Some properties of a purinergic receptor solubilized from human uterus membranes

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A purinergic receptor was identified in human myometrium membranes using 5'-*N*-[^{3}H]ethylcarboxamideadenosine ([^{3}H]NECA) as radioligand. Scatchard analysis of the binding data gave a K_d of 123 nM with 2.3 pmol ligand bound/mg protein. Displacement studies indicated that the binding site had the characteristics of the A_2 adenosine receptors and some of those of the P_2 purinoceptors since it was inhibited by two slowly degradable ATP derivatives with IC_{50} values comparable to that of NECA. The receptor was solubilized with sodium cholate and its binding properties were the same as those of the membrane-bound form. No -SH group appeared to be essential for the binding activity. By density gradient centrifugation the purinergic receptor–detergent complex was estimated to have an apparent molecular mass of 95 kDa.

Purinergic receptor *5'-N-Ethylcarboxamideadenosine* *Human uterus membrane* *Receptor solubilization*

1. INTRODUCTION

Adenosine exerts many physiological effects on a variety of tissues through modulation of the adenylate cyclase system [1]. Two distict classes of membrane adenosine receptors have been described [2,3]: Ri or A_1 sites responsible for the inhibition of adenylate cyclase and Ra or A_2 sites which mediate an increase in cAMP level. A high affinity towards adenosine analogs characterizes the inhibitory receptors at which L-PIA and CHA are more potent than 2-chloroadenosine and NECA. The stimulatory receptors are low-affinity adenosine receptors at which NECA is more potent than 2-chloroadenosine and much more potent than L-PIA and CHA. A_1 receptors have been extensively studied in crude membrane preparations and have been solubilized from bovine forebrain and rat brain stem [4,5].

The availability of a radioligand preferentially effective on A_2 receptors such as NECA and the observation during a screening study on purinergic receptors in different tissues that human myometrium contained a large number of NECA-binding sites prompted us to characterize these receptors. We report here the solubilization and some properties of the urinergic receptors in human uterus.

Abbreviations: L-PIA, L-N^6-phenylisopropyladenosine; CHA, N^6-cyclohexyladenosine; NECA, 5'-*N*-ethylcarboxamideadenosine; NEM, *N*-ethylmaleimide; p-CMPS, *p*-chloromercuriphenylsulfonic acid

2. MATERIALS AND METHODS

2,8-[^{3}H]CHA (11.5 Ci/mmol) and 2,8-[^{3}H]NECA (30.0 Ci/mmol) were obtained from New England Nuclear, Florence, Italy. Unlabeled NECA was a generous gift from Byk Gulden Lomberg, Konstanz, FRG. Other adenosine derivatives were from Sigma, St. Louis, MO. All other chemicals were of analytical grade.

Myometrial samples were collected from pre-

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menopausal women undergoing hysterectomy and were stored at −70°C until use. Crude membrane fractions were prepared as in [6]. The washed pellet was resuspended in incubation buffer [50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol] with 2 units/ml of adenosine deaminase at 37°C for 30 min to remove contaminating adenosine.

Protein was determined as in [7] using bovine serum albumin as standard.

The binding assay was performed in a final volume of 800 μl containing 300–600 μg protein, incubation buffer and [^{3}H]NECA for 45 min at 4°C. The reaction was terminated by centrifugation at 13 000 × g for 30 min at 4°C. The resulting pellet was carefully rinsed 3 times with 0.5 ml ice-cold 50 mM Tris–HCl (pH 7.4) and incubated with 150 μl Protosol (NEN) for 40 min at 45°C to allow for solubilization. Samples were counted in a Beckman LS 1800 scintillation counter at about 40% efficiency.

For solubilization of the receptor the membrane pellet was suspended in incubation buffer containing protease inhibitors and 1% Na cholate, stirred for 30 min at 0°C and then centrifuged at 105 000 × g for 30 min at 4°C. The resulting supernatant was referred to as the solubilized preparation. Incubation of the solubilized fraction was carried out for 90 min at 4°C, then 0.2 ml bovine γ-globulin (13 mg/ml) and 0.4 ml polyethylene glycol 6000 were added and the mixture was immediately centrifuged as above.

Specific binding was calculated from the total binding minus non-specific binding measured in the presence of 250 μM unlabeled NECA.

3. RESULTS

[^{3}H]NECA at a concentration (100 nM) comparable to its K_d value was found to bind to membranes from rat brain (1790 ± 350 fmol/mg protein), bovine cortex (910 ± 275 fmol/mg protein), rat muscle (38 ± 12 fmol/mg protein), bovine heart atrium (193 ± 61 fmol/mg protein) and septum (41 ± 14 fmol/mg protein), and human uterus (1278 ± 259 fmol/mg protein). When [^{3}H]CHA, a selective ligand for A_1 receptors, was used at a concentration (4 nM) 4-times its K_d value, no binding was observed in the same membrane preparations, except for rat brain (209 ± 45 fmol/mg protein).

A typical binding curve of [^{3}H]NECA to myometrial membranes is shown in fig.1A. A Scatchard plot of the data gave a K_d value of 123 nM and a B_{max} of 2.3 pmol/mg protein. [^{3}H]NECA specific binding increased linearly with protein concentration up to 800 μg per assay.

Similar kinetic analysis of [^{3}H]NECA binding was performed for the solubilized preparations (fig.1B): the K_d value was 144 nM in good agree-

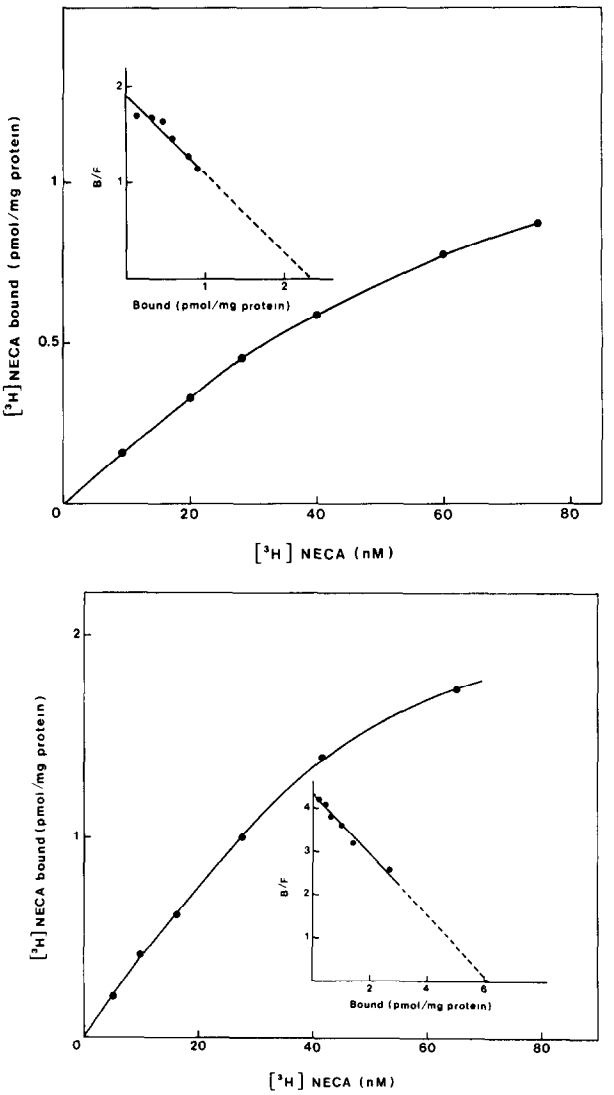

Fig.1. Specific binding of [^{3}H]NECA to human myometrium membranes (A) and solubilized preparations (B). The representative experiment, performed in triplicate as described in section 2, was repeated 3 times. (Inset) Scatchard analysis of the binding data.

ment with the value obtained for the membrane-bound sites. The B_{max} value was 6.2 pmol/mg protein. Since 10–35% of the membrane proteins were solubilized by cholate the total recovery of binding sites in the solubilized form ranged from 40 to 100%.

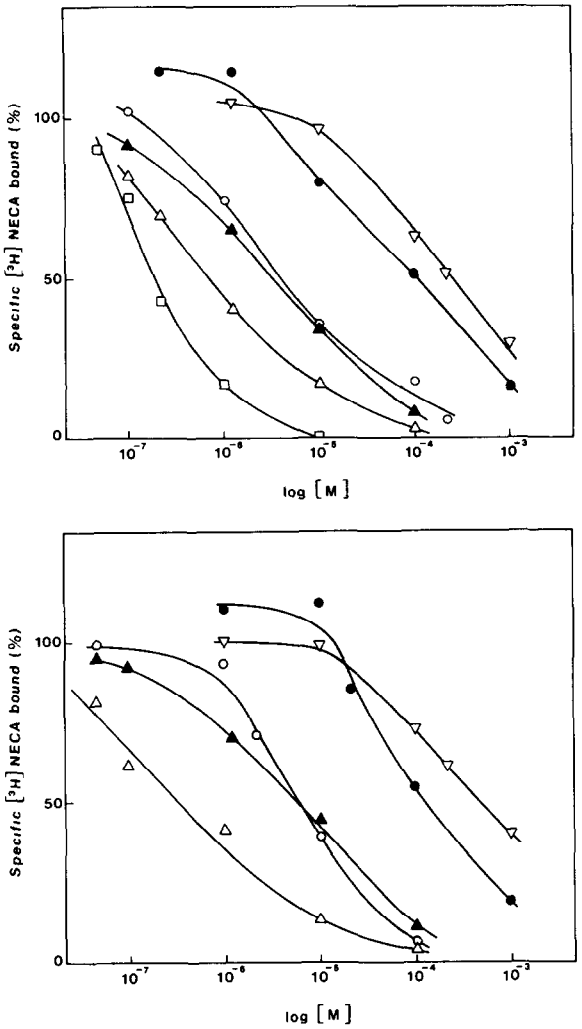

Fig.2. Displacement of membrane-bound (A) and solubilized (B) [^{3}H]NECA specific binding by adenosine derivatives. Data are the mean of 3 independent experiments performed in triplicate as described in section 2 in the presence of 10 nM [^{3}H]NECA. The IC_{50} values for β,γ-methylene ATP (△), α,β-methylene ATP (▲), 2-chloroadenosine (○), 3′-isobutyl-1-methylxanthine (●), and L-PIA (▽) were 6.7×10^{-7}, 3.1×10^{-6}, 4×10^{-6}, 1.1×10^{-4} and 2.4×10^{-4} M, respectively, at the membrane-bound sites and 3.4×10^{-7}, 6.0×10^{-6}, 6.5×10^{-6}, 1.2×10^{-4} and 4.5×10^{-4} M, respectively, at the solubilized sites. The IC_{50} value for NECA (□) reported for the membrane-bound sites was 1.9×10^{-7} M.

To verify whether the characteristics of the membrane-bound and soluble [^{3}H]NECA receptor were essentially similar a series of adenosine derivatives were examined for their ability to inhibit [^{3}H]NECA specific binding (fig.2A,B).

Myometrium membranes were kept frozen at −20°C for 1 week and at −70°C for 15 days without loss of [^{3}H]NECA binding activity. Exposure to a temperature of 65°C for 2 h almost completely abolished the membrane specific binding and preincubation with trypsin (100 μg/ml) for 3 h at 4°C decreased it by 75%. Pretreatment with 3 mM NEM or 1 mM p-CMPS had no effect. As far as the solubilized binding sites were concerned they were stable only for 1 day at 4°C. Storage for 5 days at −20°C led to a 70% loss in binding ability and brief exposure to heat (15 min at 37°C) reduced it by about 80%. NEM and p-CMPS were also without effect on the solubilized form.

Density gradient centrifugation of the solubilized produced a single peak of [^{3}H]NECA binding

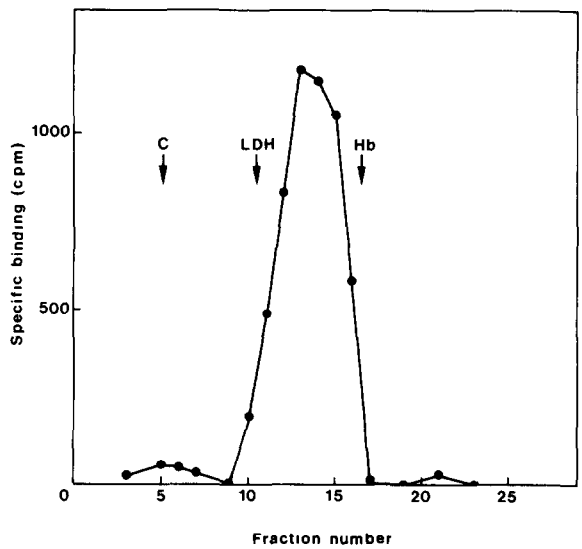

Fig.3. Sucrose gradient centrifugation of the solubilized preparation from myometrium membranes. Aliquots (0.2 ml) were layered on 5-ml, 5–20% sucrose gradients made in incubation buffer plus 0.5% Na cholate and centrifuged at 105 000 × g for 18 h at 4°C. Fractions of about 220 μl were collected and assayed for specific [^{3}H]NECA binding. Beef liver catalase (C), hog muscle lactic dehydrogenase (LDH), and beef blood hemoglobin (Hb) were run in parallel as standards.

activity corresponding to an apparent molecular mass of 95 ± 10 kDa (fig.3).

4. DISCUSSION

We have demonstrated in human myometrium the existence of a large number of binding sites with the characteristics of a purinergic receptor. The affinity constant for [^{3}H]NECA and the order of potency of adenosine analogs in displacing [^{3}H]NECA specific binding conform to the low-affinity, adenylate cyclase stimulatory adenosine receptors (A_2 or Ra) as defined in [2]. The sites do not show the characteristics of the A_1 (or Ri) inhibitory adenosine receptors when tested with [^{3}H]CHA, a more selective ligand for these receptors. α,β- and β,γ-methylene ATP, two ATP derivatives which we observed to be only slightly metabolized under our experimental conditions, were able to displace [^{3}H]NECA with IC_{50} values comparable to that of NECA, indicating that the myometrial binding sites also possess some characteristics of P_2 purinoceptors according to the classification in [8].

Adenyl compounds were shown to induce both contraction and relaxation of the uterus and the existence of two distinct adenosine receptors was hypothesized in this tissue [9]. Our observations that a high receptor recovery was obtained upon solubilization, that the binding properties are the same as in the membrane-bound form, and that a single peak with binding capacity results from density sedimentation of the solubilized extract suggest the existence of a single membrane-bound receptor.

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