The effect of neuropeptides kyotorphin and neokyotorphin on proliferation of cultured brown preadipocytes

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Abstract Stimulation of DNA and protein synthesis in brown preadipocytes by 1 μM neokyotorphin in serum-containing media was comparable with the effect of 1 μM norepinephrine. In serum-free medium a decrease and a shift of the maximal effect to lower concentration of neokyotorphin were observed. Kyotorphin had no effect on cell proliferation in either medium; however, 0.01–1 μM kyotorphin inhibited the cell proliferation stimulated by 1 μM norepinephrine. Norepinephrine and both peptides stimulated comparable Ca^{2+} rise in freshly isolated brown preadipocytes. The effects of neokyotorphin and norepinephrine were additive, whereas 0.03–0.3 μM kyotorphin blocked the action of 3 μM norepinephrine. The peptides did not affect the cAMP level in non-stimulated or norepinephrine-stimulated cultured cells. The effects of the peptides on the brown fat cell cultures indicate that peripheral tissue cells contain receptors for these neuropeptides.

Key words: Kyotorphin; Neokyotorphin; Cell proliferation; Brown adipocyte (neuropeptides, norepinephrine)

1. Introduction

Brown adipose tissue constitutes an interesting model for studies of cell proliferation and cellular differentiation. Brown adipose tissue plays a major role in production of heat necessary for newborns, hibernators and mammals living in the cold. In brown adipose tissue both the facultative thermogenic response and the long-term recruitment processes in the tissue are under positive adrenergic control. Cold exposure of an animal induces within the tissue a marked increase in mitogenic activity and in the expression of genes coding for proteins specific for brown adipose tissue function. Norepinephrine, released from sympathetic nerve fibres in the tissue, acts through β-adrenergic receptors and elevation of intracellular cAMP [1,2]. An interesting point is that although the physiological stimulus for recruitment remains switched on, the cells only respond with a limited and well-controlled hyperplasia. The regulatory factors behind the limited hyperplasia are still not defined.

A study of the regulatory factors behind the large-scale physiological changes in hibernating and cold-acclimated animals can help to clarify molecular events leading to many pathological processes in normothermic mammals. During the last years, several peptides possibly involved in regulation of hibernation have been isolated from the brain of hibernating ground squirrels [3–5]. It was found that two of them, which turned out to be the already known neuropeptides kyotorphin (Tyr–Arg, YR) and neokyotorphin (Thr–Ser–Lys–Tyr–Arg, TSKYR), had some thermoregulatory properties when intraperitoneally injected into animals [5,6]. Kyotorphin and neokyotorphin are related to 'non-classical' 'opioid-like' peptides and may be involved in pain regulation in mammals [7], but they do not bind to typical opioid receptors [8]. Kyotorphin induces analgesia by a release of Met-enkephalin in the brain [8,9] and neokyotorphin by an inhibition of a stimulated release of γ-aminobutyric acid [10].

Using a brown fat cell culture system, we have studied the ability of kyotorphin and neokyotorphin to affect cell culture growth and to modulate the adrenergic stimulation of cell proliferation. We proposed that outside the central nervous system, these peptides may not have an analgesic, but rather another function, such as regulation of mitogenic activity. In order to analyse the effect of the peptides in the absence of 'classical' growth factors, a system for the culture of brown fat cells under fully defined conditions has been developed [11]. Preliminary results were reported earlier [12,13].

2. Methods

2.1. Cell isolation and cultivation

Brown fat precursor cells were isolated from 3–4-week-old male mice of the NMRI strain principally as described earlier [1]. The brown adipose tissue depots were dissected out from each mouse, and the tissue was digested with crude collagenase type 2 (200 U/ml). The infranatant was filtered and the precursor cells were pelleted by centrifugation, suspended in culture medium and inoculated onto 6- or 12-well standard plates (density 90 000 cells/cm^2). The cells were cultivated in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new-born calf serum and insulin. For experiments in serum-free conditions, standard medium was changed on day 3 or 4 to a DMEM/F12-based serum-free medium, supplemented with 0.5% albumin [11].

The experiments were performed with cells grown in culture for 3–4 days with serum or for 5 days with 1–2 days without serum, i.e. preconfluent, proliferating cells. Peptides were added 5 min before norepinephrine. Incubation with peptides, 1 μM norepinephrine and [^{3}H]thymidine was terminated by harvesting the cells 4 h (with serum) or 20 h (serum-free medium) after addition of agents. A cell homogenate was prepared and used for protein, DNA and [^{3}H]thymidine determination as described earlier [1].

2.2. cAMP determination

The cultured cells were incubated for 5–10 min (day 3–4) or 20–30 min (day 6) with the agents, the medium was discarded and ethanol was added to each well for cAMP extraction. The samples were dried

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Abbreviations: CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxyl[-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride; IP_3, D-myo-inositol-1,4,5-triphosphate; NE, norepinephrine

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in a Speedvac centrifuge and cAMP was analysed according to the description in the Cyclic AMP [^{3}H] Assay System from Amersham.

2.3. IP_3 determination

The cultured cells were incubated for 0.5–20 min with 1 μM agents, the medium was discarded and 0.6 ml of 0.5 M $HClO_4$ was added to each well and the cells were harvested. The suspension was centrifuged (16000×g×10 min) and to 0.5 ml of supernatant 50 μl of 2.5 M K_2CO_3 in 60 mM HEPES buffer was added. After 20 min incubation and centrifugation (16000×g×3 min), 100 μl×2 aliquots were used for IP_3 determination in the D-myo-inositol-1,4,5-triphosphate [^{3}H] Assay System from Amersham.

2.4. Measurement of intracellular Ca^{2+}

Concentration of Ca^{2+} in cultured cells was measured as described earlier [14] using the fluorescent dye Fura-2. Cells were grown on coverslips in 6-well plates. On the day of the experiment cover-slips were transferred to Krebs-Ringer phosphate buffer and cells were loaded with Fura-2 for 1 h. Fluorescence was measured in Krebs-Ringer bicarbonate buffer with excitation wavelengths of 355 and 395 nm. Ionomycin (30 μM) and 2 mM $MnCl_2$ were used to estimate R_{max} and R_{min}, respectively.

Freshly isolated brown preadipocytes were loaded with Fura-2, and the fluorescence was measured in Hanks' balanced salt medium, buffered with 10 mM HEPES, pH 7.2. F_{max} was estimated by adding 25 μM digitonin; $F_{min} = F_{max}/3$. The wavelengths of excitation and registration were 337 nm and 495 nm, respectively.

2.5. Receptor binding experiments

Membranes from brown adipose tissue depots were prepared as described earlier [15]. Competition-binding experiments were performed with 0.8 nM [^{3}H]Prazosin and [^{3}H]CGP-12177, and 5×10^{-9}–10^{-3} M kyotorphin and neokyotorphin. Non-specific binding was measured in the presence of 5000-fold excess of phentolamine and alprenolol, respectively.

3. Results and discussion

The presented results indicate that the investigated neuropeptides act in opposite directions on the proliferation of cultured brown preadipocytes. A pronounced stimulation of brown preadipocyte proliferation by neokyotorphin was obtained in the presence of new-born calf serum (Fig. 1A). The effect of neokyotorphin was comparable with the effect of norepinephrine, but their actions were not additive: neokyotorphin had only a small additional effect in the presence of norepinephrine. The effect of neokyotorphin was clearly dose-dependent, $EC_{50} \sim 0.6$ μM. In serum-free conditions, the efficiency of neokyotorphin was higher, its dose response curve had a bell-shape in the range 1–100 nM (not shown). Under these conditions both norepinephrine (it was also shown earlier [11]) and neokyotorphin, had smaller effects (Fig. 1B), although the additivity was more pronounced. The shorter peptide, kyotorphin, had in itself very low inhibitory or no effect on cell proliferation, whereas kyotorphin at some concentrations abolished the simulatory effect of norepinephrine on cell proliferation (Fig. 1C,D). In the presence of serum, the most effective concentration of kyotorphin was around 1 μM, whereas in serum-free medium the effect was shifted to lower concentrations of the peptide, $IC_{50} \sim 3$ nM (Fig. 1D). In contrast to norepinephrine [1] neither peptide affected the specific labelling of DNA with [^{3}H]thymidine.

In competition-binding experiments, we checked a possible binding of the peptides to β_1- and α_1-adrenoceptors and did not find any interaction (not shown). There is little evidence in the literature for the existence of specific receptors for these peptides. The kyotorphin receptor has been identified in membranes of rat brain. The low-affinity receptor appears to be functionally coupled via G_i to stimulation of phospholipase C and an increase in IP_3 [16–18]. A receptor and a second messenger for neokyotorphin have not been identified. With a commercially available [^{3}H] assay kit for IP_3, we were only able to reliably register the effect of norepinephrine on differentiated brown adipocytes (day 6 in culture). All changes in IP_3 after addition of norepinephrine to proliferating cells were on the limit of the sensitivity of the kit. For 1 μM peptides we were unable to find any signs of their action, neither in proliferating nor in differentiated cells. This is not contradictory to the results published in [16] because they found a statistically significant accumulation of IP_3 only with 100 μM kyotorphin.

Measurements of intracellular Ca^{2+} in cultured cells were carried out with cells grown on cover-slips. Due to the low number of the cells and, therefore, low signal/noise ratio, we could not work with the cells in the early stages of proliferation but used them on day 4, when up to 30% of the cell population showed characteristics of differentiation. To exclude an effect of the peptides on the differentiated cells, we compared proliferating cells with differentiated cultured brown adipocytes and freshly isolated preadipocytes.

Principal difference in the kinetics and magnitude of Ca^{2+} response has been found between the freshly isolated and the cultured cells. The effect of norepinephrine on cultured cells was quick and high in magnitude ($t_{1/2} \sim 0.2$ min, $\Delta[Ca^{2+}]_i \sim 300$–$800$ nM, Fig. 2A) both in proliferating and differentiated cells. As a first approximation, 0.3–30 μM neokyotorphin approximately doubled $[Ca^{2+}]_i$ in the cells; this is in agreement with the effect of neokyotorphin on myocardial cells [3]. In the differentiated cells we did not find significant effects of neokyotorphin on $[Ca^{2+}]_i$. Kyotorphin did not have any clear effect on $[Ca^{2+}]_i$ in either cultured cells. The freshly isolated preadipocytes responded to norepinephrine very slowly ($\Delta[Ca^{2+}]_i \sim 2$ nM/min, Fig. 2B), all in accordance with the results of Tuchiya and Nagai [19]. Under these conditions, norepinephrine and both peptides had almost identical kinetics of Ca^{2+} increase. Kyotorphin modulated the cellular response to norepinephrine in either cells. It increased the magnitude of the stimulatory effect of norepinephrine on $[Ca^{2+}]_i$ in cultured cells, but in freshly isolated cells kyotorphin at some concentrations blocked the stimulatory action of norepinephrine (Fig. 3). The most effective concentrations of kyotorphin in modulation of the Ca^{2+} response to norepinephrine (0.03–0.3 μM) were almost identical to those that were most effective in suppression of norepinephrine-induced cell proliferation (Fig. 1C,D). Neokyotorphin did not change the response of cultured cells to norepinephrine, but in freshly isolated cells there was seen a tendency to the additivity of their effects (Fig. 2B).

The ability of adrenergic stimulation to affect the rate of DNA synthesis in mouse brown adipocyte precursor cells proliferating in primary culture has earlier been investigated [1]. It was concluded that brown fat precursor cells respond directly to norepinephrine stimulation with an increased DNA and protein synthesis and that this response is mediated via the classical β_1 receptors positively coupled to cAMP [1]. The investigated peptides did not in themselves affected cAMP accumulation, although 0.1–1 μM kyotorphin influenced cAMP accumulation stimulated by norepinephrine, by approximately 20%. This either inhibitory or stimulatory effect was usually statistically significant within an experiment. Ear-

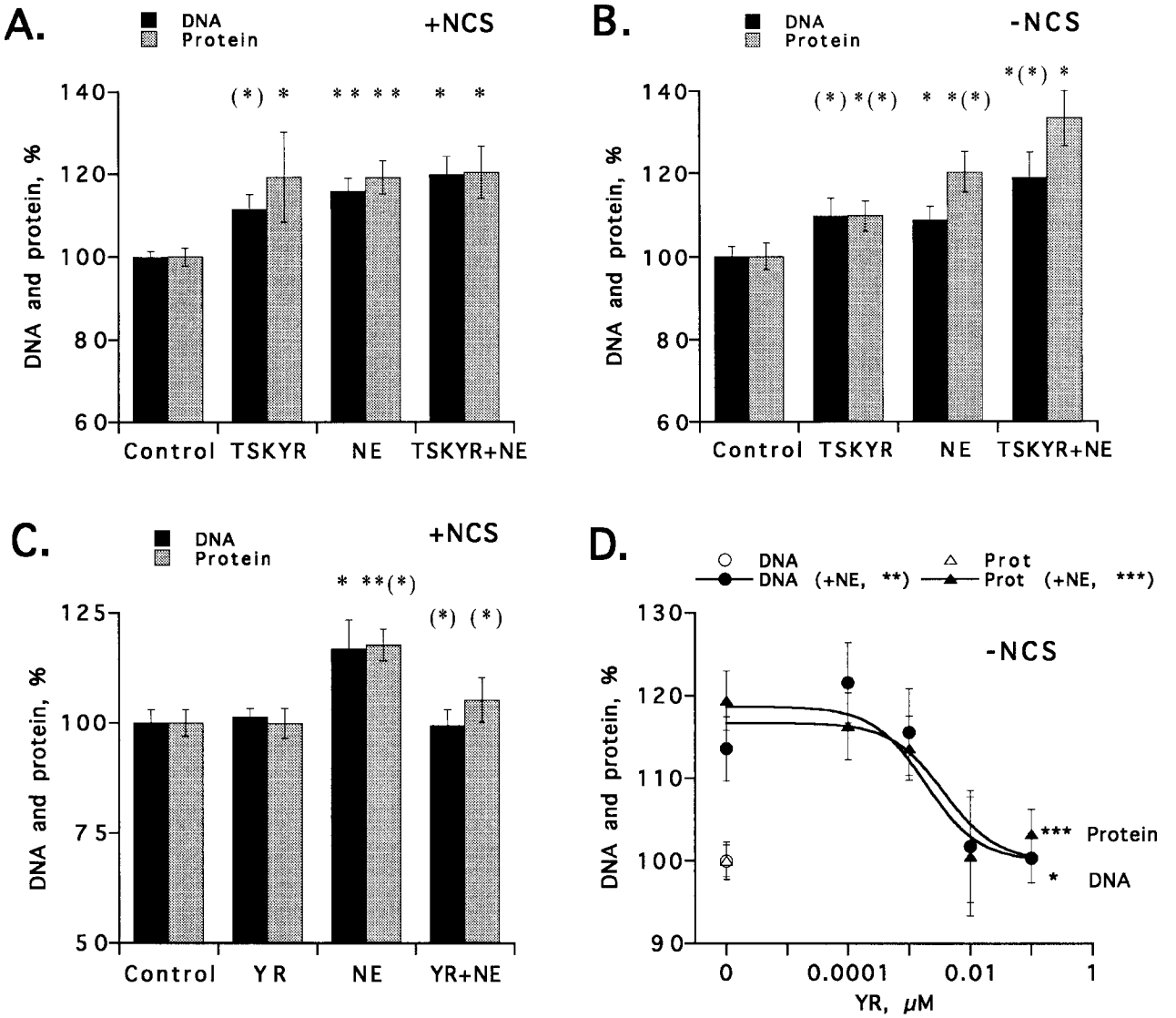

Fig. 1. Effect of neokyotorphin (A,B) and kyotorphin (C,D) on DNA and protein synthesis in cultured preconfluent proliferating brown adipocytes, stimulated and non-stimulated with norepinephrine. Cells were cultivated in standard 6-well plates either in the presence of serum (A,C) or in serum-free conditions (B,D). Concentrations: 1 μM norepinephrine, 1 μM (A) and 0.01 μM (B) neokyotorphin, 1 μM kyotorphin (C). In each experiment, the average value obtained in the control non-treated wells was set to 100% and the data were expressed in percent of the control value. The mean value corresponding to 100% varied from an experiment to experiment within 2–5 μg DNA and 30–70 μg protein. Bars are means ± SE of all samples (4–7 experiments, 2–4 samples in each). The data were analysed for significant differences from their respective control values and, where relevant (YR+NE), from the NE effects in each experiment by Student's paired *t*-test. $^{(*)}P<0.1$; $^{*}P<0.05$; $^{*(*)}P<0.025$; $^{**}P<0.01$; $^{***}P<0.001$.

lier we found that norepinephrine-stimulated DNA synthesis in proliferating cells is almost insensitive to these small variations in intracellular cAMP concentrations (G. Bronnikov et al., unpublished results). Therefore we conclude that the effect of kyotorphin on cAMP accumulation may be related to a 'side effect' and can not explain the inhibitory action of the peptide.

From our previous experiments, it is clear that cultured brown adipocytes contain Ca^{2+}-activated adenylyl cyclase and phosphodiesterase [20], and a modulation of intracellular Ca^{2+} can affect these activities. In the differentiated cells, simultaneous activation of β- and α_1-receptors was shown to lead to two opposite effects. It increased 2–3-fold the expression of uncoupling protein mRNA with cAMP as second messenger, but at the same time the α_1-mediated Ca^{2+} rise activated phosphodiesterase and therefore inhibited cAMP accumulation. The final effect depended on the relative activation of β- and α_1-receptors [20,21]. In proliferating cells, cAMP accumulation was almost insensitive to elevated Ca^{2+}, and the Ca^{2+}-ionophore, ionomycin (1 μM), had only a 10–15% inhibitory effect on cAMP level (80% in the differentiated cells) [21]. Earlier it was found that in proliferating cells, norepinephrine utilises both β- and α_1-receptors synergistically to maximally induce expression of the c-fos protooncogene [14], but nothing is known about a role of Ca^{2+} in regulation of proliferation of brown adipocytes. The presented results are the first evidence of possible participation of Ca^{2+} ions in this regulation.

The effects of the peptides on the brown adipocyte cell culture system clearly indicate that peripheral tissue cells con-

tain receptors for these neuropeptides. We would like to speculate that outside the central nervous system these peptides do not have an analgesic, but another function: regulation of mitogenic activity in peripheral tissues. Most probably, the tissues of animals generally contain some amount of these peptides and enzymes for their formation and degradation. Alternatively neokyotorphin can also be produced by proteolytic degradation of haemoglobin in erythrocytes [22]. The amino acid sequences of neokyotorphin and kyotorphin are identical to that of the five and two C-terminal amino acid residues of the haemoglobin A-chain, respectively. Brown adipose tissue is highly enriched with blood vessels and capillaries, and neokyotorphin originating from haemoglobin could play a role in brown adipose tissue recruitment under circumstances when the adrenergic system can not be used. The ratio of concentrations of these peptides may be an important parameter, because the stimulatory effect of neokyotorphin can be reversed by the cleavage of the peptide by a specific protease [23]. Very similar regulation was found for neokyotorphin recently [24]. Degradation of neokyotorphin of erythrocyte origin to neokyotorphin (1–4) resulted in formation of a novel activity: TSKY induced DNA fragmentation in tumor cells [24].

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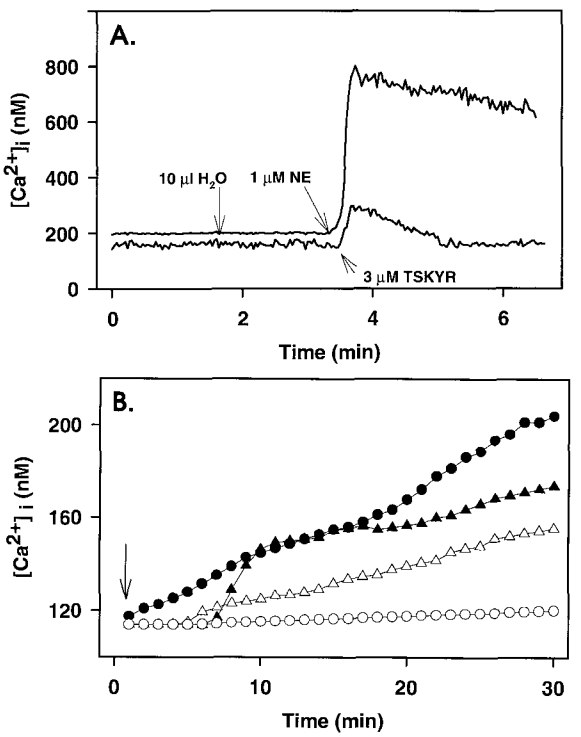

Fig. 2. Typical calcium response to norepinephrine and neokyotorphin in (A) 4-day cultured brown adipocytes and (B) freshly isolated brown preadipocytes. In (B) arrow indicates the additions: (○) control, (△) 3 µM TSKYR, (▲) 3 µM NE, (●) NE+TSKYR. Data from four experiments were summarised (not shown) and analyzed by Student's paired *t*-test. The significant differences from the control for the effects of all agents and for the effect of TSKYR from NE+TSKYR are $P \leq 0.025$.

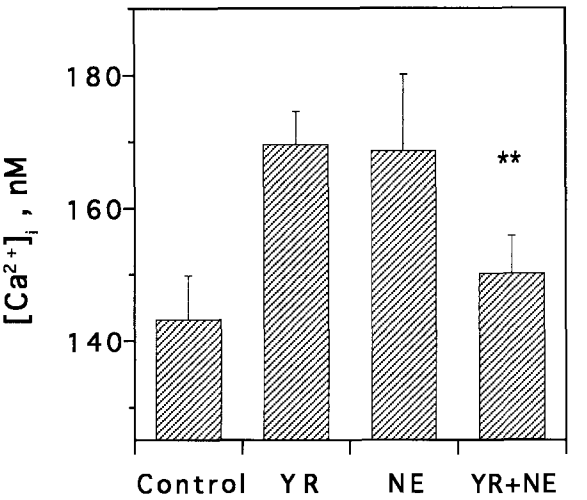

Fig. 3. Effect of kyotorphin and norepinephrine on intracellular $[Ca^{2+}]$ in freshly isolated brown preadipocytes. $[Ca^{2+}]_i$ was registered 20 min after addition of the agents. Concentrations: 0.1 µM kyotorphin, 3 µM norepinephrine. Bars are mean ± SE of 4 experiments in duplicates. Data were analyzed by Student's paired *t*-test. The significant differences from the control for the effects of all agents are $P \leq 0.01$, and for the effect of YR+NE from the effect of NE is shown on the figure.

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