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Cross-Linking of Endothelin 1 and Endothelin 3 to Rat Brain Membranes: Identification of the Putative Receptor(s)

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ABSTRACT: Affinity-labeling experiments with ^{125}I-endothelin derivatives using bifunctional cross-linking reagents were carried out in an attempt to identify the polypeptide component(s) of the endothelin/sarafotoxin receptors in rat brain tissues. In rat cerebellum, cortex, and caudate putamen, endothelin 1 specifically labeled a major component with a molecular mass of around 53 000. In the same tissues endothelin 3 specifically labeled, in addition to the 53 000 band, a band of molecular mass of 38 000. This result clearly indicates that in the brain the endothelin binding site resides within a polypeptide of apparent M_r = 53 000. The possible presence of receptor subtypes is discussed with reference also to the reported identification of endothelin receptors in chick cardiac membrane and in rat mesangial cells.

In brain and in other tissues, endothelins (ET)[1] and sarafotoxins (SRTX) have been shown to bind specifically to a class of receptors that are associated with the hydrolysis of phosphoinositides (PI) (Ambar et al., 1988, 1989; Kloog et al., 1988; Bousso-Mittler et al., 1989; Jones et al., 1989) and with the mobilization of intracellular Ca^{2+} (Miasiro et al., 1988; Van Renterghen et al., 1988). Mammalian endothelin (ET-1) (Yanagisawa et al., 1988a) and sarafotoxin *b* (SRTX-*b*) (Takasaki et al., 1988) are highly potent vasoconstrictive peptides. Other homologues of this family of peptides, all of which possess 21 amino acid residues, are the mammalian ET-2 and ET-3 (Yanagisawa et al., 1988a,b; Inoue et al., 1989) and vasoactive intestinal contractor (Saida et al., 1989) and the snake toxins SRTX-*a*, SRTX-*c*, and SRTX-*d* (Kochva et al., 1982; Takasaki et al., 1988; Bdolah et al., 1989). All of these peptides bind to the ET/SRTX receptors in rat brain and atrium and induce contractions of smooth muscles in various tissues.

Affinity labeling by means of bifunctional cross-linking reagents has proved to be an extremely useful procedure in the biochemical and pharmacological analysis of many receptors, especially when the questions to be addressed concern multiple receptor subtypes [for review, see Pilch and Czech (1984)]. Recent studies point to the possible existence of receptor subtypes for the endothelin/sarafotoxin family of peptides [Kloog & Sokolovsky, 1989; Kloog et al. (1989) and references cited therein]. This possibility was now examined by the cross-linking technique in three rat tissues: cerebellum, cortex, and caudate putamen. The ^{125}I derivatives of endothelin 1 (ET-1) and endothelin 3 (ET-3) were employed. Cross-linking was achieved by the use of two reagents, di-

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[1] Abbreviations: ET, endothelin; SRTX, sarafotoxin; DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidyl propionate).

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succinimidyl suberate (DSS) and dithiobis(succinimidyl propionate) (DSP).

Materials and Methods

[^{125}I-Tyr^{13}]ET-1 and [^{125}I-Tyr^{6}]ET-3 (~2000 Ci/mmol) were purchased from Amersham International (Buckinghamshire, England). ET-1 and ET-3 were from American Peptide Co. Inc. (Santa Monica, CA). DSS and DSP were from Pierce (Rockford, IL).

Adult male Charles River derived rats were decapitated and the required tissues removed and homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing protein inhibitors (5 units/mL aprotinin, 5 μg/mL pepstatin A, 0.1 mM phenylmethanesulfonyl fluoride, 3 mM EDTA, and 1 mM EGTA).

The homogenates were centrifuged at 40000*g* and the pellets resuspended in about 50 volumes of 10 mM Tris buffer, pH 7.4, containing 10 μM $MgCl_2$ and the above protease inhibitors. Membranes (0.3 mL, 2 mg/mL protein) were incubated in Eppendorf tubes at 25 °C for 1 h with 1 nM ^{125}I-ET-1 or ^{125}I-ET-3 (10-fold isotopically diluted) in the presence and absence of 5×10^{-7} M unlabeled peptides (ET-1, ET-3, and SRTX-*b*). The reaction mixture was centrifuged at 10000*g* for 10 min at 4 °C. The resulting membrane pellet was resuspended in 0.3 mL of ice-cold buffer, pH 7.4 (10 mM sodium phosphate containing 120 mM NaCl), and the indicated cross-linking reagent [50 mM freshly prepared stock solution in dimethyl sulfoxide (DMSO)] was added to a final concentration of 1 mM DSP or 1.5 mM DSS. Control samples received an equal volume of DMSO. Incubation was allowed to proceed for 10 min at room temperature before the reaction was terminated by the addition of 10 μL of 1.5 M ammonium acetate. The mixture was then centrifuged at 10000*g* for 10 min and the resulting pellet resuspended in 50 μL of water and 25 μL of sample buffer (3% SDS, 10% glycerol, 0.001% bromophenol blue, and 62.5 mM Tris, pH 6.8, in the absence of mercaptoethanol). Prior to electrophoresis, insoluble material was removed. The running gel was composed of 10% acrylamide. Upon completion of the electrophoretic run the gel was dried and subjected to autoradiography using Agfa Curix RP-2 film for 5–8 days at −70 °C. Molecular mass standards were run in one lane of the gel for determination of the M_r of the radiolabeled protein species. Generally, about 150–200 μg of solubilized membrane protein was applied to each lane of a gel. In addition to autoradiography, in several cases the gel was sliced into 2-mm sections, which were digested in 5 mL of Lipoluma/Lumasolve (Lumac Inc., Langraaf, The Netherlands) and counted after 24 h.

Results and Discussion

The ligands ET-1 and ET-3, labeled at high specific activity with ^{125}I at Tyr-13 and Tyr-6, respectively, are very useful for affinity cross-linking studies. They exhibit a high percentage of specific binding (~80% of total), and they bind with high affinity to brain binding sites [in rat cerebellum, cortex, and caudate putamen the affinity for ET-1, for example, was in the range of 0.2–2 nM (Ambar et al., 1989; Kloog & Sokolovsky, 1989)]. Moreover, the dissociation time for the ^{125}I-ET-1–receptor complex is slow enough (e.g., in the cerebellum $t_{1/2} > 120$ min at room temperature) to allow time for washing prior to addition of cross-linking reagent.

Specific cross-linking of the ^{125}I derivatives of ET-1 and ET-3 in membranes was achieved by binding of the ligand(s) to the receptor, washing of the complex to remove free or loosely bound ligand, and incubation with the bifunctional reagent DSS or DSP, followed by SDS–PAGE and autoradiography. In preliminary experiments we found that the two

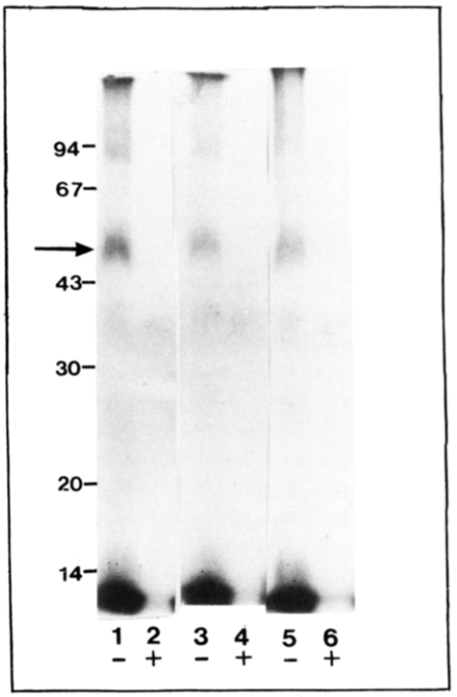

FIGURE 1: Electrophoretic analysis of ^{125}I-ET-1 cross-linked to rat brain membranes. Mebranes (200–300 μg of protein) prepared from cerebellum (lanes 1 and 2), cortex (lanes 3 and 4), and caudate putamen (lane 5 and 6) incubated with 1 nM ^{125}I-ET-1 were cross-linked with 1 mM DSP as described under Materials and Methods. The preparations were then subjected to SDS–PAGE on a 10% gel. The figure shows the corresponding autoradiographic pattern. (−) represents the total binding and (+) the nonspecific binding (in the presence of 5×10^{-7} M unlabeled ligand). The positions of the molecular mass standards are shown ($M_r \times 10^{-3}$).

peptides, when cross-linked to rat brain homogenates as outlined under Materials and Methods, labeled a peptide of $M_r = 53000$. Using this covalent incorporation as an indicator of cross-linking, we then proceeded to establish the optimal experimental conditions: we first compared the effectiveness of the two cross-linking reagents DSS and DSP after incubation for 10 min. Autoradiography revealed that, for cross-linking of ET-1 to the receptor, DSP was more effective than DSS. For ET-3 DSS was more efficient. Experimentation with several concentrations of the cross-linking reagents indicated that covalent incorporation was best observed with DSS at 1.5 mM and with DSP at 1 mM. Labeling was detected after 2 min of cross-linking; however, the most intense labeling of the $M_r = 53000$ band was observed after 8–10 min and at a protein concentration of 1.8–2 mg/mL. Specific cross-linking signals were barely detectable at ^{125}I-ET-1 concentrations of <0.5 nM, optimally seen at radiopeptide concentrations of about 1 nM, and poorly resolved at higher ^{125}I-ET-1 concentrations, largely due to high background radioactivity (not shown). Accordingly, the procedure adopted for the covalent coupling of iodinated endothelins to their receptors was as follows: membrane preparations from the various tissues were prebound with ^{125}I-ET-1 and ^{125}I-ET-3 (1 nM, 1 h, room temperature) and then exposed to the bifunctional cross-linking reagent (1 mM DSP or 1.5 mM DSS, 10 min, room temperature, 2 mg of protein/mL).

Figure 1 shows the autoradiographs obtained when membranes from rat cerebellum, cortex, and caudate putamen were cross-linked with ^{125}I-ET-1. As shown, all three tissues contain one major labeled protein with an apparent M_r of 53 000 ($n = 5$). Specificity of labeling is indicated by the finding that labeling did not occur when 5×10^{-7} M unlabeled ET-1 or SRTX-*b* was present during binding. The labeled adducts

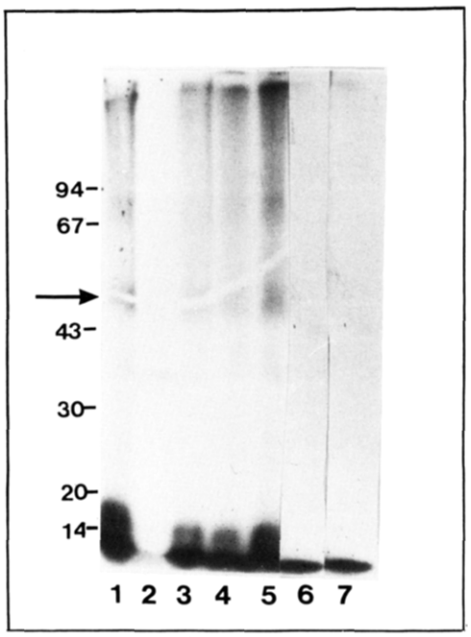

FIGURE 2: Affinity cross-linking of rat cerebellar membranes with ^{125}I-ET-1 in the presence of various concentrations of unlabeled ET-1 and 5×10^{-7} M ET-3 (lane 6) and SRTX-*b* (lane 7). Lane 1, control. The following concentrations of unlabeled ET-1 were used: lane 2, 5×10^{-7} M; lane 3, 1×10^{-7} M; lane 4, 5×10^{-8} M; lane 5, 1×10^{-8} M. Cross-linking was performed with 1 mM DSP as described under Materials and Methods.

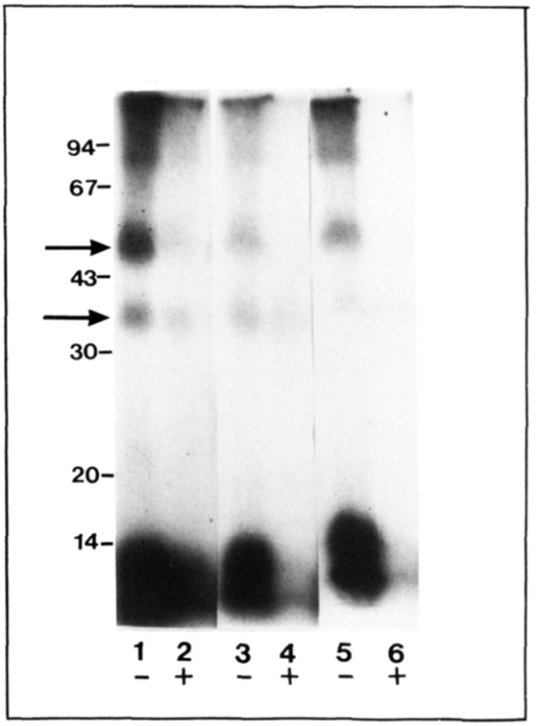

FIGURE 3: Electrophoretic analysis of ^{125}I-ET-3 cross-linked to rat brain membranes. Membranes (200–300 μg of protein) prepared from cerebellum (lanes 1 and 2), cortex (lanes 3 and 4), and caudate putamen (lanes 5 and 6) and incubated with 1 nM of ^{125}I-ET-3 were cross-linked with 1.5 mM DSS as described under Materials and Methods. The preparations were then subjected to SDS–PAGE on a 10% gel. The figure shows the corresponding autoradiographic pattern. (−) represents the total binding and (+) the nonspecific binding (in the presence of 5×10^{-7} M unlabeled ligand). The positions of the molecular mass standards are shown ($M_r \times 10^{-3}$).

were not seen when cross-linker was omitted. Also, cross-linking of ^{125}I-ET-1 alone did not produce bands corresponding to those observed upon cross-linking in the presence of membranes (not shown). It should be noted that a second band, which demonstrated weak labeling (about M_r = 40 000; see Figure 1, lane 2), is nonspecific, as indicated by its labeling with similar intensities in the total and the nonspecific lanes (compare lanes 1 and 2). Further evidence for the specificity of labeling of the receptor is provided by electrophoretic analysis of receptor–ligand complexes incubated in the presence of varying concentrations of unlabeled ET-1 (Figure 2): reduction of labeling under these conditions was dose dependent, becoming detectable at 10^{-8} M and complete at 5×10^{-7} M unlabeled ET-1 and 5×10^{-7} M ET-3 or SRTX-*b*.

On the basis of the results obtained with rat cerebellum, cortex, and caudate putamen, we are tempted to suggest that the ET-1 binding sites reside on a peptide of M_r = 53 000 and may therefore represent the high-affinity ET/SRTX receptors found both in brain and smooth muscle (Kloog et al., 1989). While this work was in progress, Watanabe et al. (1989) reported that cross-linking of chick cardiac membranes with ET-1 using disuccinimidyl tartarate yielded one major specific band having an M_r similar to that reported here for brain tissue. It should be noted that two receptors, M_r = 58 000 and M_r = 34 000, were recently identified (Sugiura et al., 1989) following the cross-linking of ET-1 with cultured rat mesangial cells. The former might be close to our M_r = 53 000. Our failure to detect the latter polypeptide with ^{125}I-ET-1 might be attributable to differences in tissues, experimental conditions, or both (see below).

On the other hand, cross-linking of ^{125}I-ET-3 with rat brain tissues shows the presence of two major bands of M_r = 53 000 and M_r = 38 000 (Figure 3). The former polypeptide accounts for 60–70% of the binding and the latter 30–40% (n = 6), as determined by both densitometry and gel slicing and counting (see Materials and Methods). Labeling of these two bands could be completely prevented by the presence of 5×10^{-7} M ET-3, ET-1, or SRTX-*b*, and again, the specificity of labeling is further evident from the fact that reduction of labeling was dose dependent, becoming detectable at 10^{-8} M and complete at 5×10^{-7} M unlabeled drug (Figure 4). The labeling pattern obtained with ^{125}I-ET-3 was similar when electrophoresis was conducted under reducing conditions, i.e., in the presence of 2-mercaptoethanol. Note that this experiment could not be conducted with DSP as a cross-linking agent (see above) because of the presence in this reagent of disulfide bonds. Thus, ET-1 labels one M_r = 53 000 polypeptide while ET-3 labels two, one of them similar in size to that labeled by ET-1 and the other of lower molecular weight. The labeling of the M_r = 53 000 polypeptide by both ET-1 and ET-3 is in accord with the demonstration that ET-3 binds with a relatively high affinity to the ^{125}I-SRTX-*b* binding sites in rat brain (Kloog et al., 1989), which are most likely represented by this polypeptide. This is not the case with the M_r = 38 000 polypeptide, which is labeled exclusively by ET-3 in rat brain.

Occasionally a few autoradiograms revealed the presence of discrete and diffuse bands in the M_r range of 80 000–90 000. These bands might represent various carbohydrate substituents of membrane proteins. However, we cannot exclude the possibility that partial proteolysis of a single peptide of M_r of around 90 000 may be responsible for the generation of the M_r = 53 000 and 38 000 bands.

The extent of incorporation achieved in this study was 8–10% of the original ligand bound for each of the ^{125}I derivatives. Since all the experiments were conducted under similar conditions, it seems unlikely that the M_r = 38 000 polypeptide represents a product of proteolysis, although the possibility that ET-3 binding to the receptor sensitizes it to

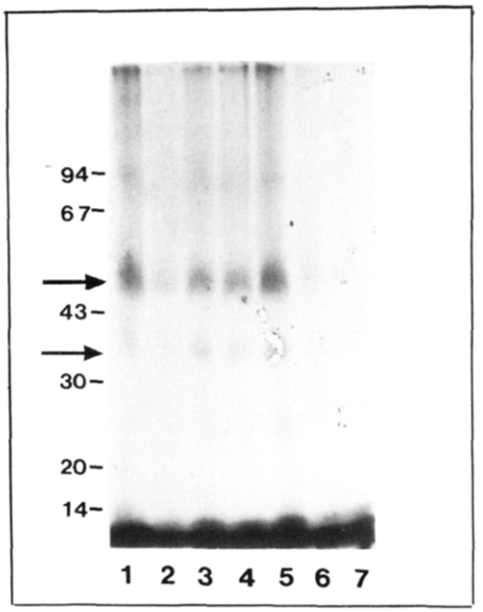

FIGURE 4: Affinity cross-linking of rat cerebellar membranes with ^{125}I-ET-3 in the presence of various concentrations of unlabeled ET-3 and of 5×10^{-7} M ET-1 (lane 6) and SRTX-*b* (lane 7). Lane 1, control. The following concentrations of unlabeled ET-3 were used: lane 2, 5×10^{-7} M; lane 3, 1×10^{-7} M; lane 4, 5×10^{-8} M; lane 5, 1×10^{-8} M. Cross-linking was performed with 1.5 mM DSS as described under Materials and Methods.

proteolysis cannot be completely discounted. Another possible reason for the heterogeneity of labeling might be attributable to the use of two different cross-linking reagents. In DSP a –S–S– bond replaces the $-CH_2-CH_2-$ bond present in DSS. The resulting small difference in molecular size together with the structural differences between ET-1 and ET-3 [as discussed in detail by Kloog and Sokolovsky (1989)] might give rise to differences between the two ligands in the proximity of their reactive components, thus leading to different patterns of labeling. For example, in ET-1 position 7 is occupied by a methionyl residue and in ET-3 by a lysyl residue; the ϵ-amino of the latter might be the reactive element that becomes cross-linked to the receptor.

The observed differences in labeling patterns might point to the existence of multiple forms of ET receptors, thus supporting previous binding and pharmacological data (Kloog & Sokolovsky, 1989; Kloog et al., 1989; Watanabe et al., 1989).

As reported by Watanabe et al. (1989) for chick cardiac membranes, ^{125}I-ET-3 labels two major bands and one minor band of M_r = 34000, 36000, and 53000, respectively. Thus, in contrast to labeling with ET-1, the results obtained with ET-3 for chick cardiac membranes differ from those obtained here for rat brain tissues. There are several possible reasons (or their combinations) for this discrepancy. First, we have systematically selected the experimental conditions for optimal results (i.e., incubation time, choice of the coupling reagent, etc.); deviations from this protocol in the other studies might alter the results. Second, the different reports employed very different membrane preparations: while we have used homogenates, Watanabe et al. (1989) employed cardiac membranes prepared from newborn chicks by sucrose density gradient centrifugation, and Sugiura et al. (1989) have used a microsomal fraction prepared from cultured rat mesangial cells. Third, the discrepancy may be attributable to the use of different tissues in the above reports—rat brain in the current study as compared to peripheral tissues such as chick cardiac preparations or rat mesangial cells. This suggestion might indicate differences in the molecular structure of the receptor–ET complex in rat brain tissue vs peripheral tissues.

We have previously suggested that the high-affinity ET-1/SRTX-*b* receptors are those that are coupled to the increase in phosphatidylinositide hydrolysis induced by these peptides (Galron et al., 1989; Kloog et al., 1989). It will be now of interest to examine whether the M_r = 38000 polypeptide is also linked to the PI system or to another second messenger pathway.

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