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Prolonged kallikrein inhibition does not affect the basal growth and secretory capacity of rat adrenal cortex, but enhances mineralo- and glucocorticoid response to ACTH and handling stress

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Summary. The effects on the pituitary-adrenocortical functions of the prolonged (7-day) blockade of endogenous bradykinin (BK) synthesis, obtained by the administration of the kallikrein inhibitor (K-I) cyclohexylacetyl-Phe-Arg-Ser-Val-Gln amide, were investigated in the rat. K-I treatment did not cause significant changes in the (i) body and adrenal weights; (ii) basal plasma levels of ACTH, aldosterone and corticosterone; and (iii) average volume of adrenocortical cells and their basal secretory capacity. Conversely, K-I administration induced a significant magnification of the *in vivo* mineralo- and glucocorticoid responses to the intraperitoneal (i.p.) *bolus* injection of ACTH. Moreover, K-I-treated rats, but not control ones, displayed a moderate and short-term adrenal secretory response to the mild stress evoked by the placebo i.p. injection. Collectively, these findings rule out the possibility that endogenous BK plays a relevant role in the control of adrenocortical function under basal conditions. However, they suggest that endogenous BK may be involved in quenching exceedingly high adrenocortical responses to ACTH and stresses.

Key words: Kallikrein inhibitor, Bradykinin, Steroid-hormone secretion, Adrenal cortex, ACTH, Rat

Introduction

Bradykinin (BK) is a vasodilatory peptide, which acts through two subtypes of receptors called B_1 and B_2. B_1 receptor is mainly expressed in the aorta, whereas B_2 subtype is widely distributed in the body. BK synthesis from α_2-globulins (kininogens) is catalyzed by kallikreins, which are distinct in plasma and tissue kallikreins (for review, see Bathon and Proud, 1991; Margolius, 1996).

Tissue-kallikrein protein and mRNA have been demonstrated in the rat adrenal gland, thereby indicating the local synthesis of BK (Scicli et al., 1991; Nolly et al., 1993). The possibility that BK may be included in that group of peptides involved in the paracrine/autocrine regulation of adrenocortical secretion (for review, see Nussdorfer, 1996; Mazzocchi et al., 1998) is suggested by the following lines of evidence. BK, acting via B2 receptors, was found to stimulate in *vitro* aldosterone production by bovine and rat zona glomerulosa (ZG) cells (Rosolowsky and Campbell, 1992; Malendowicz et al., 1995b) and corticosterone secretion by dispersed rat zona fasciculata-reticularis (ZF/R) cells (Malendowicz et al., 1995a). Moreover, BK has been reported to hamper the secretory responses of dispersed adrenocortical cells to ACTH (Malendowicz et al., 1995a,b).

In *vivo* investigations, using acute or prolonged administration of BK and B2-receptor antagonists, gave conflicting and rather disappointing results, collectively suggesting that endogenous BK does not play a relevant role in the physiological control of the adrenocortical functions under basal conditions (Rudichenko et al., 1993; Malendowicz et al., 1995c, 1996a). However, the selectivity of the BK-receptor antagonists employed, as well as their effectiveness in producing a long-term blockade of BK actions are questionable. Therefore, it seemed worthwhile to study whether the prolonged blockade of BK synthesis by a selective tissue-kallikrein inhibitor (K-I) is able to modify the secretion and growth of rat adrenal cortex.

Materials and methods

Reagents and animals

The K-I cyclohexylacetyl-Phe-Arg-Ser-Val-Gln

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amide (Burton and Benetos, 1989), ACTH(1-24), human serum albumin (HSA), dimethylsulfoxide (DMSO), and other laboratory reagents were purchased from Sigma (St. Louis, MO, USA). Medium 199 was obtained from Difco (Detroit, MI, USA), and radioimmuno assay (RIA) kits for ACTH, aldosterone and corticosterone were from Cis Bio International (Gif-sur-Yvette, France), IRE-Sorin (Vercelli, Italy) and Eurogenetix (Milan, Italy), respectively. Adult male Sprague-Dawley rats (240-260 g body weight) were purchased from Charles-River (Como, Italy). Animals were kept under a 12:12 h light-dark cycle (illumination onset at 8:00 a.m.) at 23 °C, with free access to standard chow (Charles-River) and tap water. The experiment protocol was approved by the local Ethical Committee for Animal Studies.

Animal treatment

Rats were divided into two groups, which were given daily subcutaneous (s.c.) injections of 0.5 μmol/kg K-I dissolved in 0.5 ml H_2O-DMSO (4:1) or of vehicle (control group) for 7 days. Arterial blood pressure (BP) was measured by tail cuff sphygmomanometry (BP-Recorder, Basile, Commerio, Italy) at 11:00 a.m. On the 8th day, a number of rats in each group was given an intraperitoneal (i.p.) injection of 50 pmol/kg ACTH dissolved in 0.2 ml 0.9% NaCl or of the saline vehicle. All the injections were made at 9:00 a.m., and animals were decapitated at times 0 (just after the i.p. injection), 30, 60, 120 or 240 min after the i.p. injection (6 rats for each time point). The trunk blood was collected in the presence of EDTA, and plasma was separated and stored at -30 °C. Adrenal glands of no intraperitoneally-injected rats (n=12 in each group) were promptly removed and cleaned from adherent adipose tissue.

Morphometry

The left adrenals were weighed, and then fixed in Bouin's solution, embedded in paraffin and serially cut at 6 μm. Sections were stained with hematoxylin-eosin. The volume of ZG, zona fasciculata (ZF) and zona reticularis (ZR) cells was calculated at x3,000 (Malendowicz, 1987) by computer-assisted morphometry, using a software specifically written for this purpose (Studio Casti Imaging, Venice, Italy).

Dispersed adrenocortical cells

The right adrenal glands were decapsulated to separate ZG, and dispersed ZG and ZF/R cells were obtained by sequential collagenase digestion and mechanical disaggregation (Andreis et al., 1989). The viability of isolated cells was checked by the trypan-blue exclusion test and found to be higher than 92%. Dispersed ZG and ZF/R cells obtained from three rats were pooled to obtain a single cell suspension, and 4 different cell suspensions for each incubation experiment were employed. Dispersed cells were placed in Medium 199 and Krebs-Ringer bicarbonate buffer with 0.2% glucose, containing 5 mg/ml HSA. They were incubated (10^5 cell/ml) for 90 min in a shacking bath at 37 °C in an atmosphere of 95% air -5% CO_2. The medium was collected and kept frozen at -30 °C until hormonal assay.

Hormonal assays

ACTH was extracted from plasma (Andreis et al., 1992), and its concentration determined by RIA (ACTH-RIA kit; sensitivity: 10 pg/ml; cross-reactivity: ACTH(1-24), 100%, α-MSH and ß-lipotropin, 0.1%, and other pituitary hormones less than 0.001%). Intra- and interassay variations were 6% and 9%, respectively. Steroid hormones were extracted from the plasma and incubation medium with dichloromethane. The extracts were washed twice with 0.1N NaOH and 1 μl distilled water, and then evaporated to dryness under vacuum and redissolved in 50 μl methanol. Aldosterone and corticosterone were purified by HPLC, as detailed previously (Neri et al., 1993), and their concentrations were measured by RIA (ALDO CTK2 kit; sensitivity: 5 pg/ml; cross-reactivity: aldosterone 100%, 17-iso-aldosterone and other steroids less than 0.1%. BX-RIA kit; sensitivity, 25 pg/ml; cross-reactivity, corticosterone and cortisol 100%, 11-deoxycorticosterone and progesterone 2%, and other steroids less than 0.001%). Intra- and interassay variations were aldosterone: 6% and 8%, and corticosterone: 8% and 9%, respectively.

Statistics

Data were expressed as means ± SEM, and their statistical comparison was done by ANOVA, followed by the Multiple Range Test of Duncan.

Results

Prolonged K-I treatment did not evoke significant changes either in the body and adrenal weights or in the BP (Table 1). The basal plasma concentrations of ACTH, aldosterone and corticosterone were not affected by K-I (Table 1). Neither the average volume of ZG, ZF and ZR cells, nor the basal aldosterone secretion by dispersed ZG cells and corticosterone secretion by dispersed ZF/R cells displayed significant alterations in K-I-administered rats as compared to the control animals (Table 2).

Vehicle i.p. injection did not induce any significant change in the plasma concentrations of aldosterone and corticosterone in control rats, but caused a small but significant rise in the blood level of the two hormones at 30 and 60 min in K-I-treated animals (Fig. 1). As expected, ACTH *bolus* injection resulted in marked increases in aldosterone and corticosterone plasma levels, which reached a maximus at 120 min (4.0- and 2.4-fold, respectively) and declined at 180 and 240 min. K-I administration significantly magnified both aldosterone and corticosterone response to ACTH (by

Table 1. Effect of K-I on some physical and hormonal parameters of rats. Means±SEM (n = 6).

	CONTROLS	K-I
Body weight (g)	280±16	291±15
Adrenal weight (mg)	61±7	63±9
BP	132±10	138±12
ACTH plasma concentration (pM)	17.1±1.1	15.6±1.4
Aldosterone plasma concentration (pM)	190.8±21.2	204.5±19.9
Corticosterone plasma concentration (nM)	321.8±39.6	342.1±40.7

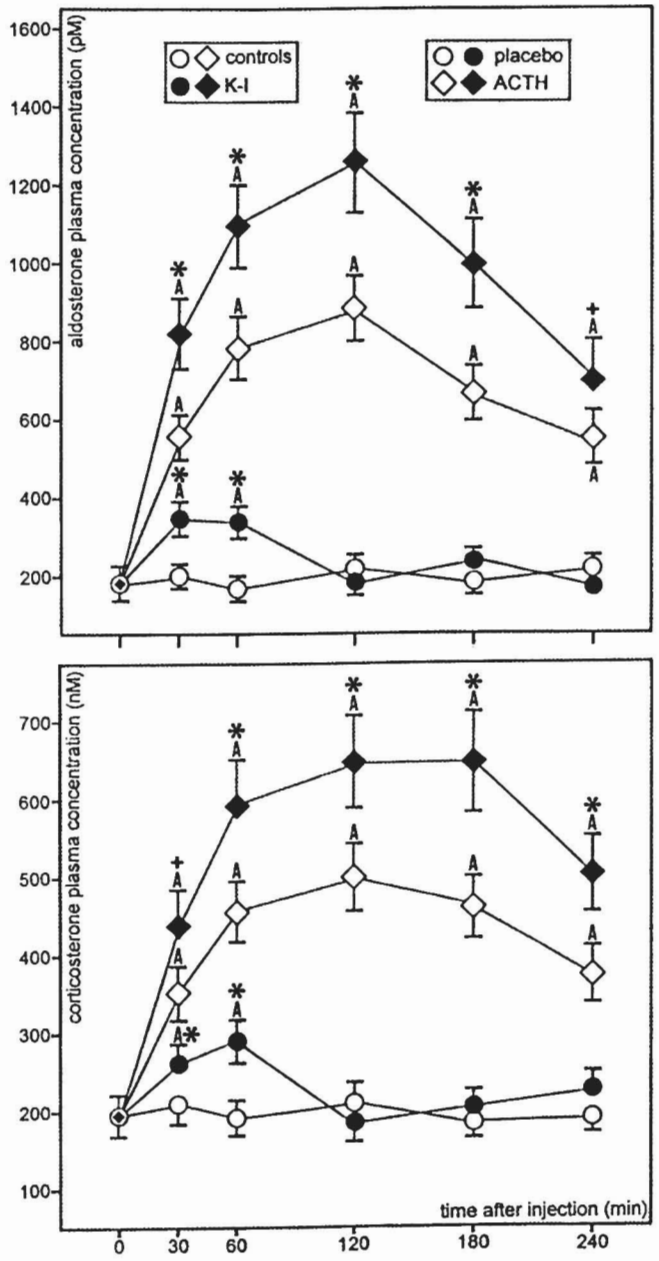

Fig. 1. Effects of ACTH and placebo i.p. *bolus* injections on aldosterone (upper panel) and corticosterone plasma concentration (lower panel) in control and K-I-administered rats. Data are means ± SEM (n=6). A; p<0.01 from 0-time group; +: p<0.05 and *: p<0.01 from the respective control group.

Table 2. Effect of K-I on the volume and secretory capacity of rat adrenocortical cells. means ± SEM (volumes, n=6; secretion, n=4).

	CONTROLS	K-I
Volume of ZG cells (μm^3)	702±98	658±81
Volume of ZF cells (μm^3)	1906±202	2019±215
Volume of ZR cells (μm^3)	923±101	870±97
Aldosterone secretion of ZG cells (pmol/10^6 cells.h)	30.3±2.9	33.8±2.5
Corticosterone secretion of ZF/R cells (pmol/10^6 cells.h)	215.6±20.7	199.8±21.2

about 40-45% and 30-35%, respectively) (Fig. 1).

Discussion

Our findings indicate that the prolonged kallikrein inhibition and the consequent blockade of BK synthesis does not affect the basal secretory activity and growth of rat adrenal cortex. This observation agrees with the contention that endogenous BK is not involved in the physiological control of adrenal function in the rat (Malendowicz et al., 1996a,b). The same also seems to apply to the possible role of endogenous BK in the regulation of the vascular tone, inasmuch as K-I did not evoke any sizeable increase in BP. We have previously reported that BK, through a B_2 receptor-independent mechanism, is able to inhibit adrenocortical secretagogue action of ACTH (Malendowicz et al., 1995a,b). Our present study provides clear-cut evidence for a role of endogenous BK in dampening exceedingly high secretory responses of adrenal cortex to ACTH. In fact, the prolonged inhibition of endogenous BK synthesis markedly enhances the adrenal aldosterone and corticosterone secretion evoked by the *bolus* administration of a maximal effective dose of ACTH. Thus, it may be calculated that the i.p. injection of 10 pmol ACTH is able to produce a blood concentration of the hormone of about 10^{-9} M (Nussdorfer, 1996), which is the maximal effective one *in vitro*.

The i.p. injection of vehicle did not evoke any appreciable response of the pituitary-adrenal axis in control rats, thereby making it likely that the animals were accustomed to handling stress by the preceding 7 s.c. injections of vehicle. However, K-I-treated rats displayed a transient secretory response of their adrenals to the placebo i.p. injection. This finding is in keeping with the possibility that the blockade of endogenous BK synthesis does not allow rats to quench the response of their pituitary-adrenal axis to a mild stress, as that evoked by the i.p. injection. In this connection, it should be recalled that BK administration was found to depress glucocorticoid responses to ether and cold stresses in rats (Malendowicz et al., 1996b).

Collectively, our present observations allow us to conclude that endogenous BK may play a physiological role in the negative modulation of the secretory

responses of adrenal cortex to its main secretagogue ACTH. The mechanism underlying this action of BK remains to be addressed by *in vitro* studies aimed at ascertaining whether it occurs up- or down-stream to the ACTH-induced adenylate-cyclase activation.

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