Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 400-407

RAPID DESENSITIZATION OF AGONIST-INDUCED CALCIUM MOBILIZATION IN TRANSFECTED PC12 CELLS EXPRESSING THE RAT NEUROTENSIN RECEPTOR

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Received December 6, 1993

Summary: The effect of neurotensin on intracellular calcium mobilization was measured in PC12 cells transfected with the cDNA sequence encoding the rat neurotensin receptor. Stimulation with nanomolar concentrations of neurotensin induced a rapid increase of the $[Ca^{++}]_i$. This response was transient and the $[Ca^{++}]_i$ returned to basal level within 2 minutes, despite the continuous presence of the agonist. The response was also observed in the absence of extracellular calcium, indicating the intracellular origin of the released calcium. Successive stimulations with the same concentration of the peptide failed to produce similar responses. ATP was also found to mediate the release from the same intracellular store of calcium in PC12 cells. The amplitude of the response to ATP was not affected by previous stimulation with neurotensin. These results demonstrate the existence of a rapid and homologous desensitization of the neurotensin-induced calcium release in PC12 transfected cells.

The tridecapeptide neurotensin (NT) binds to high affinity NT receptors present in the brain and in different cultured cell lines (1). The NT receptors belong to the broad family of G-protein coupled receptors and their stimulation lead to the activation of phospholipase C. Increase in cytosolic IP_3 and $[Ca^{++}]_i$ induced by NT have been described in both primary cultures of rat neurons (2) and in various cultured cell lines (3-5).

As most of G-protein coupled receptors, the NT receptor becomes desensitized after stimulation. This agonist-induced receptor desensitization occurs through different mechanisms. Desensitization at the molecular level constitutes the faster regulating process and is responsible for the transient response to agonist, despite a continuous stimulation of cell surface receptors, as well as for the diminished response to

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successive stimulations (6). In the case of the NT receptor, such desensitization process has been reported in HT29 human adenocarcinoma cells (7), in neuroblastoma x glioma hybrid NG108-15 cells (5) and in cultured neurons (2). Internalization and down-regulation are regulating processes occurring at the cellular level and are responsible for redistribution or degradation of cell membrane receptors, respectively. Receptor internalization has been reported *in vitro*, in both cultured neurons (8) and cell lines (7,9), but also *in vivo*, in the dopaminergic neurons of rat striatum (10).

The cDNA sequence encoding NT receptor has been cloned in the rat brain (11) and the human HT29 cell line (12). Transfection of the rat sequence in CHO cells allowed the stable expression of NT receptors functionally coupled to the activation of phospholipase C (13,14). In the present study, we report the stable expression of the NT receptor in PC12 cells and the characterization of the NT-induced intracellular calcium mobilization followed by receptor desensitization.

MATERIALS AND METHODS

Cell culture and transfection. PC12 cells were cultured in Dulbecco's modified Eagle medium with 10 % foetal calf serum, 5% horse serum, 100 IU/ml Penicillin and 100 IU/ml Streptomycin. All materials and media were from Gibco. Cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 - 95 % air. For intracellular calcium measurements, cells were cultured on glass coverslips. The cDNA sequence encoding the rat NT receptor, cloned in the pSVK3 expression vector (14), was used in stable transfection of PC12 cells using the calcium phosphate precipitation method.

Radioligand binding assays. Binding of [^{3}H]NT (spec. act.: 101 Ci/mmol in ethanol, from New England Nuclear) on cell homogenates was performed as previously described (14). In displacement experiments, [^{3}H]NT was used at 1 nM concentration. Non specific binding was determined in the presence of 1 μM unlabelled NT (Sigma Chemical Co).

Determination of the $[Ca^{++}]_i$. Cells grown on glass coverslips were incubated for 1 hr with 5 μM fura-2-AM (Molecular Probes). Coverslips were then washed with incubation buffer (Hepes 10 mM, pH 7.4; NaCl 150 mM; KCl 5 mM; $MgCl_2$ 1.2 mM; $CaCl_2$ 1.2 mM; glucose 10 mM) and mounted in a thermostated 1 ml cuvette placed in the light path of a inverted Nikon microscope equipped with an epifluorescence illumination. Fluorescence of delimited cell focies (3-4 cells) was monitored on a Deltascan spectrofluorimeter (Photon Technology International) and stored on a personal computer. $[Ca^{++}]_i$ was estimated from the 340/380 nM ratio according to (15) using 224 nM as the K_d of Fura-2 for Ca^{++}. The cuvette was continuously perfused at 37°C with incubation buffer at 1-1.5 ml/min. For experiments conducted in the absence of extracellular calcium, $CaCl_2$ was omitted from the incubation buffer and replaced by EGTA 0.1 mM. Calibration of the fluorescence signals was performed at the end of the assays after permeabilization of the cells with 5 μM ionomycin (Sigma Chemical Co) to obtain minimal and maximal fluorescence in the absence (supplemented with 2 mM EGTA) and in the presence of extracellular calcium (18 mM), respectively. Autofluorescence background was measured after calibration by quenching with 2 mM $MnCl_2$.

RESULTS AND DISCUSSION

Cell transfection and [^{3}H]NT binding on cell homogenates. Transfection of CHO cells with the cDNA sequence encoding the rat NT receptor cloned in the pSVK3 plasmid was previously reported (14). The same construction was used for the transfection of PC12 cells using the calcium phosphate precipitation method. Saturation curves of the [^{3}H]NT specific binding on transfected PC12 cell homogenates revealed a K_d value of 0.32 ± 0.03 nM (n=3), similar to that found in cultured neurons and a B_{max} value of 503 ± 88 fmol/mg of protein (n = 3; figure 1A). Displacement of [^{3}H]NT specific binding was achieved using NT itself and the non peptide NT antagonist (SR48692) (16) with IC_{50} values (2.89 ± 0.62 and 7.17 ± 1.00 nM respectively, n = 3), in good agreement with those reported in the rat brain (figure 1B).

Increase of intracellular [Ca^{++}]. As shown on figure 2A, basal $[Ca^{++}]_i$ in transfected PC12 cells was 54.15 ± 11.64 nM (n = 35) and stimulation with 100 nM NT produced a rapid and important increase in the $[Ca^{++}]_i$, reaching about 680 ± 58 nM (n = 8) after 30 seconds. A significative response was already observed with 1 nM and maximal stimulation was obtained with 100 nM NT (figure 2C). Interestingly, the EC_{50} value (4.35 ± 0.31 nM) was 13 fold higher than the K_d value found in binding experiments. Similar observations were previously reported in primary cultured neurons (2) and probably result from differences in the experimental conditions (binding experiments are performed at equilibrium on cell homogenates). The $[Ca^{++}]_i$ increase was also

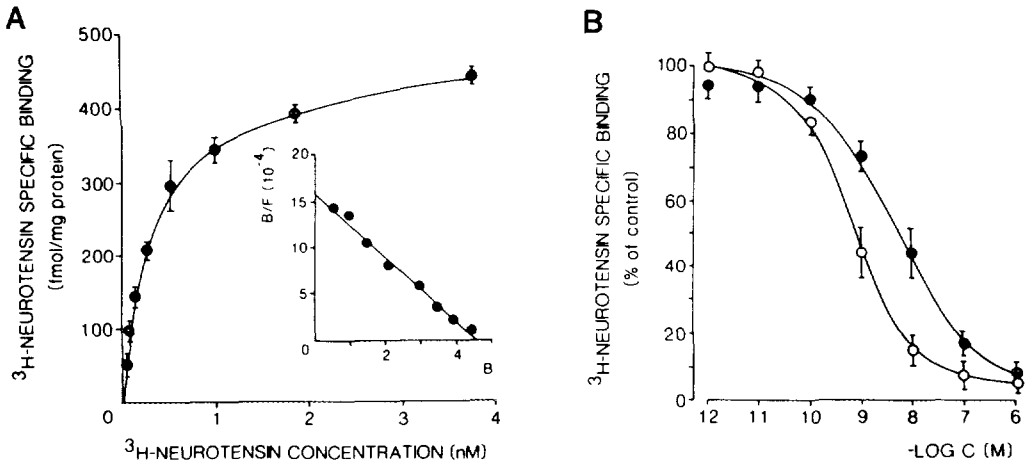

Fig.1. Binding of [^{3}H]-NT to transfected PC12 cell membranes. Panel (A) shows a typical (n=3) saturation curve of the [^{3}H]-NT specific binding. Scatchard analysis of the specific binding of [^{3}H]-NT is also shown. Panel (B) shows displacement of [^{3}H]-NT specific binding by NT (○) and SR48692 (●). Values are given in percent of [^{3}H]-NT specific binding. The data correspond to mean values ± SD of 3 different experiments performed in triplicate.

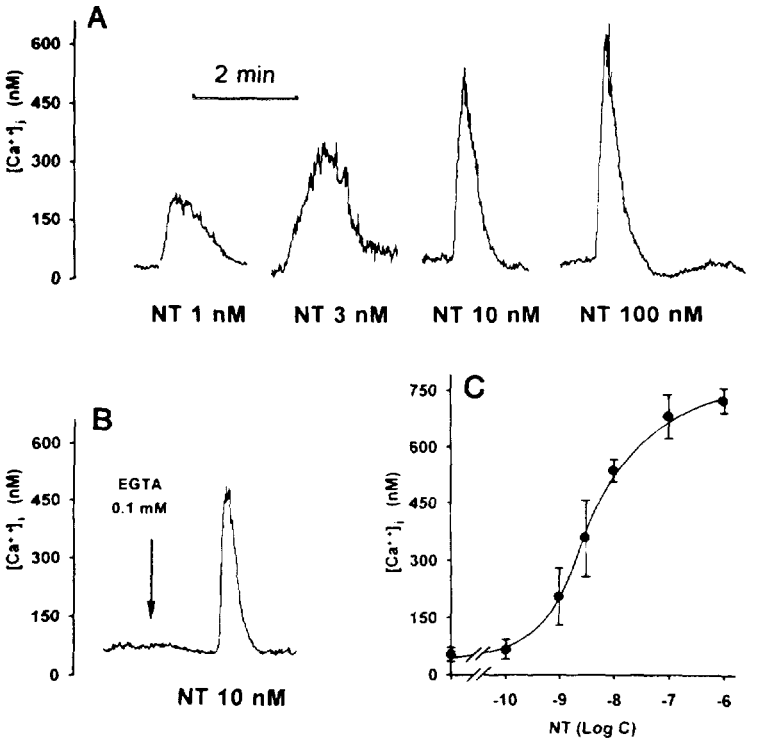

Fig. 2. Changes in $[Ca^{++}]_i$ in transfected PC12 cells in response to various stimulations with NT: **A)** independent stimulation with 1, 3, 10 and 100 nM of NT in calcium containing buffer (data correspond to typical curves representative of at least 5 different experiments); **B)** stimulation with 10 nM NT in calcium free buffer; **C)** concentration-response curve to NT (mean data of at least 5 separate determinations).

observed when stimulations were carried out in the absence of extracellular calcium, indicating the involvement of IP_3-induced calcium release from intracellular stores in response to NT (figure 2B). No response was obtained in non transfected PC12 cells with supramaximal concentrations of NT (100 nM) (not shown). The increase of the $[Ca^{++}]_i$ induced by NT was followed by a rapid decrease, and the basal level was reached within 2 min after stimulation despite the continuous presence of the agonist. After extensive washing of the agonist (perfusion at 6-7 ml/min for 3 min), a second stimulation with the same concentration of NT only resulted in a slight response which was even weaker and sometimes absent in a third stimulation (figure 3).

PC12 cells constitutively express purinergic receptors (ATP receptors). Although these receptors are linked to multiple intracellular signalling pathways, a complete pharmacological characterization of these receptors has never been described. Influx of extracellular calcium triggered by ATP receptor stimulation has been shown to play an important role in the transient $[Ca^{++}]_i$ increase (17), but the mobilization of intracellular calcium stores resulting from the activation of phospholipase C by ATP

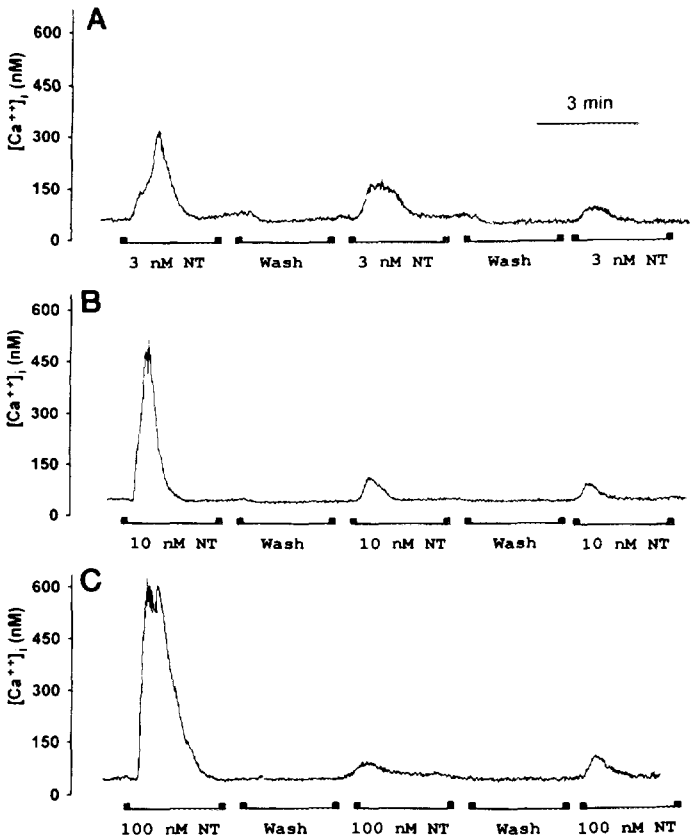

Fig.3. Responses to successive 3 min stimulations with 3 (A), 10 (B) and 100 nM NT (C). 3 min stimulations were separated by 3 min washes in calcium containing buffer. Data correspond to typical curves representative of at least 5 different experiments.

was also demonstrated in these cells (18,19). In agreement with this, we observed that stimulation of the transfected PC12 cells with ATP (50 μM) resulted in a rapid and transient increase of the $[Ca^{++}]_i$ (figure 4A). Though less important, this $[Ca^{++}]_i$ increase was also observed in calcium free buffer, indicating a contribution of the phosphoinositides pathway in response to ATP receptor stimulation. After a first stimulation with NT in calcium free buffer, the response to a subsequent stimulation with ATP was practically absent (figure 4B) .This indicates that both ATP and NT mobilize calcium from the same intracellular store, which can be rapidly chelated by extracellular EGTA (20). However, when stimulations with NT were performed in a calcium containing buffer, the response to ATP could still be measured after desensitization of the neurotensin receptor (Fig. 4C). This response was also observed when the stimulation with ATP was performed in a calcium free buffer, demonstrating that the NT response desensitization does not results from the depletion of intracellular calcium stores. In addition, these results show that the stimulation of the transfected PC12 cells with NT specifically induces the homologous desensitization of the NT receptor.

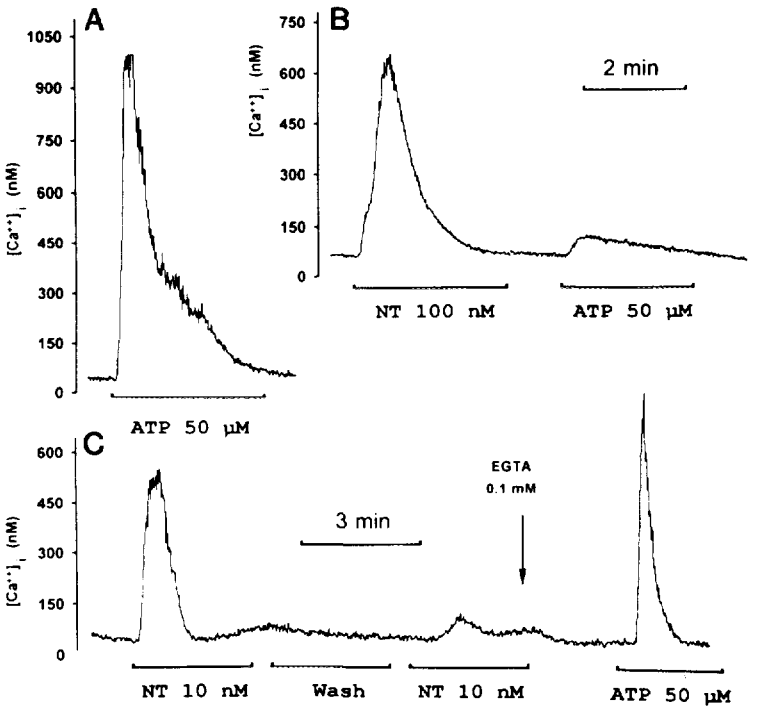

Fig.4. Changes in $[Ca^{++}]_i$ in transfected PC12 cells in response to various stimulations with ATP and NT. Panel A shows the response to ATP (50 µM) in calcium containing buffer. In B, the cells were succesively stimulated with NT (100 nM) and ATP (50 µM) in calcium free buffer. In C, the cells were stimulated twice with NT (10 nM) in calcium containing buffer, thereafter, the calcium buffer was replaced with calcium free (EGTA containing) buffer and the cells were stimulated with ATP (50µM). Data correspond to typical curves representative of at least 5 different experiments.

Both in vivo and in vitro experiments have shown that NT plays an important role in the control of the synthesis and the release of dopamine (21,22). Since the neurotransmitter release is calcium dependent, the rapid NT receptor desensitization measured in the present study probably accounts for the desensitization of the dopamine release observed after successive stimulations of rabbit striatum slices with NT (23). Rat pheochromocytoma PC12 cells constitute a commonly used model for the study of catecholamine biosynthesis and release (24,25). Although some receptors have been expressed in PC12 cells (26,27), these cells do not constitute a widely used model for the stable expression of G-protein coupled receptors because they constitutively express several membrane receptors, like bradykinin, VIP, nicotinic, angiotensin, and multiple growth factors receptors. As far as we known, NT binding sites have never been described on these cells, and this is in agreement with the absence of NT response in non transfected cells. The present paper indicates that NT binding sites expressed in PC12 cells after transfection show pharmacological and biochemical properties similar to those reported for the rat brain NT receptor. Therefore, transfected PC12 cells constitute an appropriate model to study the

pharmacology of the NT receptor found in neuronal cells and the interaction of neurotensin with dopamine at the molecular level.

ACKNOWLEDGMENTS

We thank J. Lambert for excellent technical assistance. This study was supported by a grant from the Fonds National de la Recherche Scientifique (FRSM LN 9.4511.89) and by the Ministère de l'Education et de la Recherche Scientifique (Action concertée 89/95-135). E. Hermans, Ph. Gailly are Research assistants and J.N. Octave is Research associate of the Fonds National de la Recherche Scientfifique (FNRS). SR48692 (batch number 9200VI) was kindly provided by Dr. D. Gully, (Sanofi Recherches, Toulouse, France).

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