to gel filtration on TSK-HW65(S) (16 mm i.d. × 500 mm) which was equilibrated with 10 mM ammonium acetate (pH 6.0), 0.1% Triton X-100, 0.1 M NaCl, at a flow rate of 1 ml/min at 4°C. The elution volume of the receptor was determined by HA binding assay. Protein standards were (a) thyreoglobulin (669 kDa), (b) apoferritin (443 kDa), (c) β-amylase (200 kDa), (d) alcohol dehydrogenase (150 kDa), (e) BSA (68 kDa), and (f) carbonic anhydrase (29 kDa).

specific cell commitment in hydra [4]. In dose–response experiments using stable synthetic HA analogues, it was found that HA has differential effects depending on its active concentration [3,4]. At concentrations as low as 10^{-13} M, HA stimulated mitosis in interstitial and epithelial stem cells. At higher concentrations (10^{-11} M) and longer incubation times the same cells became committed, in the case of interstitial cells, to nerve cell differentiation, and in the case of epithelial cells, for head-specific differentiation.

In a previous study [3] we characterized the binding of a biologically active iodinatable HA derivative ([^{125}I]YHA-HA) to hydra cells. Scatchard analyses revealed biphasic binding characteristics, suggesting two types of binding sites. The K_d values were 0.9×10^{-9} M for the low-affinity site and 1.25×10^{-11} M for the high-affinity site. These results were confirmed by association and dissociation experiments. From these results it was concluded that hydra cells contain two receptor subtypes with different affinities for HA, and that HA may trigger its different biological effects by binding to different receptors.

In the present study we show the isolation of the low-affinity subtype of HA receptor, which is more abundant on hydra cells, by a factor of 100 times, than the other receptor. Since the receptor remained in the membrane even after alkaline treatment the receptor protein had to be solubilized with high detergent concentrations (1% Triton X-100 and 2.5 M urea). The solubilized HA receptor could be purified to homogeneity by anion-exchange chromatography and affinity chromatography. The success of this purification procedure was mainly due to the strong interaction of the receptor with the HA affinity resin. The disadvantage of this strong binding was that extremely acidic conditions were necessary to elute the receptor protein from the affinity matrix. This led to a loss of HA binding activity, resulting in a relatively low enrichment factor of 243.

The molecular mass of the purified receptor was 96 kDa, as determined by SDS-PAGE. A similar value of 113 kDa was measured with the native receptor by gel filtration.

So far only very few neuropeptide receptors have been characterized biochemically after protein purification. One of them is the receptor for the vasoactive intestinal peptide (VIP) [9]. The VIP receptor was purified by a one-step affinity chromatography to homogeneity. As in our study, the solubilized VIP receptor bound very strongly to the affinity resin and could be eluted under acidic conditions after the non-specifically bound proteins were washed from the column.

For a structural comparison of the HA receptor with other peptide receptors which were characterized using molecular genetic methods [10–13] the characterization

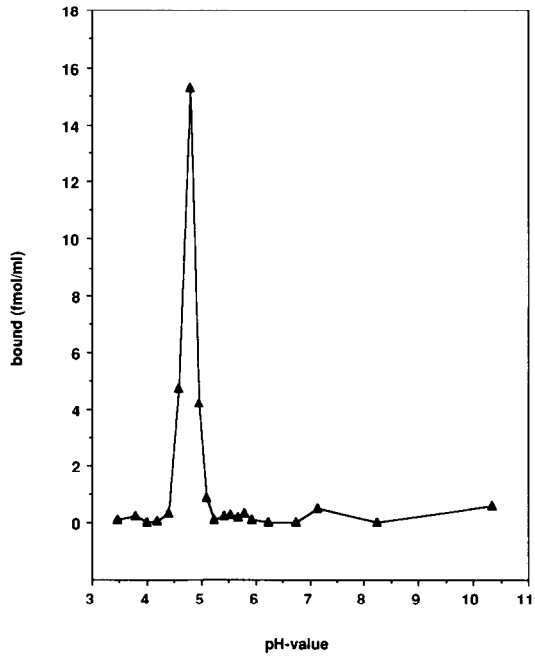

Fig. 7. Liquid-phase isoelectric focusing. The supernatant fluid of a membrane solubilization (500 mg protein) was focused using a rotofor cell (Bio-Rad) in a pH range from 3 to 10 as described. After dialysis each fraction was assayed for HA binding activity.

of the HA receptor gene will be necessary. Thus the aim of our future work will be the microsequencing of HA receptor fragments and subsequent cloning of the receptor cDNA.

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