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Inhibitory effect of imipramine on the human corticotropin-releasing-hormone gene promoter activity operates through a PI3-K/AKT mediated pathway

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Abstract

Antidepressant drugs inhibit the corticotropin-releasing-hormone (CRH) gene promoter activity in the differentiated Neuro-2A cells, but a molecular mechanism of their action has been poorly recognized. The aim of the present study was to elucidate the involvement of some intracellular signal transduction pathways in imipramine-induced inhibition of CRH gene activity in the differentiated Neuro-2A cells, stably transfected with a human CRH promoter fragment linked to the chloramphenicol acetyltransferase (CAT) reporter gene. It was found that wortmannin (0.1 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3-K) and forskolin (10, 25 μM), an activator of adenylate cyclase enhanced the basal activity of CRH gene promoter, whereas inhibitors of protein kinase A, calcium/calmodulin kinase (CaMK) and mitogen-activated protein kinase (MAPK) had opposite effects. Moreover, wortmannin at a low concentration (0.01 μM) significantly reversed the inhibitory effect of imipramine on CRH-CAT activity, whereas other protein kinase inhibitors were inactive or even enhanced the imipramine effects. The involvement of PI3-K/Akt pathway in the imipramine action was confirmed by Western blot study, which showed that this drug increased phospho-Ser-473 Akt level, but had no effect on total Akt and glycogen synthase kinase (GSK-3β) levels. These results indicate that the inhibitory effect of imipramine on the CRH gene promoter activity in Neuro-2A cells is mainly connected with enhancement of PI-3K/Akt pathway.

Keywords: Imipramine; hCRH gene promoter activity; Differentiated Neuro-2A cells; Stable transfection; Protein kinases

1. Introduction

Apart from the disturbed monoaminergic neurotransmission, hyperactivity of hypothalamic–pituitary–adrenal (HPA) axis is the main biochemical change observed in the patients suffering from a major depression (Holsboer et al., 1985; Linkowski et al., 1985). High incidence of depression in Cushing's syndrome and antidepressant effects of adrenocortical enzyme inhibitors and an antagonist of corticotropin-releasing-hormone (CRH) receptors (Jeffcoate et al., 1979; Murphy, 1997; O'Brien et al., 2001; Zobel et al., 2000) support the hypothesis that hyperactivity of HPA axis may be involved in pathogenesis of depression. The elevated CRH concentrations in the cerebrospinal fluid in depressed patients and increased number of CRH-expressing neurons in the hypothalamus strongly

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suggest that the aberrant activity of the HPA axis in major depression is evoked by hypersecretion of CRH (Nemeroff et al., 1984; Raadsheer et al., 1994).

Antidepressant drugs are known to normalize HPA axis activity, while lack of such an effect is often associated with the risk of relapse or the lack of clinical improvement. In experimental animals, long-term treatment with antidepressants decreased ACTH and corticosterone concentrations in blood and the CRH mRNA level in the rat hypothalamus, although some negative results were also reported (Brady et al., 1991, 1992; Stout et al., 2002).

The delayed therapeutic effects of antidepressant drugs (2–3 weeks) and a recent preclinical evidence suggest that long-term adaptive changes in the neuronal system, possibly at a genomic level, may be important to their mechanism of action (Manier et al., 2002; Schwaninger et al., 1995; Tamura et al., 2002). Our previous studies have shown that antidepressant drugs (imipramine, amitryptyline, desipramine, fluoxetine, citalopram, mianserin, venlafaxine, reboxetine, mirtazapine and milnacipram) inhibited hCRH gene promoter activity in the differentiated Neuro-2A cells, stably transfected with a human CRH promoter fragment linked to the chloramphenicol acetyltransferase (CAT) reporter gene (Budziszewska et al., 2004). The effect of these drugs on CRH gene transcription was observed only after longer time of their presence in culture medium, which suggests that antidepressants do not act directly on the CRH-CAT reporter gene, but rather affect a downstream processes involved in the regulation of CRH gene activity. The regulation of the human CRH promoter gene has been a subject of several in vitro investigations, but only the involvement of PKA, PKC and glucocorticoids was confirmed (Guardiola-Diaz et al., 1994, 1996; Itoi et al., 1996; Malkoski and Dorin, 1999; Rosen et al., 1992). However, a possible participation of this signal transduction pathway in the mechanism of antidepressant drug effect on the CRH gene regulation has not been studied so far.

The aim of the present study was to examine an involvement of protein kinase A (PKA), protein kinase C (PKC), protein kinase B (PKB; Akt), Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and mitogen-activated protein kinase (MAPK) (e.g. enzymes which are known to be changed in depression and are affected by antidepressant drugs) (Dwivedi et al., 2001; Einat et al., 2003; Hsiung et al., 2003; Mann et al., 1995; Maragnoli et al., 2004; Nalepa and Vetulani, 1991; Silver et al., 1986) in inhibitory action of imipramine on CRH gene promoter activity.

We chose imipramine for this study, because its effects on second messengers, protein kinases and transcription factors are the best recognized among antidepressants. Furthermore, since our previous studies have shown that antidepressants acted more strongly and were effective at lower concentrations in the differentiated than in undifferentiated Neuro-2A cells or pituitary cells (Budziszewska et al., 2004), we used the differentiated Neuro-2A cells.

2. Materials and methods

2.1. Cell culture conditions

Neuro-2A (mouse neuroblastoma) cells, obtained from the American Type Culture Corporation (ATCC), were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 50 units/ml of penicillin and 50 μg/ml of streptomycin (Sigma Chemical Co.), in the atmosphere composed of 5% CO_2/95% O_2 at 37 °C. Stably transfected cells were cultured in the medium with zeocin (150 μg/ml; Symbios), instead of penicillin and streptomycin. For in vitro differentiation, the cells were grown for 3 days before experiment in the medium containing 1% serum.

2.2. Plasmid construction and cell transfection

Syntheses of hCRH-CAT plasmid and stable transfection of Neuro-2A cells were performed as described previously (Budziszewska et al., 2002, 2004). Human DNA was isolated from blood using Blood DNA Prep Kit (A&A Biotechnology, Poland). Based on the known human CRH gene promoter sequence (GenBank Accession No. AF48855), the specific primers from the region −663 to +124 bp were synthesized for PCR amplification. The forward primer was: CRHKpn 5′ *CGC GGT ACC* GAG AGA CGT CTC CGG GGG C 3′ (28 nt, containing a *Kpn*I recognition site), and the reverse primer was: CRHBgl 5′ GCG *AGA TCT* GGC TCA TAA CTC CTT TAT GTG CTT GC 3′) (35 nt, containing a *Bgl*II recognition site). The reaction mixture consisted of: 50 ng of the human genomic DNA, 2 μl (10 μM) of each primer, 5 μl (10 mM) of dNTPs, 5 μl of 10× PCR buffer (100 mM Tris–HCl, pH 8.9, 500 mM KCl, 20 mM $MgCl_2$, 1% Triton X-100), 2 U of Pwo DNA polymerase (Dąbrowski and Kur, 1998). The fragment was amplified by 7 cycles performed with the following temperature profile: 30 s at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and then 33 cycles (30 s at 94 °C, 1 min at 70 °C, and 1 min at 72 °C). The amplification product was analyzed by electrophoresis on a 1% agarose gels stained with ethidium bromide. Specific, approximately 800 bp long, PCR product was obtained (0.2 μg), which was purified using Clean-Up Kit (A&A Biotechnology, Poland), and digested with *Bgl*II and *Kpn*I, and then isolated from an