Bioorganic & Medicinal Chemistry Letters, Vol.2, No.6, pp. 609-612, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

SYNTHESIS OF FLUORESCEINYL-NEUROKININ-A, A BIOLOGICALLY ACTIVE PROBE FOR NK_2 RECEPTORS

Karin Ceszkowski and Andre Chollet *
Glaxo Institute for Molecular Biology S.A., chemin des Aulx 14
CH-1228 Plan-les-Ouates, Switzerland

(*Received* 2 *April* 1992)

Abstract.- The tachykinin peptide neurokinin-A has been selectively labelled at a single site with fluorescein isothiocyanate. The fluorescein-labelled neurokinin-A (F-NKA) retained full binding affinity for the NK_2 receptor as well as functional activity. F-NKA was used as a tool to study NK_2 receptor in cell lines by flow cytometry.

Neurokinin-A (NKA, substance K) is a member of the tachykinin peptides family. Tachykinins are closely-related mammalian neuropeptides including substance P (SP), NKA and neurokinin B (NKB) that display a variety of biological activities in the central and peripheral nervous systems [1]. Tachykinins recognize specific cell-surface receptors expressed in many tissues. Three classes of receptors have been identified pharmacologically and cloned and characterized subsequently: NK_1, NK_2 and NK_3 receptors displaying some selectivity toward SP, NKA and NKB respectively [2]. The first step in the physiological action of NKA is assumed to be the binding to NK_2 receptors. The availability of an appropriately labelled NKA reagent would greatly facilitate the study of NK_2 localization, structure and function. In this paper we report the first preparation and characterization of a bioactive fluorescent NKA analogue (F-NKA). We demonstrate that F-NKA is a useful tool for NK_2 receptor recognition in cell lines.

The decapeptide NKA was derivatized with fluorescein by reaction with 1.2 equivalent of the amino group selective reagent fluorescein isothiocyanate in 0.02 M sodium borate pH 9.0 containing 5% of N,N-dimethylformamide at 4°C for 1h. The crude mixture was purified by hydrophobic interaction ('reverse-phase') HPLC [3] to give two fractions, distinct from NKA, in a 78:22 ratio. The major fraction eluted first and contained monosubstituted NKA (F-NKA, 74% yield) by mass spectrometry analysis [4]. The minor fraction was NKA substituted with two fluoresceinyl groups. The presence of fluorescein in the two fractions was further confirmed by visible spectrometry at 442 nm.

NKA has two amino groups with different pKa : N-α amino group on His1 and N-ε amino group on Lys2. Under the reaction conditions used we found preferential attachment of fluorescein to the N-terminal position of His1. This was indicated by the mass spectrometry fragmentation pattern which showed peak corresponding to the loss of fluoresceinyl-histidine [4]. This was further confirmed by comparative Edman degradation of F-NKA and NKA and analysis of the PTH-derivatives at each cycle. At cycle 1, His-PTH was recovered for NKA only, whereas at cycle 2 both the F-NKA sample

and NKA gave the expected lysylphenylthiohydantoin derivative.

The fluorescein-labelled peptide F-NKA was assayed for NK_2 binding affinity by competitive displacement binding analysis with ^{125}I-NKA on CHO cells transfected with bovine NK_2 [5] or human NK_2 receptors [6] (CHO/NK_2)(Figure 1). We found that F-NKA bound to NK_2 receptors with the same affinity as the natural ligand NKA (D_{50} = 1 x 10^{-9} M). This results indicate that the N-terminal part of the decapeptide NKA does not play a direct role in receptor binding. This also confirms several observations on structure-activity relationships on NKA [7].

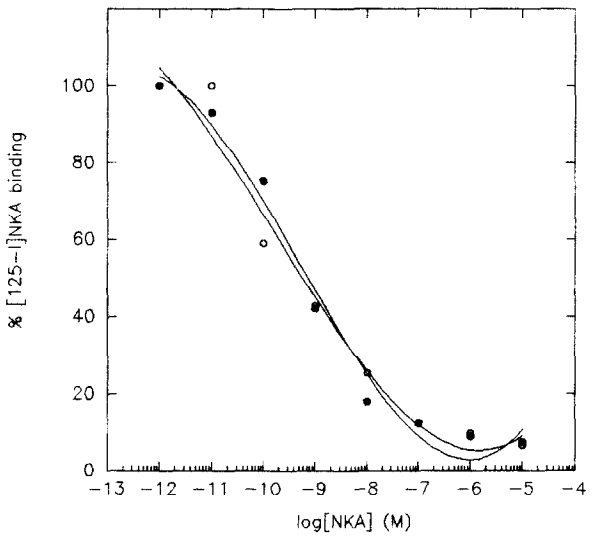

FIGURE 1:
Competitive inhibition of [^{125}I]NKA binding to CHO/NK_2 by NKA peptides; o = NKA, ● = F-NKA.

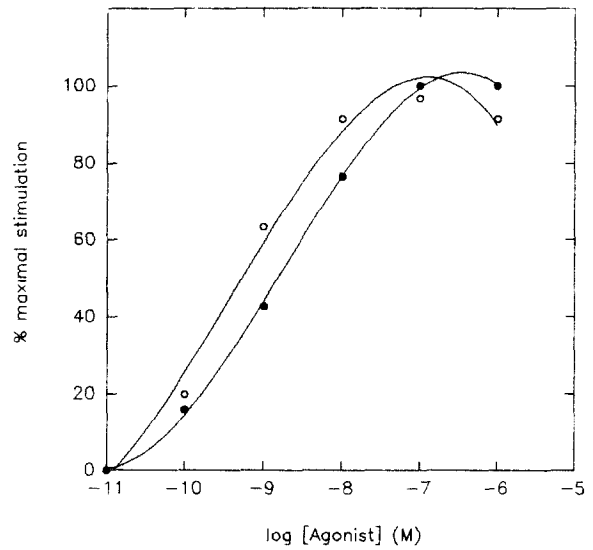

FIGURE 2:
Calcium mobilization in CHO/NK_2 cells as a function of increasing concentrations of either NKA (o) or F-NKA (●).

The functional activity of F-NKA was assessed by the ability to mobilize Ca^{2+} from intracellular stores in CHO/NK_2 cells (Figure 2). Intracellular Ca^{2+} concentrations were determined as described previously [8] by using the fluorescent Ca^{2+} chelating agent fura-2 except that the fluorescence was recorded at 480 nm to avoid overlap with the fluorescein fluorescence. F-NKA stimulated a Ca^{2+} response with an EC_{50} of 3.0 ± 0.2 nM compared to an EC_{50} of 0.8 ± 0.5 nM for NKA. This indicates that F-NKA acts as an agonist at the NK_2 receptor. The slight decrease in potency for F-NKA compared to NKA may suggest some minor involvement of the N-terminal part of these peptides in the conformation change associated with the activation of the NK_2 receptor.

TABLE I :
Fluorescence analysis of binding of F-NKA to CHO/NK_2 cells. MFI of total cells are shown on a linear scale in the presence of various concentrations of a) F-NKA, b) F-NKA + 10 µM NKA, c) F-NKA + 10 µM antagonist R 396 [10]. MFI represents the difference a) - b).

[F-NKA] (nM)	MFI a)	MFI b)	MFI c)	MFI a)-b)
Medium	4.9	-	-	-
0.5	6.5	4.9	4.7	1.6
1	8.8	4.8	5.0	4.0
2	12.6	5.0	5.0	7.6
5	18.8	4.9	5.2	13.8
10	28.3	5.2	5.6	23.1
20	36.2	5.3	5.8	30.9

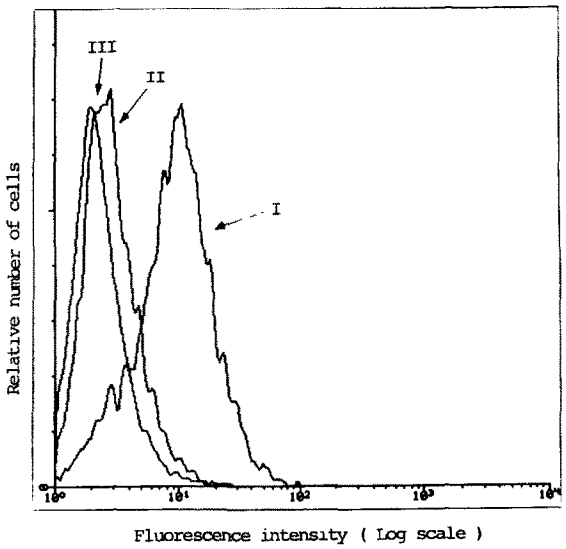

FIGURE 3:
Flow cytometry analysis of NK_2 receptors on CHO/NK_2 cells using F-NKA. I = F-NKA, II = F-NKA + excess NKA, III = autofluorescence of the cells.

The ability of F-NKA to recognize NK_2 receptors was analyzed by flow cytometry on stably transfected CHO cells expressing either bovine NK_2 (21,300 receptors/cell) [5] or human NK_2 (28,000 receptors/cell) [6]. The degree of fluorescence was measured as mean fluorescence intensity (MFI) on total cells [9] (Table I). MFI was measured as a function of the F-NKA concentration (Table I) and the lower limit of significant detection was found at about 10^{-9} M. A typical fluorescence histogram is shown in Figure 3. Below 10^{-8} M, F-NKA binding was specific for NK_2 and no interaction with the other tachykinin receptors NK_1 and NK_3 in stable cell lines was detected.

These results demonstrate that F-NKA is a useful reagent for the analysis of NK_2 receptors by flow cytometry, at least in cells expressing several thousands NK_2 receptor molecules. In summary, we describe a fluorescent and biologically active ligand specific for the NK_2 receptor. F-NKA could be a useful tool for studying NK_2 receptors in real tissues as well as for probing receptor structure and dynamics using fluorescence and fluorescence-transfer measurements.

Acknowledgement.- We thank Charles Bradshaw, Gerardo Turcatti and Dr Keith Rose for skillful assistance.

References and Notes

1. Maggio. J.E. *Annu. Rev. Neurosci.* **1988**, *11*, 13.
2. Nakanishi, S. *Annu. Rev. Neurosci.* **1991**, *14*, 123.
3. Aquapore RP300, 100x4.6 mm , 7 µm column (Bromlee Labs) using an increasing gradient of acetonitrile + 0.1% trifluoroacetic acid in water + 0.1% trifluoroacetic acid. Fraction I contained F-NKA (78%) and fraction II contained difluoresceinyl-NKA (22%). Unreacted NKA was found only in trace amounts (<1%). Fractions were evaporated to dryness and taken up in water-acetonitrile7:1 at a concentration of 1 mM and stored at -20°C.
4. F-NKA was analyzed by positive ion fast-atom bombardment mass spectrometry (FAB/MS) using glycerol/thioglycerol as matrix (Dr K Rose, University of Geneva). F-NKA gave the expected protonated molecular ion at m/z 1522. Signals due to fragmentation appeared at m/z 996 (assigned as $+H_2$- NKA(2-10)) and 1133 ($+H_2$-NKA).
5. Eistetter, H.R.; Church, D.J.; Mills, A.; Godfrey, P.P.; Capponi, A.M.; Brewster, R.;Schulz, M.-F.; Kawashima, E.; Arkinstall, S.J. *Cell Regulation* **1991**, *2*, 767.
6. Arkinstall, S.J.; Chollet, A.I.; Ceszkowski, K.; Gillieron, C.; Chollet, A.; Kawashima, E.; *in preparation.*
7. Rovero, P.; Pestellini, V.; Rhaleb, N.-E.; Dion, S.; Rouissi, N.; Tousignant, C.; Telemaque, S.; Drapeau, G.; Regoli, D. *Neuropeptides* **1989**, *13*, 263.
8. Capponi, A.M.; Lew, P.D.; Jornot, L.; Valloton, M.B. *J. Biol. Chem.* **1984**, *259*, 8863.
9. Cells (10^6) were incubated in phosphate buffered saline (PBS) pH 7.2, 3 mM $MnCl_2$, bovine serum albumin (0.2 mg/mL) in the presence of 10^{-8}M F-NKA. The cells were washed twice with cold PBS pH 7.2 and kept on ice. The cell fluorescence was recorded with a Becton-Dickinson FACSCAN instrument using either linear or logarithmic modes with excitation/emission at 288/518 nm. The specific fluorescence was measured with 10^{-8}M F-NKA + 10^{-5}M NKA or 10^{-5}M Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396).
10. Maggi, C.A.; Patacchini, R.; Giuliani, S.; Rovero, P.; Dion, S.; Regoli, D.; Giachetti, A.; Meli, A. *Br. J. Pharmacol.* **1990**, *100*, 588.