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Receptor mediated internalization of neurotensin in transfected Chinese hamster ovary cells

Abstract—After association with intact Chinese hamster ovary (CHO) cells expressing the rat neurotensin receptor, tritiated neurotensin was rapidly internalized. Internalization was maximal after 30 min and accounted for about 90% of the total associated ligand. Neurotensin internalization was not observed at 0–4° and was inhibited by an excess of unlabelled neurotensin or by the neurotensin non peptide antagonist, SR 48692. Moreover, the incubation of intact cells for 30 min with 10 nM neurotensin resulted in a significant decrease in the number of the cell surface neurotensin receptors. These results indicate that the endocytosis of membrane bound neurotensin in transfected CHO cells resulted from the internalization of the ligand–receptor complex inside the cell, through an agonist-induced process.

Key words: neurotensin, neurotensin receptor, internalization, CHO cells, transfection

NT* is a tridecapeptide neurotransmitter mainly found in the central nervous system and in the gastrointestinal tract of various mammalian species. In rat and human brains, NT was found to specifically bind to high affinity membrane receptors which were also characterized in different cultured cell lines. The stimulation of these receptors results in the appearance of intracellular second messengers including inositol phosphates [1] and cyclic GMP [2]. Recently, the isolation of cDNA clones encoding the rat and human NTRs has been reported [3, 4]. The NTR belongs to the broad family of G-protein coupled receptors which share the common structure of seven hydrophobic transmembrane domains joined together by intracellular and extracellular hydrophilic loops. We report here on the internalization of NT and its receptor in CHO cells expressing the rat NTR.

Materials and Methods

CHO cells were transfected with the complete cDNA sequence encoding the rat NTR using the calcium phosphate precipitation method. The specific binding of [^{3}H]NT in cell homogenates was measured as described previously [5]. Binding on intact cells was performed in 24-well plates. Cells were incubated in the presence of 1 nM [^{3}H]NT in binding buffer (Ham-F12 culture medium containing 0.2 mM bacitracin and 0.1% bovine serum albumin). After binding, cells were washed three times with PBS or with acidic PBS pH2.5) for the determination of total and internalized ligand, respectively. The non-specific binding, representing less than 5% of the total binding, was determined in the presence of 1 μM unlabelled peptide. After washing, the cells were dissolved in 500 μL deoxycholic acid 1%, pH 11.3 and the radioactivity contained in 200 μL was counted by liquid scintillation. When the effect of unlabelled NT or SR 48692 was tested on the internalization, these drugs were added to the dishes 15 min before [^{3}H]NT.

For receptor internalization assays, transfected cells grown in 24-well plates were incubated in the binding buffer for 1 hr in the presence of 10 or 100 mM of unlabelled NT. At the end of the incubation, the plates were transferred to an ice bath to stop the reaction, washed three times with ice-cold PBS and once with the binding buffer. After equilibrating for 2 hr in the binding buffer at

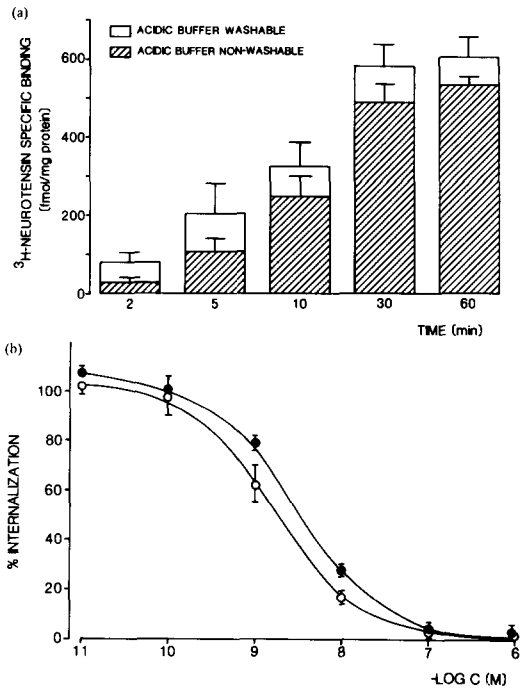

Fig. 1. (a) Internalization of [^{3}H]NT in intact transfected CHO cells. Cells were incubated for 2, 5, 10, 30 and 60 min in the presence of 1 nM [^{3}H]NT at 37°. Thereafter, the cells were washed with neutral PBS (total binding) or acidic PBS (internalized ligand). Data represent mean values ± SD of five different experiments performed in quadruplicate. (b) Inhibition of the internalization of [^{3}H]NT by unlabelled NT or SR 48692. The cells were incubated at 37° for 30 min in the presence of [^{3}H]NT and increasing concentrations of NT (○) or SR 48692 (●). Thereafter, cells were washed with acidic PBS in order to determine the amount of [^{3}H]NT internalized. Results are expressed as per cent of control internalization which represent about 90% of the cellular total associated [^{3}H]-NT. Data represent mean values ± SD of two different experiments performed in quadruplicate.

* Abbreviations: NT, neurotensin; NTR, neurotensin receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

0–4°, the binding was measured directly on the monolayer with 5 nM [^{3}H]NT for 1 hr at 0–4°. Cells were washed and radioactivity was measured as described above.

Results and Discussion

Binding of [^{3}H]NT in homogenates of CHO cells, transfected with the complete cDNA sequence encoding the rat NTR, revealed the presence of high affinity NT binding sites. Scatchard analysis of the saturation binding curves revealed a K_D value of 0.45 ± 0.08 nM. Displacement of the specific binding was observed using NT itself, the active fragment of NT (NT8–13), neuromedin N and SR 48692, recently described as a non-peptide antagonist of NT [6]. The K_i values, determined on the basis of inhibition curves (0.66 ± 0.2, 0.13 ± 0.04, 7.07 ± 2.42 and 3.29 ± 0.05 nM for NT, NT8–13, neuromedin N and SR 48692, respectively), were in good agreement with the values previously described in rat brain homogenates [5, 6]. Finally, the transfected NTR was found to activate phospholipase C after stimulation with agonists [7].

After binding to the intact CHO cells at 37°, [^{3}H]NT was rapidly internalized into the cells as indicated by the inability to remove the cell-associated radioactivity by extensive washing with acidic PBS (Fig. 1a). Internalization was maximal after 30 min and accounts for about 90% of the total associated ligand. This process was not observed in non-transfected cells and was inhibited by unlabelled neurotensin or by SR 48692. As shown on Fig. 1b, the abilities of NT and SR 48692 to inhibit [^{3}H]NT internalization were in agreement with their respective affinities for the transfected receptor. These results indicate that the endocytosis of the peptide was specifically mediated by the NTR. No internalization of the radioligand was observed after incubation for 1 hr at 4°, because all active internalization processes were inhibited.

In a previous study, we have reported the internalization of NT with its receptor in primary cultures of rat neurons [5]. Since there is an endocytosis of NT in intact transfected CHO cells, we measured the amount of NTR remaining at the surface of intact cells after incubation in the presence of unlabelled NT. As shown on Fig. 2, the incubation of intact cells for 1 hr with 10 nM of NT resulted in a dramatic decrease of the cell surface NTR number, in a similar fashion to what was observed on the cultured neurons. On the transfected cells, the loss corresponded to about 80% of the cell surface receptors measured before the incubation with NT. Such a decrease was not observed when the incubation with unlabelled NT was performed at 0–4°. These results strongly suggest that the endocytosis of membrane bound NT resulted from the internalization of the ligand–receptor complex inside the cell.

Internalization of both a ligand and the corresponding receptor is a common feature of numerous membrane receptors and constitutes an important mechanism of membrane receptor regulation, involved in the control of the cellular response to agonist stimulation [8]. In the case of NTR, ligand-induced receptor regulation has been reported in different cultured cell types. The regulation of the NTR has been described in HT29 [9] and N1E-115 cells [10, 11] whereas an internalization process was found in primary cultured rat [8] and murine neurons [12]. In these models, it was shown that after binding to cell surface receptors, there was a rapid endocytosis of NT through a receptor-mediated mechanism. For many G-protein coupled receptors, it has been shown that internalization of the receptors in the cells was followed by receptor degradation after fusion of internalized vesicles with lysosomes. As a rule, prolonged incubation with NT has been shown to result in a decrease of the total cell receptor number [10, 11]. In some experimental models, NTR internalization was shown to lead to other biological responses before degradation. It was recently described that after internalization in the terminals of the dopaminergic neurons in the striatum, NT was actively transported to the cell body [13]. In the same way, NT was found to be closely associated with the cell nucleus after internalization in cultured neurons [12]. Moreover, it was found that the transport in the nigrostriatal pathway was followed by specific activation of gene transcription after possible nuclear translocation [14]. However, the specific roles played by the internalization and nucleus translocation of NT in the increase of gene expression remain to be defined. Internalization might constitute the first step of this transfer, from the cell surface to the nucleus. The molecular determinants for the internalization of the NTR are still unknown. The transfected cells expressing the wild type or mutagenized forms of the NTR provide an interesting pharmacological tool for the identification of receptor domains specifically involved in physiological response and in the receptor internalization process.

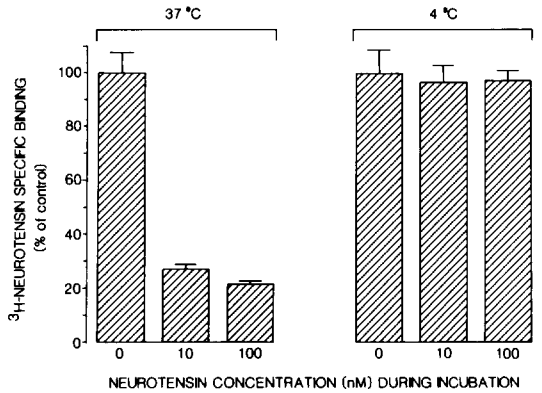

Fig. 2. Loss of membrane receptor after preincubation of transfected CHO cells with 10 and 100 nM NT at 0–4° or 37°. Intact CHO NTR cells were incubated for 30 min in the presence of 10 or 100 nM unlabelled NT at 0–4 or 37°. After removal of membrane bound NT, the cell surface specific binding of [^{3}H]NT was measured at 0–4°. Results are expressed as per cent of control binding. Data represent mean values ± SD of four different experiments performed in quadruplicate.

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