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Isolation and N-terminal amino acid sequence of an octopamine ligand binding protein

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An octopamine receptor photoaffinity probe was used to label membranes from the light organs of *Photinus pyralis*, a tissue highly enriched in octopamine receptors. Labeling was concentrated in a glycoprotein of 75±2 kDa with lesser labeling of a 79±2 kDa component. Labeling could be displaced by prior incubation with octopamine, mianserin, cyproheptadine, phentolamine or propranolol, with a relative potency that correlated with the ability of these same agents to modulate light organ octopamine-sensitive adenylate cyclase. The 75 kDa binding protein was isolated and its N-terminal amino acid sequence was determined.

Adrenergic receptor; Adenyl cyclase, octopamine-sensitive; Norsynephrine; Octopamine receptor; (*Photinus pyralis*)

1. INTRODUCTION

OA is a neurotransmitter, neurohormone, and neuromodulator in invertebrates, fulfilling a role analogous to that which norepinephrine and epinephrine fulfill in vertebrates [1–3]. An OA receptor, coupled to the activation of adenylate cyclase, was first described over 15 years ago [4], and since then, OA receptor subtypes have been defined on the basis of pharmacological criteria [5]. However, in contrast to recent advances in the isolation of vertebrate amine neurotransmitter receptors, little progress has been made in the isolation of OA receptor proteins. In part, this has been due to a lack of any high-affinity or irreversible OA ligands, a problem particularly relevant to isolation of the lower-affinity OA receptor(s) coupled to the activation of adenylate cyclase.

We have recently synthesized NC-5Z [6], a compound which mimics OA's actions physiologically and is 50- to 100-fold more potent than OA in activating OA-sensitive adenylate cyclase in a number of invertebrate tissues [7]. NC-5Z can be photoactivated bo bind irreversibly to tissue membranes and such binding can be reduced by prior incubation with OA agonists or antagonists in a manner indicating that binding is occurring at OA receptors [7].

Using NC-5Z, we have found that the OA-innervated light organs of fireflies are enormously enriched in OA receptors [7], containing a density of receptors (33–200 nmol/g protein), comparable to that found for cholinergic receptors in the electric organ of *Torpedo* [8], where nicotinic receptors constitute 5% of membrane protein. The high density of OA receptors in the light organ is consistent with the very high specific activity of OA-activated adenylate cyclase in the firefly tissue (1–4 nmol/mg protein per min), which is among the greatest reported for any excitable tissue in the aminal kingdom. In this paper, using NC-5Z and membranes from the light organ, we describe the isolation and N-terminal amino acid sequence of an NC-5Z binding protein which may represent an OA receptor.

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Abbreviations: OA, octopamine; NC-5Z, 2(4-azido-2,6-diethylphenylimino)imidazolidine; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Con A, concanavalin A; G_s, guanine nucleotide binding protein mediating stimulation of adenylate cyclase; N-glycanase, peptide-N^4-[N-acetyl-β-glucosaminyl]asparagine amidase

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number Y07523

2. MATERIALS AND METHODS

2.1. *Tissue preparation*

Light organs from the firefly, *Photinus pyralis*, were prepared as described [7] and homogenized in 6 mM Tris-maleate buffer, pH 7.4. For receptor isolation studies, one-fifth volume of 3 M NaCl was added, and tissue was incubated at 25°C for 5 min. The salted homogenate was diluted with 30 ml 6 mM Tris-maleate (pH 7.4), centrifuged at 120000 × *g* for 30 min, the pellet rehomogenized in 30 ml of buffer, and recentrifuged. The final pellet (P_2 fraction) was resuspended in a volume of 6 mM Tris-maleate, pH 7.4 (containing 10 mM glutathione and 0.1% BSA) equivalent to the starting amount used for homogenization and maintained at 0°C until use. For adenylate cyclase measurements, the initial tissue concentration was 5 mg wet weight/ml and for receptor labeling experiments, 20–50 mg/ml.

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2.2. *Adenylate cyclase assay*

Adenylate cyclase activity was measured, as described in detail in [7], in tubes containing (in 0.3 ml) 80 mM Tris-maleate, pH 7.4; 10 mM theophylline; 8 mM $MgCl_2$; 0.1 mM GTP; 0.5 mM EGTA; 2 mM ATP; 0.06 ml of P_2 fraction; and the compounds to be tested. For antagonist studies, various concentrations of antagonist were tested against a fixed concentration (10 μM) of OA. Inhibitory constants (K_i) for antagonists were calculated as described previously [7].

2.3. *Photoaffinity labeling*

For affinity labeling, membranes were added to tubes containing the reaction mixture described above for the adenylate cyclase assay. Displacing agents were added as described in section 3 and the mixture was incubated in dim light at 4°C for 15 min, following which [^{3}H]NC-5Z (17.4 or 34.4 Ci/mmol) [6] was added to a final concentration of 0.5 to 3 μM and tubes were incubated for an additional 30–60 min in the cold. New ATP and GTP was then added and the contents of the tubes were transferred to quartz (far UV) minicuvettes (1 mm path length, total volume 0.30 ml). Photolysis was carried out at 4°C for 15 min at a distance of 3 cm from three 15 W Sylvania GTE germicidal lamps (G15T8). Cuvettes were turned over every 2 min during the exposure and following photolysis, the contents were transferred to tubes containing 10 ml of 6 mM Tris-maleate, pH 7.4. Tubes were spun at 120 000 × g for 30 min, the pellet resuspended in 10 ml of buffer, and washed twice more. The final pellet was solubilized (20 μg original wet weight of lantern/μl) in 63 mM Tris (pH 6.8) containing 2% SDS and 10% (v/v) glycerol, and placed in a boiling water bath for 2 min. After a 15-h incubation at 25°C, the protein content was determined, and 1/20 volume each of β-mercaptoethanol and 0.1% Bromophenol blue was added to the solubilized protein. In some cases, prior to electrophoresis, solubilized proteins were deglycosylated with N-glycanase (Genzyme) (10 U/ml) for 15 h at 37°C in the presence of 10 mM 1,10-phenanthrolin hydrate to inhibit proteolysis.

2.4. *Electrophoresis*

Aliquots (75–100 μg) of the solubilized proteins were subjected to SDS-PAGE [9], using a 12% separating gel and a 4% stacking gel and running at constant current for 6 h. For greater resolution and for blotting, 9% gels were electrophoresed until 30 kDa proteins were run off the gel. Gels were stained with 0.05% Coomassie brilliant blue R, then destained, soaked in 2.5% glycerol for 1 h, then in Enlightening (Dupont) containing 1% glycerol for 30 min, rinsed briefly in dH_2O, and photographed. Dried gels were mounted on X-ray film for fluorography at −90°C. [^{3}H]NC-5Z-labeled protein bands on the developed autoradiograms were quantitated using a Helena Labs Quick Scan. Labeled peaks were cut out and area was determined by weight. For pharmacological studies of displacement of [^{3}H]NC-5Z from the receptor protein, the area of an [^{3}H]NC-5Z-labeled peak run in the presence of a displacing agent was expressed as a percentage of control ([^{3}H]NC-5Z alone) area.

2.5. *Amino acid sequencing*

Western blots were made from gels run as above except that a 1.5% agarose stacking gel was used and the separating gel was prerun for 12 h in Laemmli running buffer containing 0.1 mM thioglycolic acid. Gels were blotted onto polyvinylidine difluoride (Immobilon, Millipore) membranes in a Hoefer TE-42 transfer apparatus run at constant voltage (7 V/cm) for 1 h using a transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 10% methanol. Some membranes were incubated with Con A (2.3 μg/ml) and Con A binding was visualized with a biotinylated anti-Con A antibody using avidin/biotin peroxidase staining (Vector Labs) with appropriate controls. The remaining membranes were stained with 0.2% Coumassie blue, and areas of membranes containing identified proteins were cut out and subjected to N-terminal amino acid sequencing at the Harvard Biology Dept. Microchemical Core Facility using an Applied Biosystems Model 470A Gas-phase Sequencer. Phenylthiohydantoin amino acid fractions were identified on-line with an ABI 120A HPLC using a C-18 column. Sequencing of the A2 protein was carried out twice.

3. RESULTS AND DISCUSSION

Fluorographs of [^{3}H]NC-5Z-labeled light organ membranes demonstrated two labeled bands: a prominent band (A2) which corresponded to a protein of M_r 75 ± 2 kDa (mean ± SD, n = 14 experiments), and a faint band (A1) which corresponded to a protein of M_r 79 ± 2 kDa (n = 13 experiments) (fig.1). Labeling of both proteins was displacable when prior to photolysis, light organ membranes were incubated with [^{3}H]NC-5Z together with OA or with mianserin, a potent OA receptor antagonist (fig.1). [^{3}H]NC-5Z binding to membranes was not decreased by norepinephrine, dopamine, serotonin or histamine (see [7]).

To quantitate the pharmacological characteristics of binding to A2 and A1, a series of displacement experiments was carried out with various concentrations of OA and several antagonists known to block the light organ OA receptor with differing degrees of potency. Densitometric scans of A2 labeling on fluorograms were integrated and are shown in fig.2A,B. (The pharmacology of binding to the A1 protein was qualitatively similar but, because of its fainter labeling, quantitation was not possible.) In other experiments, the drugs used

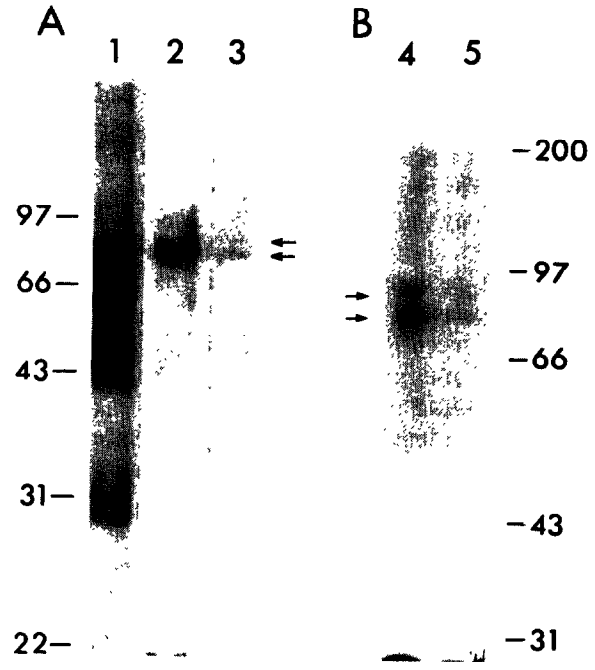

Fig.1. Labeling by [^{3}H]NC-5Z of firefly light organ membrane proteins separated by SDS-PAGE on either a 12% gel (A) or a 9% gel (B). Protein staining (1); fluorogram of [^{3}H]NC-5Z labeling showing two bands, A1 (top arrow) and A2 (bottom) (2,4); displacement of label with 1 mM OA (3) or 1 mM mianserin (5). Molecular mass markers (kDa) are shown on either side.

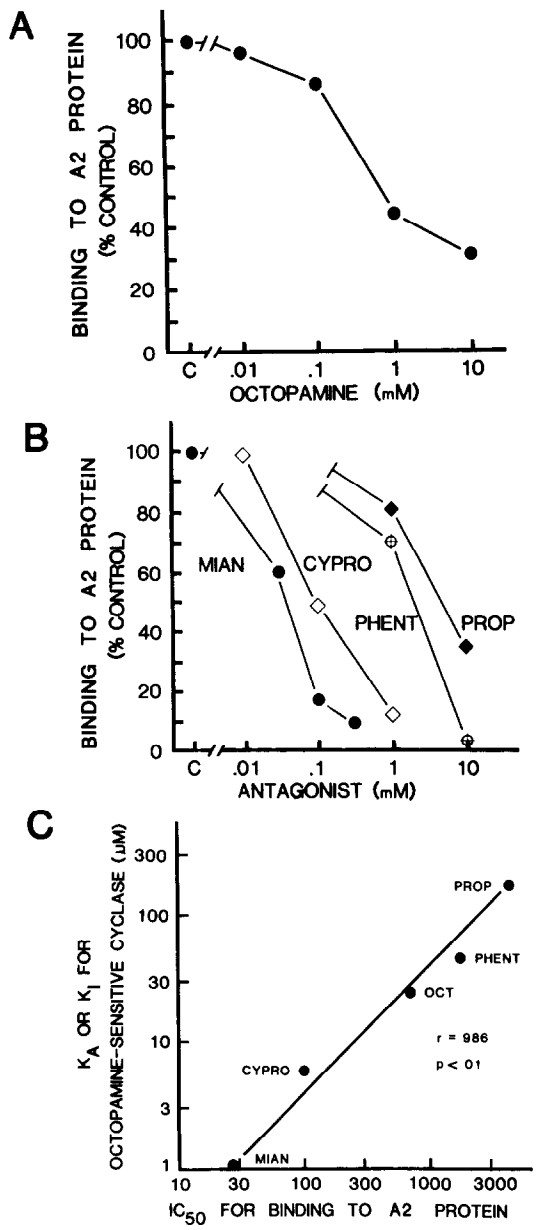

Fig.2. Displacement of [^{3}H]NC-5Z binding from A2 protein by preincubation with increasing concentrations of OA (A) or (in B) by antagonists, mianserin (MIAN), cyproheptadine (CYPRO), phentolamine (PHENT), and propranolol (PROP). The IC_{50} values (in μM) for displacing A2 binding by these agents are plotted in (C) versus the K_a or K_i values for the same agents in activating or inhibiting light organ OA-sensitive adenylate cyclase.

for displacement were evaluated for their effects on light organ OA receptors coupled to adenylate cyclase. The K_a value for activation of adenylate cyclase by OA and the K_i values for inhibition of OA activation by the various antagonists are shown in fig.2C. These values are plotted against the IC_{50} values (determined from fig.2A,B) for the same compounds in displacing labeling of A2. The two sets of values are highly correlated ($r = 0.986$; $P < 0.01$), indicating that the pharmacology of [^{3}H]NC-5Z binding to the A2 protein is consistent with the properties of light organ OA receptors coupled to adenylate cyclase activation. Furthermore, as with the known very large enrichment of OA receptors in light organs, both A1 and A2 were visualized by protein staining in light organ membranes but could not be seen in membranes from firefly head, thorax or abdomen (minus light organ).

Because neurotransmitter receptor proteins are usually N-glycosylated at one or more asparagine residues [10], we subjected light organ membranes to deglycosylation with N-glycanase. Of all the light organ proteins visualized by Coomassie staining on SDS-PAGE, only the A1 and A2 proteins showed a shift in molecular mass, with a single new band appearing at an apparent molecular mass of 72–74 kDa (fig.3). This finding, along with the fact that on Western blots, both proteins labeled heavily with Con A (data not shown) indicates that A1 and A2 are glycoproteins.

Membranes derived from light organs of fireflies which had been frozen for various periods of time showed a decrease in protein staining of A1 and A2 and an increased prominence of a band (B) of M_r 73 ± 2 kDa ($n = 8$ experiments) (fig.4A). In addition, incubation of intact, fresh light organs with either OA or cholera toxin (which raise cyclic AMP levels in light organs [7,11]), or with a lipid-soluble cAMP derivative, markedly increased staining of a protein located in the position of B (fig.4B). In still other experiments (not shown), when both A1 and A2 were electroeluted from gels, concentrated, and then reelectrophoresed, a new band appeared at the position of B (along with the purified A1 and A2 bands). These results suggest that

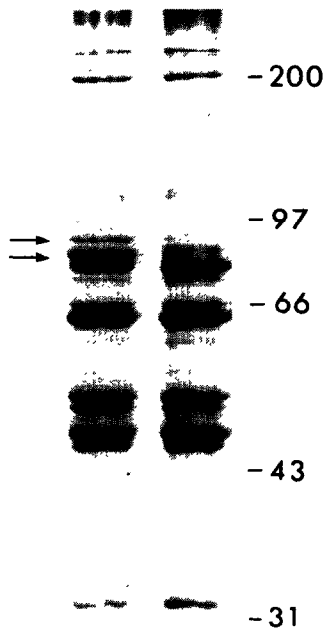

Fig.3. A1 and A2 protein bands (left) are shifted to a new band of lower molecular mass following deglycosylation with N-glycanase (right).

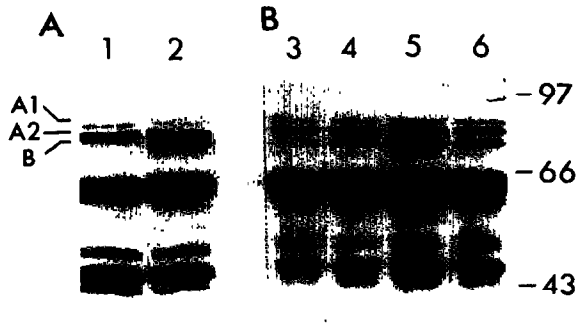

Fig.4. (A)Protein staining of A1, A2, and B bands in fresh (1) and frozen (2) light organs. (B) Change in pattern of Coomassie staining of A1, A2, and B seen following 45 min incubation of fresh, intact light organs (3) with 0.3 mM *p*-chlorophenylthio cyclic AMP (4), 0.4 μg cholera toxin (5), or 1 mM OA (6).

under some conditions, A2 and/or A1 may partially break down to a protein of M_r similar to that of B. Although we observed [^{3}H]NC-5Z labeling of B itself in one experiment, this was not a consistent finding; thus, whether B represents or is derived from an OA ligand binding protein is not clear.

On Western blots, A2 was obtained in sufficient quantity to allow amino acid sequencing. The N-terminal was found to be unblocked, and the sequence obtained (fig.5) did not match any existing proteins in the NBRF protein data base. Although it facilitated sequencing, the observed abundance of A2 (3.7 ± 1.4% of salt-extracted membrane protein (mean ± SD, $n = 7$)) exceeded the figure (1.5% of membrane protein) which would have been expected given the molecular mass of A2 and the previously estimated number (up to 200 nmol/g protein) [7] of G_s-linked OA receptors in the light organ. Assuming that there are no spare receptors undetected by binding studies, this discrepancy

NH_2-Asp-[Asp]-Ile-Lys-Leu-Ser-Gln-Gln-[Tyr]-Asp-
Val-Leu-Asp-Leu-Phe-Lys-Tyr-Met-[His]-Gln- . . .

Fig.5. N-terminal amino acid sequence of A2. Amino acids in brackets indicate a probable but not definitive sequence.

raises a question about the possible relationship of A2 to the G_s-linked OA receptor. On the other hand, supporting a possible relationship are the glycoprotein nature of A2, its pharmacology of binding, its marked enrichment in the light organ versus other firefly tissues, and the known specificity of NC-5Z for OA receptors [7]. Hopefully, use of the data in fig.5 in future cloning studies should help to resolve A2's relationship to OA receptors.

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REFERENCES

[1] Orchard, I. (1982) Can. J. Zool. 60, 659–669.
[2] Axelrod, J. and Saavedra, J.M. (1977) Nature (Lond.) 270, 257–259.
[3] Juorio, A.V. and Robertson, H.A. (1977) J. Neurochem. 28, 573–579.
[4] Nathanson, J.A. and Greengard, P. (1973) Science (Wash. DC) 180, 308–310.
[5] Evans, P.D. (1981) J. Physiol. (Lond.) 318, 99–122.
[6] Nathanson, J.A. and Kaugars, G. (1989) J. Med. Chem. 32, 1795–1799.
[7] Nathanson, J.A. (1989) Mol. Pharmacol. 35, 34–43.
[8] Sobel, A., Weber, M. and Changeux, J.-P. (1977) Eur. J. Biochem. 80, 215–224.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Dowd, B., Lefkowitz, R. and Caron, M. (1989) Annu. Rev. Neurosci. 12, 67–83.
[11] Nathanson, J.A. (1985) J. Cycl. Nucleotide Res. 10 (2), 157–166.