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Kinetic and cross-linking studies indicate different receptors for endothelins and sarafotoxins in the ileum and cerebellum

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Kinetics of ligand/receptor interactions using ET-1, ET-3 and SRTX-b were studied and cross-linking experiments carried out in guinea pig ileum and rat cerebellar preparations. Dissociation studies indicate that the two regions are characterized by different receptor subtypes and different modes of ligand binding. Autoradiographic patterns obtained following cross-linking of ET-1 and ET-3 to the different tissues support these conclusions.

Receptor subtype; Endothelin-1; Endothelin-3; Rate of dissociation; Affinity labeling; Brain; Peripheral; Sarafotoxin

1. INTRODUCTION

Data obtained from binding studies and affinity cross-linking experiments suggest the possibility of heterogeneity among the endothelin/sarafotoxin (ET/SRTX) receptors in certain tissues [1–3]. On the other hand, a recent study of the contractile effects of the ET/SRTX family of peptides in the ileum and the binding of these peptides to guinea pig ileum suggests the presence of only one cell surface receptor subtype in this preparation [4]. These findings raise the possibility that the receptors present in the guinea pig ileum are different from those found in brain preparations. In order to investigate this possibility we conducted kinetic studies aimed at determining the rate constants that characterize the binding of three ligands, ET-1, ET-3 and SRTX-b, to their receptors in two different tissue preparations and examined their affinity cross-linking to the membranal target. One of the tissue preparations used, namely membranes prepared from guinea pig ileum, presumably contains homogeneous receptor binding sites [4], while the other, from rat cerebellum, is thought to contain multiple receptor subtypes [5].

2. MATERIALS AND METHODS

2.1. *Materials*

[^{125}I-Tyr^{6}]ET-3 (~2000 Ci/mmol) was purchased from Amersham International (UK). Iodinated [^{125}I]STRX-b and ET-1 were prepared as described previously [6]. ET-1 and ET-3 were from American Peptide Co. (Santa Clara, CA). DSS and DSP were from Pierce (Rockford, IL).

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2.2. *Tissue preparation*

Cerebellar tissue (from adult male Charles River derived rats) and excised guinea pig ileum were washed and homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing protein inhibitors (5 units/ml aprotinin, 5 μg/ml pepstatin, 0.1 mM phenylmethanesulfonyl fluoride, 3 mM EDTA and 1 mM EGTA).

The cerebellar homogenates were centrifuged at 40000 × g and the pellets resuspended in about 50 vols of 10 mM Tris buffer containing 10 μM $MgCl_2$ and the above protease inhibitors. The guinea pig homogenates were filtered through three layers of cheesecloth and centrifuged twice at 30000 × g for 20 min. The final pellet was resuspended in Tris buffer containing the same protease inhibitors.

2.3. *Binding experiments*

Binding of [^{125}I]peptides (ET-1, ET-3 and SRTX-b) to the membranes was carried out at 25°C for 1 h as previously described [4,6].

2.3.1. Dissociation of [^{125}I]peptides

Receptor/ligand complexes were formed by preincubation of membrane preparations for 1 h at 25°C with 10 nM [^{125}I]peptides (ET-1, ET-3 or STRX-b). The membranes were pelleted out by centrifugation (15000 × g, 20 min), washed with ice-cold buffer, and resuspended in the original volume of buffer (5 ml). Dissociation reactions were initiated by the addition of unlabeled peptide (1 μM final concentration). Reactions were terminated either immediately upon addition of the unlabeled peptide (zero time) or at the indicated times.

Specific cross-linking of [^{125}I]derivatives of ET-1 and ET-3 was performed as described in detail previously [5], employing DSP (1 mM) and DSS (1.5 mM), respectively, as the cross-linking reagent.

3. RESULTS AND DISCUSSION

Data obtained previously from binding isotherms in guinea pig ileum mebranes indicate that a single receptor binds ET-1, ET-3 and SRTX-b with similar affinities (K_d values of 0.7, 0.85 and 0.73 nM,

respectively) [4]. In cerebellar preparations the corresponding K_d values were 10, 12, and 3 nM. Hiley et al. [7] also found in rat cerebellum rather similar affinities for these ligands, although the affinities they report are somewhat higher, probably due to the use of different experimental protocols [2,6]. Similar K_d values do not, however, necessarily reflect site identity, and a more accurate indication is provided by kinetic analysis of binding. One such parameter, which is relatively simple to measure, is dissociation rate.

Dissociation of bound ^{125}I-labeled peptides from their preformed receptor/ligand complexes was initiated and the rates measured as described in section 2. As shown in Fig. 1, the dissociation rates for all three ligands were significantly lower in the cerebellar than in the ileum preparations. In addition, in both preparations the rates of dissociation of ET-3 from the receptor were significantly lower than those of ET-1 or SRTX-b. The $t_{1/2}$ values in the ileum are about 18 min for ET-1 and 7 min for SRTX-b, in contrast to more than 2 h for ET-3; in the cerebellum the $t_{1/2}$ values are more than 2–3 h for SRTX-b and ET-1, while for ET-3 the rate of dissociation from the receptor is negligible (under these experimental conditions). The fact that for each of the three ligands the dissociation rate in the cerebellar preparation was significantly different from that in the ileum supports the suggested existence of different receptor subtypes in these two regions. Alternatively, these findings might be explained in terms of interactions of the same receptor with different membrane components in the different tissues. Also worth noting are the differences in dissociative behavior between ET-3 vs ET-1 and SRTX-b in both the cerebellum and the ileum. These differences could stem from dissimilarities in the nature of the receptor/ligand complexes (i.e. different modes of binding) [8]. Recent kinetic studies of the binding of ET-1 and ET-2 to Swiss 3T3 fibroblasts [9] disclosed different rates and extents of dissociation for the two isoforms. It should be noted that the dissociation curves (Fig. 1) were not monophasic. Such a behavior is indicative of either site heterogeneity or ligand induced conformational changes in the receptor, or both. These possibilities are currently under investigation in our laboratory.

Specific cross-linking of the [^{125}I]derivatives of ET-1 and ET-3 in membranes was achieved by binding of the ligand to the receptor (1 nM, 1 h, room temperature), washing of the complex to remove free or loosely bound ligand, and incubation with the bifunctional reagent DSS (1.5 mM) or DSP (1 mM) (1 min, room temperature, 2 mg protein/ml), followed by SDS-PAGE and autoradiography [5]. Autoradiographs from guinea pig ileum membranes in which [^{125}I]ET-1 and [^{125}I]ET-3 were cross-linked are shown in Fig. 2. In both cases, densitometry disclosed one major labeled protein (60–70%), with an apparent M_r of 74–75 kDa. Also detected were two additional bands, which demonstrated weaker labeling at 53 kDa (10–15%) and at 38 kDa (10–15%). The labeling was specific, as indicated by the fact that it did not occur when unlabeled ligand (5×10^{-7} M) was present during binding. The labeled adducts were not seen in the absence of cross-

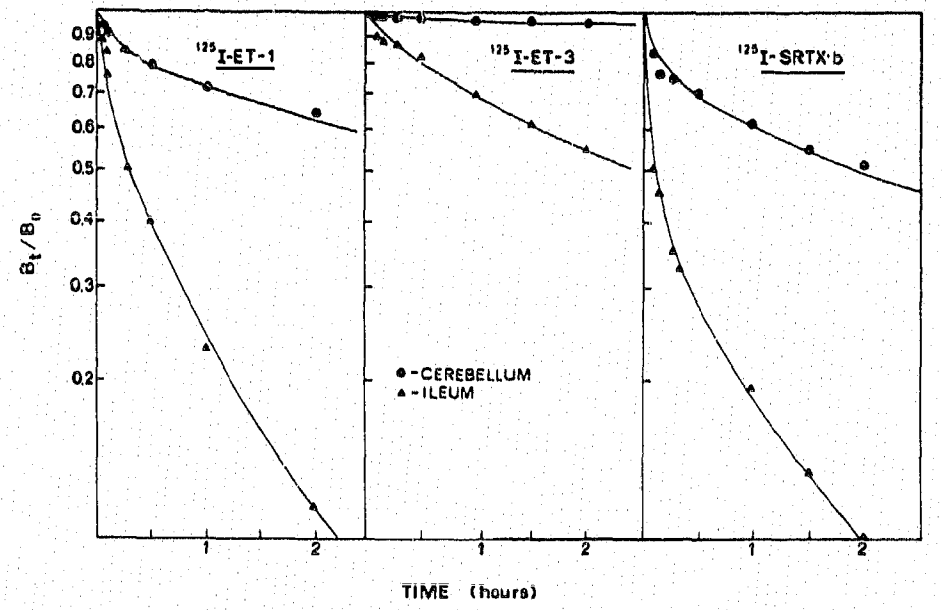

Fig. 1. First-order plots of dissociation of iodinated ET-1, ET-3 and SRTX-b receptor complexes. Dissociation of the preformed complexes was initiated by the addition of 1 μM unlabeled peptide (see section 2). B_0 = amount of radioligand bound at zero time; B_t = amount of radioligand bound at time t. Data are the mean values from four experiments, in which individual data points were obtained in triplicate. Standard deviation was 5–10% of the measured values.

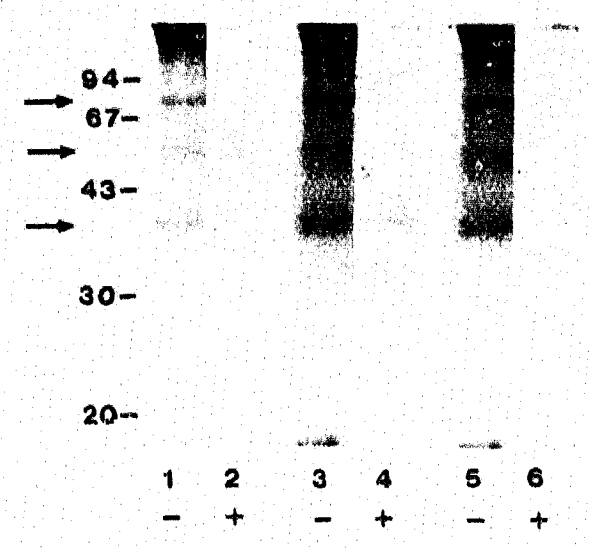

Fig. 2. Autoradiographic patterns derived from electrophoretic analysis of [^{125}I]peptides cross-linked to guinea pig ileum membranes. Membranes (200–300 μg of protein) incubated with 1 nM [^{125}I]ET-1 (lanes 1 and 2), [^{125}I]ET-3 (lanes 3 and 4), and [^{125}I]SRTX-b (lanes 5 and 6) were cross-linked as described in section 2 and then subjected to SDS-PAGE on 10% gels. (–) Total binding; (+) nonspecific binding (in the presence of 5×10^{-7} M unlabeled ligand). The positions of molecular mass standards are shown (M_r 10^{-3}).

linking. Cross-linking of iodinated peptides alone did not produce bands corresponding to those observed upon cross-linking in the presence of the membranes (not shown). Two recent reports have described the existence of an endothelin receptor with an apparent M_r of about 70000 [3,10]. The former report describes the existence of two receptor subtypes having different molecular weights, i.e. 73000 and 60000.

In our previous study [5] conducted in rat cerebellar preparations labeled with [^{125}I]ET-1 we observed one major labeled band with an apparent M_r of 53 kDa (80–90%). Cross-linking of [^{125}I]ET-3 with cerebellar tissue showed the presence of two bands of M_r 53000 and 38000; densitometry disclosed that the former polypeptide accounts for 60–70% of the binding and the latter for 30–40%.

Cross-linking experiments performed at 4°C (not shown) yielded results identical with those obtained at 25°C. In view of this, and because the two types of tissue preparations were labeled simultaneously and under identical conditions, it seems unlikely that the M_r 38000 polypeptide represents a product of proteolysis. A number of possible explanations for the different labeling patterns obtained by various laboratories have been presented elsewhere ([5] and references therein).

These results clearly indicate that in the ileum the majority of endothelin-binding sites are located on a polypeptide of apparent M_r 70000, while in the cerebellum the receptor polypeptides are smaller, having apparent M_r of 50000 and 35000. They therefore support the assumption derived from the kinetic data, namely that binding in the two tissues is associated with different receptor(s). This could imply, as we have suggested elsewhere [2,5,8], that the molecular structure of the receptor/ET complex in brain tissue differs from that in peripheral tissues. One cannot, however, discount the alternative or additional possibility of interspecies differences in the receptor system (e.g. [11]). The fact that in the ileum ET-1 and ET-3 yielded similar labeling patterns but different dissociation data strongly supports the existence of different receptor/ligand complexes resulting from structural differences between the two ligands (as discussed in detail by Kloog and Sokolovsky [1]).

Despite the evidence that the majority of receptor subtypes in the ileal preparation have a molecular weight of around 70 kDa, one cannot rule out the possibility that the physiological response is also mediated by one or both of the minor bands (50 or 38 kDa).

The cloning of two endothelin receptors was reported recently. Arai et al. [12] cloned the receptor from a bovine lung cDNA library, and reported a sequence of 427 amino acids, corresponding to a molecular mass of 48 kDa. The other receptor, cloned from a rat lung cDNA library [13], corresponds to a polypeptide of 415 amino acids (47 kDa). It is not yet known whether these two receptors represent the same subtype; comparison of the reported sequences indicates differences. The differences between the predicted molecular masses and those reported earlier by affinity labeling ([3,4,10] and references cited therein) could reflect different degrees of glycosylation and/or other factors affecting the estimation of molecular mass, e.g. by SDS-PAGE, as suggested earlier [13]. In view of the above, the endothelin receptor subtype identified in the cerebellum might be equivalent to one of these cloned receptors. As for the receptor identified in the ileum, the molecular weight and different pharmacological properties indicate that it is a different subtype of the ET/SRTX receptor. However, at present one cannot discount the possibility that it is basically similar to the cerebellar receptor but undergoes a heavier glycosylation. For example, in the case of muscarinic acetylcholine receptors, glycosylation was shown to be responsible for 25–28% of the estimated molecular mass [14,15].

The physiological findings [4] demonstrate that compared with ET-3, ET-1 is significantly more potent and its removal by washing from the ileum segment occurs more slowly. Since the rate of removal by washing is usually related to the dissociation rate, one might expect ET-3 to dissociate more rapidly than ET-1. This, however, is clearly not the case (Fig. 1). There are several possible explanations for this phenomenon, for example: (i) differences between the preparations employed in the two types of studies (an intact ileal seg-

ment vs. homogenate); (ii) involvement of a desensitization process with different kinetics for ET-1 and ET-3. These and other possibilities are currently under investigation in our laboratory.

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