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Studies on the involvement of endogenous neuropeptides in the control of thymocyte proliferation in the rat

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Summary. The possible involvement of endogenous vasoactive intestinal peptide (VIP), cholecystokinin (CCK) and neurotensin (NT) in the control of thymocyte proliferation has been investigated *in vivo* in the immature rat. For this task, we have studied the effects of the administration of selective antagonists of the receptors of the three neuropeptides on the mitotic index (‰ of metaphase-arrested cells after vincristin injection) of thymocytes. Both CCK- and TN-receptor antagonists were ineffective. In contrast, two VIP receptor antagonists (VIP-As) enhanced the mitotic index of thymocytes. VIP reversed the effect of VIP-As, but when administered alone it did not alter the mitotic activity of thymocytes. In light of these findings, we conclude that endogenous VIP exerts a maximal tonic inhibitory influence on the basal proliferative activity of rat thymocytes, while endogenous CCK and NT do not play a relevant modulatory role in this process.

Key words: Thymus, Cell proliferation, Vasoactive intestinal peptide, Cholecystokinin, Neurotensin, Rat

Introduction

Several regulatory neuropeptides and their receptors are present in the various components of the immune system, where they are able to modulate some important steps of the immune response. One of these steps is the proliferation of immune competent cells, among which are thymocytes (Savino et al., 1990; Dardenne and Savino, 1994; Head et al., 1998).

The characteristic cytoarchitecture of the thymus is responsible for the maintenance of a unique microenvironment, which seems to be essential for the correct development of T cells (Brelinska and Warchol, 1997). This makes the use of the cell culture techniques unsuitable in the studies on the control of thymocyte proliferation. Moreover, the *in vivo* administration of exogenous neuropeptides do not provide reliable information on the possible physiological role of their endogenous counterparts in the control of thymocyte proliferation.

Bearing these limitations in mind, we designed experiments where the involvement of endogenous vasoactive intestinal peptide (VIP), cholecystokinin (CCK)/gastrin and neurotensin (NT), three neuropeptides contained in the immune system (see Discussion), in the control of thymocyte proliferation has been investigated by administering rats with their selective receptor antagonists.

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Materials and methods

Animals and reagents

Immature (20-day-old) female Wistar rats were kept under a 12:12 h light-dark cycle (illumination onset at 8:00 a.m.) at 23±1 °C, and maintained on a standard diet and tap water *ad libitum*.

VIP(1-28) rat, the VIP antagonists neurotensin(6-11)/VIP(7-28) (Moody et al., 1993) (VIP-A1), and [Ac-His[1],D-Phe[2],Lys[15],Arg[16]]VIP(3-7)GRF(8-27)-NH_2 (VIP-A2) (Gourlet et al., 1997), and pentagastrin (PG) were purchased from Bachem (Bubendorf, Switzerland). The pituitary adenylate cyclase-activating polypeptide (PACAP) antagonist PACAP(6-38) (Dickinson et al., 1997) was obtained from Peninsula (St. Helens, UK). The CCK receptor A and B antagonists PD140,548 (CCKA-A) and PD135,158 (CCKB-A), respectively (Hughes et al., 1990; Higginbottom et al., 1993) were obtained from Research Biochemical International (Natick, Mass., USA). NT and the NT antagonist (NT-A) [D-Trp[1]]-NT (Quirion et al., 1980), as well as other laboratory reagents were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Vincristin was obtained from Gedeon-Richter (Budapest, Hungary).

Experimental procedures

Groups of immature rats (n=6) were given three subcutaneous injections (28, 16 and 4 h before sacrifice) of the following chemicals dissolved in 0.2 ml 0.9% NaCl: (i) VIP and/or VIP-A1, VIP-A2 and PACAP(6-38) (20 nmol/kg); (ii) CCKA-A or CCKB-A alone or with PG (20 nmol/kg); and (iii) NT and/or NT-A (40 nmol/kg). Control rats were injected with the saline vehicle. All groups of animals received an intraperitoneal injection of vincristin (0.1 mg/100 g) 180 min before the autopsy. Rats were decapitated at 11:00 a.m.

Cell-proliferation assay

Thymuses were promptly removed, and capsule-adjacent fragments were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Araldite. Then, 0.5 μm-thick sections were cut, and stained with toluidine blue. The mitotic index (‰ of metaphase-arrested cells) was calculated at x400, by counting 5,000 cells in the subcapsular zone (4-5 layers of cells) of each thymus.

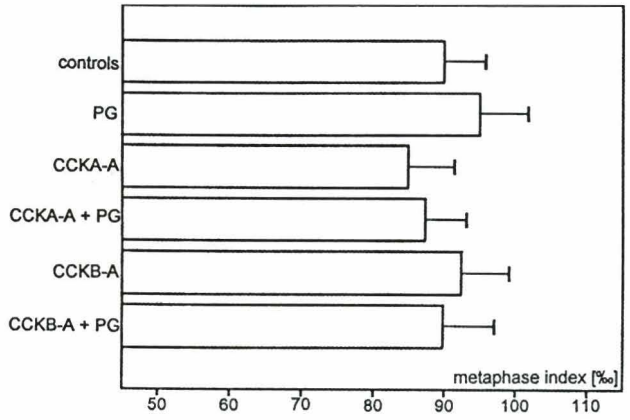

Fig. 1. Lack of effects of endogenous CCK and PG on the proliferative activity of immature rat thymocytes. Bars are means ± SEM (n = 6).

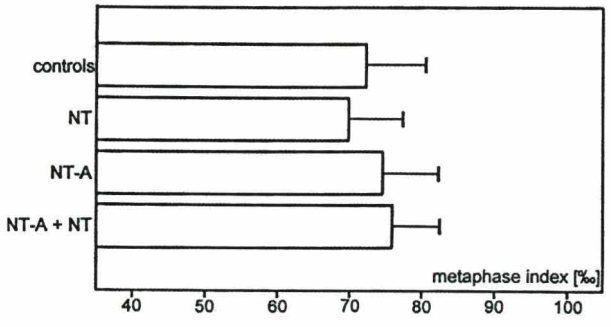

Fig. 2. Lack of effect of endogenous NT on the proliferative activity of immature rat thymocytes. Bars are means ± SEM (n=6).

Statistical analysis

Individual results were averaged per experimental group, and SEM was calculated. The statistical comparison of the data was done by ANOVA, followed by the Multiple Range test of Duncan.

Results

Neither PG nor CCKA-A and CCKB-A, administered alone or together, affected the proliferative activity of rat thymocytes (Fig. 1). Likewise, NT and/or NT-A did not change thymocyte mitotic index (Fig. 2). VIP-A1 and VIP-A2 evoked a marked increase in the mitotic index of thymocytes, and the effect was prevented by the simultaneous injection of VIP. VIP and PACAP(6-38) were ineffective (Fig. 3).

Discussion

The present results indicate that of the three neuropeptides investigated only VIP plays a physiological role in the control of thymocyte proliferation.

CCK is a 33-amino acid residue peptide, that is widely distributed in the central nervous system and peripheral organs and tissues, where it acts through two main subtypes of receptors, named CCKA and CCKB

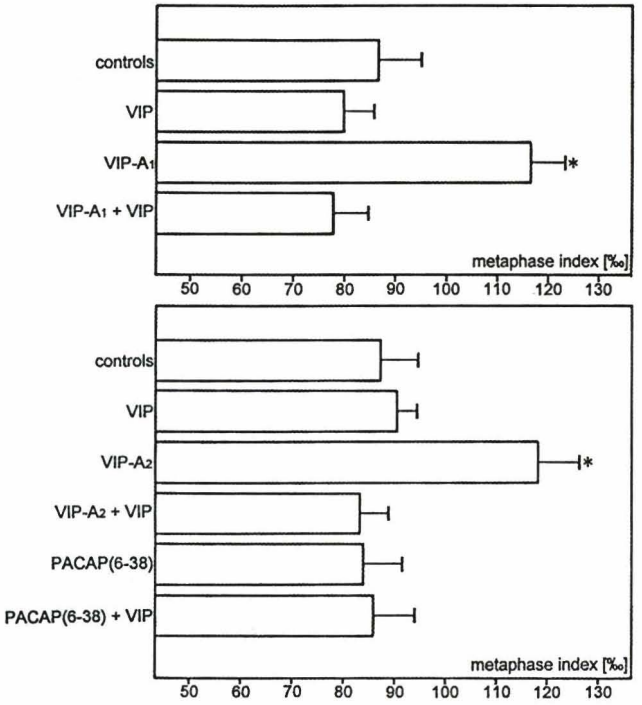

Fig. 3. Effect of endogenous VIP on the proliferative activity of immature rat thymocytes. Bars are means ± SEM (n=6). *: p<0.01 from the respective control group.

receptors belonging to the G protein-coupled receptor superfamily. PG is a selective and potent agonist of CCKB receptors (Wank et al., 1992; Pisegna et al., 1993; Crawley and Corvin, 1994). The following evidence indicates that CCK is involved in immunomodulation. CCK and its receptors are present in the lymphoid tissue, including thymus (Felten et al., 1985; Weinberg et al., 1997), and CCK restores thymus-dependent immune response in thymectomized mice and stimulates IgM-plaque forming cells (Belokrylov et al., 1990; Molchanova et al., 1992). Soder and Hellstrom (1987) reported that neither CCK nor PG affect the proliferative activity of human thymocytes or guinea pig T and B lymphocytes cultured *in vitro*. In contrast, De la Fuente et al. (1998) demonstrated an inhibitory effect of CCK on mitogen-induced proliferation of murine lymphocytes *in vitro*. Our previous *in vivo* studies (Malendowicz et al., 1999) showed a potent CCKA receptor-mediated stimulatory effect of exogenous CCK on the mitotic activity of rat thymocytes. However, our present findings strongly suggest that this effect of CCK has to be considered pharmacological in nature. In fact, neither CCKA-A and CCKB-A nor PG administration significantly affect thymocyte proliferation, thereby ruling out the possibility that endogenous CCK and gastrin play a relevant physiological modulatory role in this process.

NT, a 13-amino acid residue peptide that acts through G protein-coupled receptors (Vincent et al., 1999), is contained in the immune system. NT-immunoreactivity has been detected in the chicken (Atoji et al., 1996) and human thymus (Vanneste et al., 1997), and NT receptor mRNA in the medulla and epithelial cells of the chicken thymus (Atoji et al., 1996). NT was found to stimulate [^{3}H]-thymidine incorporation in human thymocytes and to inhibit it in guinea pig lymph node cells (Soder and Hellstrom, 1987). Our present findings cast doubts on the involvement of endogenous NT in the regulation of thymocyte mitogenic activity in the rat, inasmuch as neither NT nor NT-A affect it. This observation appears to accord well with the reported lack of NT-immunoreactivity in the rat thymus (Muller and Weihe, 1991; Atoji et al., 1996).

VIP is a 28-amino acid residue peptide, that displays a remarkable amino acid-sequence homology with PACAP (Nussdorfer and Malendowicz, 1998). VIP and PACAP act through G protein-coupled receptors, named VIP-PACAP receptors. Three subtypes of VIP-PACAP receptors have been identified so far: the PAC1, VPAC1 and $VPAC_2$ receptors (Harmar et al., 1998). Their binding potency is as follows: PAC1, PACAP>>>VIP; VPAC1, VIP>PACAP; and VPAC2, VIP=PACAP (Nussdorfer and Malendowicz, 1998). VIP is abundant in the immune system (Martinez et al., 1999) and VIP-ergic fibers are present in the subcapsular cortex, interlobular septa and accompanying vasculature of the thymus (Gomariz et al., 1990, 1993; Kendall and Al-Shawaf, 1991; Müller and Weihe, 1991; Bellinger et al., 1997). Lymphocytes and thymocytes are provided with $VPAC_1$ and $VPAC_2$ receptors (Delgado et al., 1996). VIP has been reported to inhibit [^{3}H]-thymidine uptake by non-activated and activated human thymocytes, as well as basal DNA synthesis in rat spleen cells (Soder and Hellstrom, 1987; Yiangou et al., 1990; Boudard and Bastide, 1991; De la Fuente et al., 1996; Ganea, 1996; Pankhaniya et al., 1998).

The present demonstration that VIP-As enhance the mitotic activity of rat thymocytes, while VIP per se is ineffective strongly suggests that endogenous VIP exerts a maximal tonic inhibition of the thymocyte proliferative activity. This tonic inhibition is conceivably mediated by the VPAC1 receptor subtype. In fact, the selective antagonist of VPAC1 receptors VIP-A2 (Gourlet et al., 1997) raises thymocyte proliferation, while the selective antagonist of PAC1 receptors PACAP(8-36), that also interacts with VPAC2 receptors (Dickinson et al., 1997), is ineffective. The physiological and physiopathological relevance of this VIP effect on the rat thymus growth remains to be investigated.

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