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SHORT COMMUNICATION

Desensitization of Neurotensin-Induced Phosphoinositide Hydrolysis in Transfected CHO Cells

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ABSTRACT. The regulation of neurotensin-induced phosphoinositide turnover was studied in transfected CHO cells expressing the rat neurotensin receptor. Stimulation of these cells with neurotensin resulted in an important, but transient, increase in inositol phosphate cell content. Preincubation of the cells with neurotensin dramatically decreased their response to further stimulation. This diminution, which was time-dependent and not related to the availability of phospholipase C substrate, is though to reflect a progressive homologous desensitization of the recombinant neurotensin receptor. BIOCHEM PHARMACOL 51;12:1749–1752, 1996.

KEY WORDS. neurotensin receptor; phosphoinositide hydrolysis; desensitization

NT† is a tridecapeptide neurotransmitter that is present in both the central nervous system and in the peripheral tissues. It elicits a broad range of biological activities, after specific interaction with high-affinity NT receptors that have been biochemically and pharmacologically characterized in various mammalian tissues and in multiple cell lines. The NT receptor belongs to the broad family of G-protein coupled receptors [1]. PPI hydrolysis constitutes the major second messenger pathway associated with NT-receptor stimulation and NT-induced IP accumulation has been correlated with an increase in intracellular Ca^{2+} concentration [2].

Like the majority of G-protein-coupled receptors, the NT receptor present in cultured cell lines or in primary cultured neurons is subjected to homologous regulation by means of receptor desensitization or internalization [2–4]. In a recent study, transfected CHO cells expressing the rat NT receptor were used to characterize the regulation of the recombinant NT receptor [5]. Despite rapid receptor internalization, the intracellular calcium rise induced by NT did not show any sign of desensitization, as indicated by a sustained response during prolonged stimulation and by reproducible responses upon successive NT applications. Therefore, in the present study, attempts were made to study the regulation of NT-induced IP production, which constitutes a more sensitive gauge of receptor desensitization because PPI hydrolysis biochemically precedes calcium mobilization.

MATERIALS AND METHODS

Cell Culture

Transfected CHO cells expressing the NT receptor [6] were cultured in Ham-F12 medium supplemented with 10% (v/v) foetal calf serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 –95% air. For PPI hydrolysis assays, 10^5 cells were seeded as monolayer cultures in 6 multiwell dishes and grown for 3–4 days before the experiment. Cell culture media, foetal calf serum, and cell culture plastic ware were from Gibco (Paisley, U.K.).

PPI Hydrolysis

PPI hydrolysis was measured on intact cells labelled for 18 hr with [^{3}H]inositol (New England Nuclear, Boston, MA, U.S.A.) in serum and inositol-free RPMI medium. After stimulation, cells were disrupted in 500 μL deoxycholic acid 1% pH 11.3 and scraped from the dishes. PPI were extracted after the successive addition of 940 μL chloroform/methanol/HCl (50/100/1) and 313 μL chloroform. Total [^{3}H]IP present in the aqueous phase as measured after separation on Dowex AG1X8 columns (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as previously described [7]. Desensitization of PPI hydrolysis was studied by measuring the consequence of preincubation with NT in the absence of lithium on a subsequent accumulation of IP in the presence of lithium ions. In a first set of experiments, an initial incubation in the presence or absence of NT for increasing

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† *Abbreviations*: NT, neurotensin; IP, inositol phosphates; PPI, phosphoinositide.

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periods of time (desensitization step) was followed by a second incubation with NT for 30 min in the presence of LiCl (30 mM) (accumulation step). In a second set of experiments, a 40-min preincubation with NT was followed by stimulation with NT in the presence of LiCl for different periods of time.

RESULTS AND DISCUSSION

According to our previous study [6], the stimulation of transfected CHO cells expressing the NT receptor with NT (10 nM) resulted in an important increase in the total intracellular IP content, as shown in Fig. 1A. After prolonged stimulations of the cells with NT in the presence of lithium ions, the accumulation of IP tends to saturate progressively, indicating a dramatic decrease in NT-induced PPI hydrolysis. This accumulation of IP was correlated with a progressive decrease in radioactivity measured in the inorganic extract (Fig. 1B), indicating a diminution of the PPI pool that constitutes the phospholipase C substrate. However, this loss of phospholipase C substrate was not sufficient to explain the decrease in the IP response observed after prolonged stimulations with NT. In the absence of lithium ions, the NT-induced accumulation of IP was only observed during the first minutes of stimulation. Thereafter, the cell content of IP progressively decreased. Under these conditions, a substantial decrease in intracellular PPI was also observed.

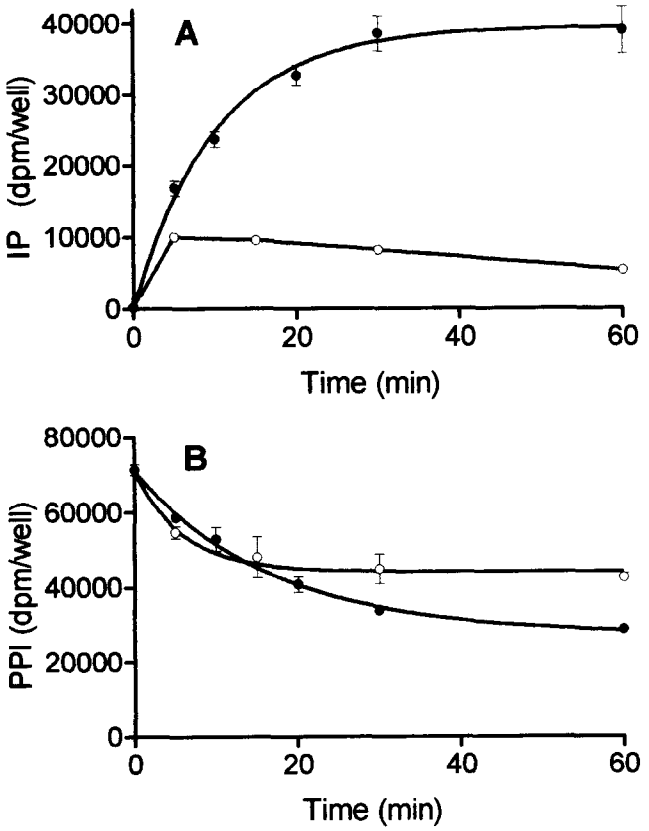

FIG. 1. A. Time-course of IP accumulation in transfected CHO cells stimulated with 10 nM NT in the presence (closed symbols) or in the absence (open symbols) of LiCl 30 mM. B. PPI cell content during stimulation with NT. Data presented correspond to mean values ± SD of the same typical experiment performed 3 times in triplicate.

In the presence of intact cells at 37°C, NT may be rapidly inactivated by extracellular peptidases. To verify the efficacy of NT in eliciting inositol phosphate production during prolonged application, the solutions containing NT were incubated in the presence of intact cells in a first set of dishes, before being transferred to a second set in which the responses were measured. As shown in Fig. 2, no significant decrease in the ability of NT to induce IP accumulation was observed after a 30-min preincubation. Therefore, the progressive decrease in the response of the cells to NT application observed in Fig. 1 could not be attributed to a significant degradation of the peptide in the incubation buffer.

As detailed in the experimental section, the desensitization of NT-induced PPI hydrolysis was measured by incubating the cells in the presence of NT in the absence of lithium ions before a subsequent stimulation step in the presence of lithium. During the desensitization step, the absence of lithium ions would allow the reincorporation of [^{3}H]inositol into phospholipids, thereby avoiding a critical loss of the phospholipase C substrate. In addition, [^{3}H]inositol was maintained in the medium during the preincubation step. However, even under these conditions, preincubation with NT also resulted in a partial decrease in radioactivity measured in the organic extract (approximately 50% decrease after 2 hr, not shown). Therefore, to study the response desensitization independently of phospholipase C substrate availability, the absolute production of IP during the accumulation step was divided by the PPI con-

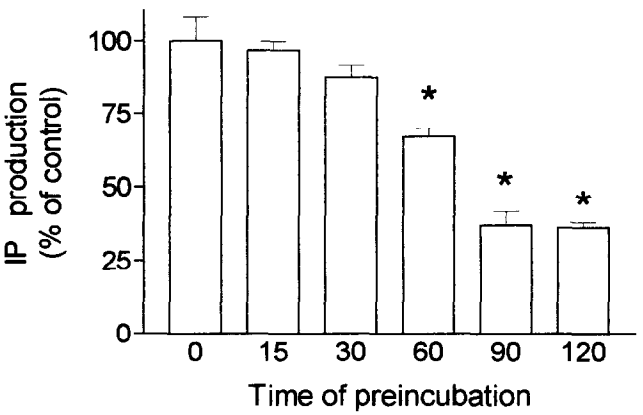

FIG. 2. Decrease in the activity of NT in the incubation buffer. Cells were stimulated for 30 min using a 10 nM NT-containing buffer that was previously incubated with intact CHO cells from a sister culture for various periods of time. Data present the accumulation of IP measured in the cells after stimulation and are expressed in percent of the response obtained using a fresh solution of NT. Maximal IP accumulation correspond to 49,464 ± 3948 dpm. Data shown correspond to mean values ± SD of 3 experiments performed in triplicate.

tent measured after the preincubation step. As shown in Fig. 3A, preincubation of the cells with NT led to a dramatic decrease in the further accumulation of IP, supporting evidence for desensitization. This desensitization of NT-induced IP accumulation was time-dependent. At a concentration of 10 nM NT during preincubation, the half-maximal response decrease was observed after approximately 10 min and maximal desensitization concerned 85% of the control response. Kinetic experiments clearly indicated that the decrease in the response measured in preincubated cells resulted from a decrease in the rate of IP accumulation. The IP level detected after stimulation of the control cells for 5 min was only reached after a 25-min stimulation of the cells preincubated for 40 min with NT (Fig. 3B).

Despite the abundant literature concerning the regulation of the NT receptor in cultured neurons [3, 4, 8], in cell lines [9], and in transfected cells [2, 7], the homologous desensitization of NT-induced PPI hydrolysis was only reported in HT29 cells [10]. Accordingly, the present results demonstrate the existence of an homologous desensitization of the recombinant NT receptor in transfected CHO cells. Such desensitization contrasts with our recent work concerning NT-induced calcium mobilization in these transfected CHO cells, where a lack of desensitization of the response triggered by NT was reported [5]. Together, these results indicate that, according to our previous hypothesis, the lack of calcium mobilization desensitization does not result from the absence of receptor desensitization by itself, but is more likely to be related to the high efficiency of the NT receptor-signalling pathway in the transfected CHO cells [5]. Indeed, if low levels of inositol trisphosphate are sufficient to assure maximal cytosolic calcium mobilization, this mobilization could be maintained despite receptor regulation and decreased IP formation. Accordingly, the absence of calcium mobilization desensitization observed for the recombinant histamine H_1 receptor expressed in CHO cells correlates with the high efficiency of histamine in mediating the response in these cells [11], whereas rapid desensitization of the histamine receptor was frequently reported in other cell lines [12]. Calcium mobilization constitutes a major intracellular messenger in cell response to NT. In view of the absence of desensitization of NT-induced calcium mobilization, the role of receptor desensitization and internalization in the control of intracellular signalling in the model of CHO cells is rather questionable. However, in contrast to what was observed in the CHO cells, studies performed in cultured neurons or in transfected PC12 cells expressing the NT receptor showed that, in these models, calcium mobilization triggered by NT was rapidly desensitized [3, 7]. In N1E-115 neuroblastoma cells, NT-induced cGMP production that is secondary to phospholipase C activation also desensitized after receptor stimulation with agonists [13]. In addition, some physiological responses induced by NT were found to decay after prolonged stimulations [14, 15]. On the contrary, absence

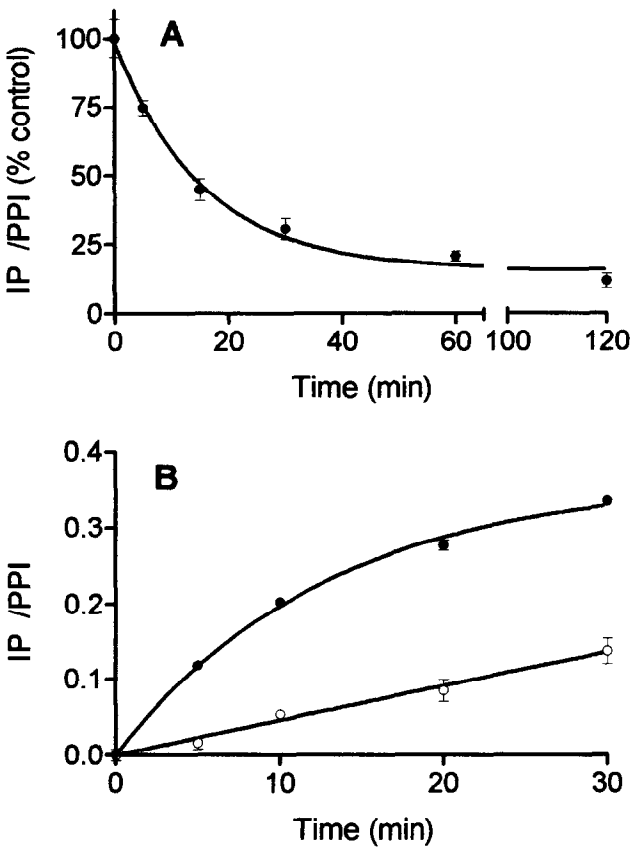

FIG. 3. A. Time course of NT-induced PPI hydrolysis desensitization in transfected CHO cells. Cells preincubated with NT (10 nM) for 0–120 min in the absence of LiCl were stimulated with NT 10 nM for 30 min in medium containing LiCl 30 mM. To avoid an influence of PPI availability in the cells at the time of stimulation, the amount of IP measured at each point was divided by the PPI content measured before the 30-min stimulation step. Data are expressed as percent of control value (nondesensitized cells) and correspond to mean values ± SD of a representative experiment performed 3 times independently in triplicate. The initial content of radioactivity in the organic phase (PPI) was 120,512 ± 3593 dpm. In control cells (0 min preincubation), the IP accumulation observed during the stimulation step was 51,936 ± 4061 dpm.

B. Time-course of NT (10 nM)-induced IP accumulation in the presence of LiCl 30 nM in control cells (closed symbol) or in cells previously exposed for 40 min to NT 10 nM (open symbol). Data correspond to the amount of IP accumulated above basal level during stimulation, divided by the PPI content measured before stimulation. Data correspond to mean values ± SD of a representative experiment performed 3 times independently in triplicate. PPI content was 134,903 ± 2675 dpm and 70,600 ± 11,172 dpm for control and desensitized cells, respectively. The inositol phosphate accumulation measured after 30 minutes was 45,154 ± 1013 dpm and 9717 ± 1735 dpm for control and desensitized cells, respectively.

of desensitization of the response elicited by NT was also reported in various models [16, 17]. Together, these data support a cell-type or tissue-dependent profile of response desensitization that may be independent of the desensitization of the NT receptor itself.

The molecular and cellular mechanisms involved in the modulation of the intracellular signal triggered by NT are still obscure. For some G-protein coupled receptors, it has been frequently proposed that the desensitization at the second messenger level may result from a molecular uncoupling of the receptor from its G-protein after phosphorylation of the receptor [18]. From this point of view, desensitization of cellular signalling may precede receptor internalization. Internalization of the NT receptor in transfected CHO cells was previously reported [5, 19]. The kinetics of desensitization measured in the present study and the kinetics of cell surface receptor decrease are very similar and did not permit us to completely distinguish their mechanisms. This question was addressed by the group of Turner using HT29, where desensitization was found to precede internalization [10]. In addition, recent studies conducted with other G-protein coupled receptors led their authors to suggest that internalization could be involved in the resensitization of the agonist-induced desensitized receptor [20]. Concerning the NT receptor, further experiments regarding an eventual agonist-induced receptor phosphorylation and/or uncoupling will help clarify the relationship between receptor desensitization and internalization.

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