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Short communication

Urocortin: slower dissociation than corticotropin releasing factor from the CRF binding protein

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Abstract

We report on a comparison of the kinetics of [^{125}I][Tyr^{0}]corticotropin releasing factor (CRF) and [^{125}I][Tyr^{0}]urocortin binding to the CRF binding protein (CRF-BP) at physiological temperature. The association rates of [^{125}I][Tyr^{0}]urocortin or [^{125}I][Tyr^{0}]CRF binding to the CRF-BP were similar. The half time of association for [^{125}I][Tyr^{0}]urocortin was 3.2 min and for [^{125}I][Tyr^{0}]CRF, 2.6 min. [^{125}I][Tyr^{0}]urocortin dissociated from the CRF-BP but the rate of dissociation was slower than for [^{125}I][Tyr^{0}]CRF. The half time for dissociation of [^{125}I][Tyr^{0}]urocortin was 131 min and for [^{125}I][Tyr^{0}]CRF, 64 min. This slower dissociation indicates that the CRF-BP may be more effective in clearing urocortin than CRF.

Keywords: Urocortin; CRF (corticotropin releasing factor); CRF binding protein

1. Introduction

Corticotropin releasing factor (CRF) and urocortin bind to the CRF binding protein (CRF-BP). The CRF-BP was first purified from human plasma (Behan et al., 1988). The cDNA encoding the CRF-BP has been identified from several species including human, rat, mouse, sheep and xenopus (Potter et al., 1991; Cortright et al., 1995; Behan et al., 1996; Brown et al., 1996). The ~37-kDa CRF-BP is broadly distributed in rat brain and pituitary. The function of the central CRF-BP is not defined, however, it is thought that its major role is to bind and clear released CRF and urocortin, analogous to the uptake systems that clear several monoamine and amino acid transmitters (Behan et al., 1995). The CRF-BP has also been proposed to serve as a mechanism to present bound agonist to post-synaptic targets (Kemp et al., 1998). In humans, the CRF-BP is also expressed in liver and placenta where it is thought to play a major role in modulating CRF levels and, especially, in protecting the maternal pituitary gland from elevated plasma CRF levels found in late pregnancy (Linton et al., 1988).

We recently reported minimal dissociation of [^{3}H]urocortin from the CRF-BP (Ardati et al., 1998) which is in contrast to previous reports using CRF as the radioligand (e.g., Behan et al., 1995). However, we used sub-physiological temperature (ambient room temperature) in our previous study, which would result in a slower rate of ligand dissociation than at physiological temperature and, indeed, could make a slowly dissociating ligand appear to be irreversible. In order to explore further the profile of urocortin and CRF binding, we now report on a series of kinetic studies designed to compare directly kinetics of [^{125}I][Tyr^{0}]CRF and [^{125}I][Tyr^{0}]urocortin binding to the recombinant human CRF-BP at physiological temperature. Our findings indicate important differences between the two known endogenous ligands for this protein.

2. Materials and methods

The cDNA of the human CRF binding protein was obtained from the I.M.A.G.E. Consortium cDNA database (Lennon et al., 1996). Clone #266336 harbouring the full length cDNA was sequenced and subcloned and stably transfected into the human embryonic kidney-293 cell line (HEK-293) and the CRF-BP harvested from the medium from these cells as previously described (Ardati et al., 1998).

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The CRF-BP binding assays for [^{125}I][Tyr^0]rat/human CRF (50 pM; New England Nuclear, 2000 Ci/mmol), [^{125}I][Tyr^0]rat urocortin (50 pM; Amersham, 2000 Ci/mmol) and [^{3}H]rat urocortin (0.6 nM; Amersham, 104 or 145 Ci/mmol) were carried out in polystyrene 96-well microtitre plates (Beckman), as described previously (Ardati et al., 1998). In brief, the binding assay consisted of 10 μl of medium from HEK-293 cells expressing the CRF-BP and [^{125}I][Tyr^0]CRF or [^{125}I][Tyr^0]urocortin (50 pM) in a final volume of 300 μl of buffer A containing 50 mM Tris–HCl (pH 8.0) and 0.1% bovine serum albumin (Sigma, St. Louis, MO). Non-specific binding was defined by the inclusion of human urocortin (300 nM; hurocortin; F. Hoffmann-La Roche).

The association rate of [^{125}I][Tyr^0]CRF or [^{125}I][Tyr^0]urocortin was determined by incubation for various lengths of time (2–120 min) at 37°C following the addition of 10 μl of medium from HEK-293 cells expressing the soluble CRF-BP. Assays were terminated by addition of buffer B (containing 50 mM Tris–HCl [pH 8.0], 10% w/v activated charcoal and 2% bovine serum albumin) followed by centrifugation of the microtitre plates for 2 min at 2000 × g at room temperature. Preliminary experiments (not presented) revealed that free [^{125}I][Tyr^0]CRF and [^{125}I][Tyr^0]urocortin bound avidly to activated charcoal, which was precipitated by centrifugation. Radioligand bound to the CRF-BP remained in the supernatant (for a review of the use of activated charcoal in such assays, see Strange, 1992). A sample of supernatant (100 μl) was removed from each well and transferred to a 96-well Packard Picoplate. Microscint 40 (200 μl) was added and the wells sealed. Radioactivity was measured by liquid scintillation spectroscopy in a Packard Topcount scintillation counter.

For determination of dissociation kinetics, assays were allowed to incubate for 60 min at 37°C. Cold human urocortin (300 nM) was added to the assay (although in some experiments human/rat CRF (Neo-system) was used) and the incubation continued for various lengths of time before assays were terminated as described above. Both ligands were followed until approximately 80% of specific binding had dissociated. Longer times were used for [^{125}I][Tyr^0]urocortin (300 min) than [^{125}I][Tyr^0]CRF (120 min) since [^{125}I][Tyr^0]urocortin dissociated more slowly. All assays were performed in quadruplicate and repeated at least three times. Results were analysed using the program KINETIC (Biosoft, Cambridge, England; McPherson, 1985).

Table 1
On and off rates for [^{125}I][Tyr^0]CRF and [^{125}I][Tyr^0]urocortin binding to the CRF-BP. Results are the means ± S.E.M. of 3–6 individual experiments. A concentration of 50 pM was used for both radioligands. Association (K_{+1}) and dissociation (K_{-1}) rate constants were calculated using the program KINETIC. Since bound radioligand was < 10% of total ligand, a pseudo first order reaction was assumed

Radioligand	K_{+1} (M^{-1} min^{-1})	K_{-1} (min^{-1})	Kinetic K_d (M)
[^{125}I][Tyr^0]urocortin	$4.2 \pm 0.8 \times 10^9$	$5.3 \pm 0.5 \times 10^{-3}$	1.3×10^{-12}
[^{125}I][Tyr^0]CRF	$5.2 \pm 1.0 \times 10^9$	$1.1 \pm 0.1 \times 10^{-2}$	2.1×10^{-12}

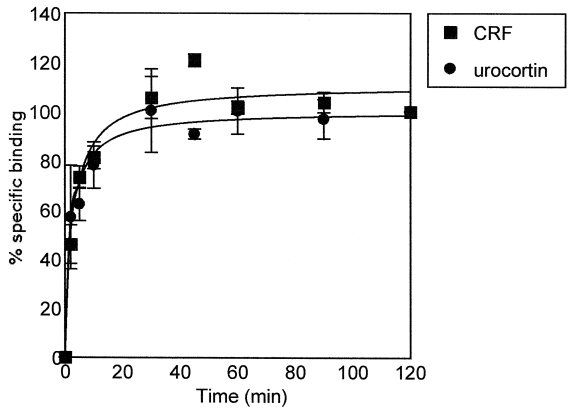

Fig. 1. The association of [^{125}I][Tyr^0]CRF and [^{125}I][Tyr^0]urocortin binding to the CRF-BP. Results are the means ± S.E.M. of three individual experiments. A concentration of 50 pM was used for both radioligands.

3. Results

The association rates of [^{125}I][Tyr^0]urocortin or [^{125}I][Tyr^0]CRF binding to the CRF-BP were similar (Table 1 and Fig. 1) with equilibrium being achieved for both ligands after approximately 30 min. The half time of association for [^{125}I][Tyr^0]urocortin and [^{125}I][Tyr^0]CRF were 3.2 and 2.6 min, respectively. Analysis using the program KINETIC revealed best fit to a single site model for both ligands.

The dissociation rate of [^{125}I][Tyr^0]urocortin binding to the CRF-BP was markedly slower than for [^{125}I][Tyr^0]CRF (Table 1, Fig. 2). The half time for dissociation of

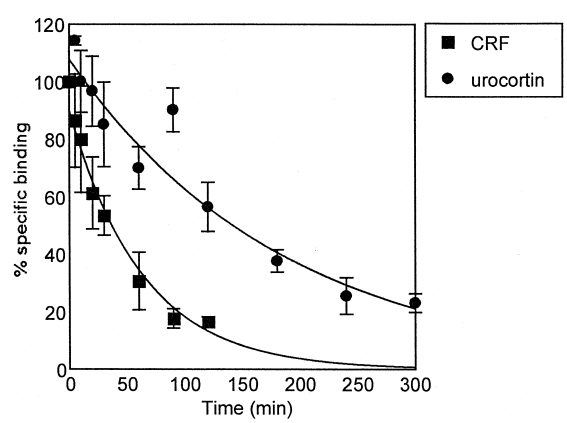

Fig. 2. The dissociation of [^{125}I][Tyr^0]CRF and [^{125}I][Tyr^0]urocortin binding to the CRF-BP. Results are the means ± S.E.M. of 3–6 individual experiments. A concentration of 50 pM was used for both radioligands. Dissociation was initiated by addition of urocortin after 60 min incubation at 37°C.

[^{125}I][Tyr^0]urocortin was 131 min and for [^{125}I](Tyr^0)-CRF, 64 min. At the longest times employed (120 min for [^{125}I][Tyr^0]CRF and 300 min for [^{125}I][Tyr^0]urocortin), approximately 80% of specific binding had dissociated. Longer times were not examined, however, there was no suggestion of a plateau being reached at this level of inhibition. Analysis using the program KINETIC revealed best fit to a single site, fully reversible model for both ligands. We have also conducted the [^{125}I][Tyr^0]CRF dissociation experiments using CRF as the displacing agent and saw an identical dissociation rate (K_{-1} $1.1 \pm 0.1 \times 10^{-2}$ min^{-1}; mean ± S.E.M., $n = 3$). In additional experiments, we examined dissociation using [^{3}H]urocortin as the radioligand. A similar profile was obtained compared to that seen with the iodinated ligand ($K_{-1} = 7.7 \pm 0.9 \times 10^{-3}$ min^{-1}; mean ± S.E.M., $n = 3$).

4. Discussion

In our previous study (Ardati et al., 1998), no dissociation of [^{3}H]urocortin binding to the CRF-BP was seen over a 2-h period. In the current study, the data are consistent with slowly dissociating but fully reversible binding of [^{125}I][Tyr^0]urocortin to the CRF-BP. The explanation for the difference profile in the two studies is that a higher temperature was used (37°C vs. room temperature) in the present study. An increase in assay temperature routinely results in faster on and off rates for a ligand and this is reflected in it here being possible to observe dissociation of urocortin from the CRF-BP at 37°C. It is very unlikely to be due to an irreversible interaction at room temperature and a reversible interaction at 37°C.

The dissociation rate that we observed for [^{125}I][Tyr^0]urocortin is considerably slower than that seen for [^{125}I][Tyr^0]CRF. Whilst their association rates were similar. The affinity of the radioligands calculated from the kinetic data revealed a slightly lower K_d (higher affinity) for [^{125}I][Tyr^0]urocortin than for [^{125}I][Tyr^0]CRF. This is consistent with literature data calculated in competition experiments (K_i values: Vaughan et al., 1995). However, the absolute values for the K_d's calculated kinetically here ($1.3–2.1 \times 10^{-12}$ M) are somewhat lower than published K_i or K_d values calculated by other means ($1.0–2.5 \times 10^{-10}$ M; Behan et al., 1995; Vaughan et al., 1995; Ardati et al., 1998). The reasons for such differences are not clear but may result from the different temperatures employed in our study (37°C) compared to those normally employed (room temperature) since temperature can have a profound effect on ligand affinities (see, e.g., Kilpatrick et al., 1986).

There are a number of points to consider before extrapolating the different profiles of CRF and urocortin to the physiological situation. First, we have used urocortin as the displacing agent for both radioligands. This could produce a different profile for the two ligands if they do not bind to the same site on the CRF-BP. However, we have also conducted the [^{125}I][Tyr^0]CRF dissociation experiments using CRF as the displacing agent and saw an identical dissociation rate. Second, our experiments were conducted at pH 8.0 in an ion free buffer because superior signal to noise ratios were observed under these conditions. However, preliminary experiments in HEPES–Krebs buffer at pH 7.4 also revealed a slower dissociation rate for urocortin ([^{125}I][Tyr^0]CRF, K_{-1} 1.6×10^{-2} min^{-1}; [^{125}I][Tyr^0]urocortin K_{-1} 8.1×10^{-3} min^{-1}; results from individual experiments performed in parallel). Thirdly, in this study, we have used [Tyr^0] iodinated versions of the radioligands. Since the addition of such a large group may effect the binding properties of a ligand, as a control, we also examined dissociation using [^{3}H]urocortin as the radioligand. A similar profile was obtained compared to that seen with the iodinated ligand. These considerations lead us to conclude that the differences observed are not experimental artifacts and are likely to reflect the physiological situation.

There is a dearth of data on the kinetics of ligand binding to the CRF-BP and, to our knowledge, this is the first time that a full study has been conducted and such differences reported. The differences in dissociation rate between CRF and urocortin may be physiologically important. Based on current hypotheses, there are two potential consequences. If the major role of the CRF-BP is to clear released CRF or urocortin, a slower dissociation rate could result in urocortin being cleared more efficiently than CRF. Thus the actions of CRF would predominate in areas where both transmitters are present, such as the lateral septum. One can also speculate that there may be selective stimulation of CRF_1 receptors since urocortin displays equal affinity at CRF_1 and CRF_2 receptors whereas CRF has a clearly higher affinity at CRF_1 receptors (Vaughan et al., 1995). Alternatively, if as proposed by Kemp et al. (1998), the CRF-BP serves to present released CRF or urocortin to postsynaptic receptors, the slower dissociation rate of urocortin may allow it to stimulate CRF receptors more effectively than CRF. It remains to be established which of these situations represents the true physiological state.

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