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OLIGOPEPTIDES INTERFERING WITH CALCIUM CHANNELS INHIBIT PROLACTIN AND GROWTH HORMONE RELEASE BY CULTURED ANTERIOR PITUITARY CELLS OF THE RAT

IVÁN NAGY,* GÁBOR B. MAKARA,†‡ VERA GARAMVÖLGYI,† SÁNDOR BAJUSZ,§ ERZSÉBET SZÉLL,§ and GYÖRGY RAPPAY†

*Heim Pál Pediatric Hospital; †Institute of Experimental Medicine, Hungarian Academy of Sciences; and §Institute for Drug Research, Budapest, Hungary

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Abstract—A number of oligopeptides, protected at their N termini and possessing an aldehyde residue at their C terminal animo acids, are able to inhibit $^{45}Ca^{2+}$ influx into anterior pituitary cells grown in monolayer culture and depolarized with high extracellular potassium concentration. In addition, the same oligopeptides interfere with hormone release, especially with that produced by lactotrophs. Our findings imply that oligopeptides may represent a new class of calcium channel ligands, and the pituitary cells are sensitive targets for them.

The mechanisms of stimulus-secretion coupling in the prolactin (PRL) and growth hormone (GH) producing anterior pituitary cells are essentially influenced by the Ca^{2+} influx through voltage-dependent Ca^{2+} channels [1–3]. Such channels in skeletal muscle are composed of integral membrane proteins in radial arrangement of cylindrically-shaped membrane spanning subunits [4, 5], and it seems reasonable to postulate that similar structures exist in the anterior pituitary cell membrane. The permeability of the channels might be influenced by calcium antagonists as well as agonists which act directly within the channel infrastructure and thereby modulate ion entry [5]. Recently, an endogenous peptide which can modulate voltage dependent calcium channels was isolated from brain tissue [6–8].

In earlier studies we demonstrated that monolayers derived from rat anterior pituitary cell suspensions release large amounts of GH and PRL [9] which can be considerably suppressed with non toxic peptide aldehydes of proteinase inhibitory properties [10]. Searching for the site of action of such compounds it has been established that they may act at a site beyond cAMP formation [11] most probably affecting voltage-sensitive calcium channels [12].

In the present work we furnish evidence that peptide aldehydes of diverse primary structure can inhibit calcium flux (as shown by $^{45}Ca^{2+}$ uptake) and as a consequence GH and PRL release by anterior pituitary cells grown in monolayer cultures.

MATERIALS AND METHODS

Cell cultures. The anterior pituitary glands of decapitated female rats were dissected under aseptic conditions and placed in tissue culture medium 199 (P199, National Institute of Public Hygiene, Budapest, Hungary). The glands were then minced and the cells were dispersed by trypsin as described previously [7]. The dispersed cells were counted, and appropriate numbers were placed in P199 supplemented with 10% newborn calf serum (Human, Budapest, Hungary). Two-milliliter aliquots containing 0.5–0.8 × 10^5 cells/mL were placed in Petri dishes (35 mm o.d.) or 5–8 × 10^4 cells/well in 96-well microplates (Linbro, Hamden, U.S.A.), which were maintained in air with 5% CO_2 at 37° in an ASSAB CO_2 incubator. The cultures were washed every other day.

Experimental protocol for Ca^{2+} *uptake.* As described previously [12], the culture medium of 6-day-old cultures grown in Petri dishes was removed and replaced by 1 mL of prewarmed (37°) HEPES-buffered, isotonic salt solution (HBSS: 18 mmol/L HEPES, 1.2 mmol/L $MgSO_4$, 5.9 mmol/L KCl, 130.6 mmol/L NaCl, 0.2 mmol/L $CaCl_2$, 11.8 mmol/L glucose) with the pH adjusted to 7.35 by the addition of TRIS base. Fifteen minutes later the medium was quickly replaced by 1 mL of $^{45}Ca^{2+}$-containing HBSS or HBSS-K (with K^+ raised to 59 mmol/L, replacing equimolar Na^+) with or without calcium channel modulators. The uptake of $^{45}Ca^{2+}$ (sp. act 10^7 GBq/g, Izinta, Budapest) was stopped by quickly aspirating the solution and washing the cultures with 1 mL of ice-cold HBSS containing 0.5 mmol/L $LaCl_3$. The wash was repeated 10 sec later. The $^{45}Ca^{2+}$ non-specifically bound to the surface of the cells and the Petri dish probably was removed by this procedure. The cells were lysed with 0.5 mL of distilled water after the second wash, kept on ice for 30 min and homogenized with a KONTES ultrasonic cell disrupter. Twenty microlitres of the homogenate was used for protein determination [13], the rest was transferred into scintillation vials. The culture dish was rinsed with 0.5 mL of distilled water which was also added to the

‡ Correspondence to: Dr G. B. Makara, Institute of Experimental Medicine, H-1083 Budapest, Szigony u. 43., Hungary.

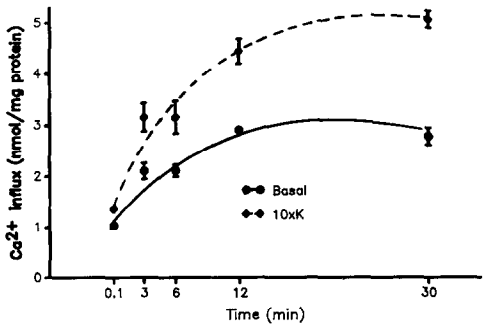

Fig. 1. Time course of $^{45}Ca^{2+}$ uptake by 6-day-old anterior pituitary cells incubated in HEPES-buffered isotonic salt solution (HBSS) or in HBSS-K (with K^+ raised to 59 mmol/L) for up to 30 min. Each value is the mean ± SE of three independent experiments.

corresponding vial. The radioactivity of the $^{45}Ca^{2+}$ was measured by liquid scintillation after the addition of 1 mL 96% ethanol and 10 mL of dioxan-based scintillation cocktail.

Experimental protocol for hormone release investigations. Seven-day-old cells grown in 96-well microplates were first washed twice with P199 supplemented with 1% bovine serum albumin, then incubated for 2 hr with or without a peptide at various concentrations. The media were collected, centrifuged and the supernatants were frozen until radioimmunoassay for GH and PRL.

Test substances. The oligopeptide derivatives were prepared by conventional stepwise synthesis starting from the appropriate derivative of the C-terminal residue [14]. Peptide aldehydes were obtained by $LiAlH_4$ reduction of the corresponding 2,5-dimethylpyrazolides [15]. Peptide alcohols were made by $NaBH_4$ reduction of the aldehydes. Their purity was checked by TLC and HPLC and was better than 95%. Z-Gly-Gly-Chloromethyl ketone, *N*-*p*-Tosyl-Phe-Chloromethyl ketone, *N*-α-Tosyl-L-Lys-Chloromethyl ketone were purchased from Bachem (Bubendorf, Switzerland), Nifedipine from Sigma (Munich, F.R.G.), and Bay K8644 from Bayer (Leverkusen, F.R.G.).

Statistical analysis. The data were subjected to one-way analysis of variance and the means ± SE are presented.

RESULTS

Calcium flux studies

To determine the time course of $^{45}Ca^{2+}$ uptake by 6-day-old anterior pituitary cells parallel cultures were incubated either in HBSS or HBSS-K, both containing the tracer for up to 30 min. Under basal condition the $^{45}Ca^{2+}$ influx reached its maximum at 12 min of incubation (Fig. 1), whereas after potassium stimulation the uptake was faster with a tendency to be saturated at 30 min (Fig. 1). In the subsequent experiments incubation with the tracer was 3 min.

Normal pituitary cells possess voltage-sensitive calcium channels which is known to be inhibited by dihydropyridine calcium antagonists [16]. Indeed, potassium-stimulated $^{45}Ca^{2+}$ uptake by 6-day-old pituitary cells in culture was inhibited by nifedipine in a dose-dependent manner (37% inhibition with 10^{-7} mol/L and 65% inhibition with 10^{-6} mol/L).

In a preliminary report we have shown [12] that a tripeptide aldehyde, BOC-D-Phe-Phe-Lysinal (BOC-D-Phe-Phe-Lys-H) inhibited both basal and high potassium-induced $^{45}Ca^{2+}$ influx into cultured pituitary cells. Since then a large number of oligopeptides (see Fig. 2) have been tested. Basal calcium uptake was inhibited by 20–35% with four peptides which were markedly inhibitory on potassium stimulated calcium uptake. In two typical experiments BOC-D-Phe-Phe-Phenylalaninal and Z-D-Phe-His Leu-Valinal (Fig. 3) interfered with potassium-stimulated $^{45}Ca^{2+}$ uptake: the influx stimulated by depolarization decreased gradually when the concentration of the drugs was increased. The IC_{50} concentrations were about 3×10^{-5} mol/L and 7.4×10^{-6} mol/L, respectively.

The oligopeptides were serially tested in dose–response experiments using 6-day-old potassium-stimulated cultures. In 10^{-4} mol/L concentration many peptides markedly inhibited $^{45}Ca^{2+}$ uptake of stimulated cells (Fig. 2). Their inhibitory effect was comparable with that of the nifedipine in 10^{-6} mol/L concentration. Some of them had a rather weak (D-Phe-Pro-Agmatine, TLCK) or no effect (BOC-D-Phe-Pro-Arginal) on $^{45}Ca^{2+}$ influx. The most potent compounds (BOC-D-Phe-*X*-Phe-H, where *X* may be Phe, Pro or Leu) decreased the $^{45}Ca^{2+}$ uptake between 20 and 40% also in 10^{-5} mol/L concentration (Fig. 4).

Reversibility of the inhibition was studied in the case of Boc-D-Phe-Phe-Lysinal. When this compound was washed out after a 6 min exposure, the inhibitory effect gradually decreased with time and was undetectable by 2 hr after exposure (data not shown).

The potent calcium channel agonist, Bay K8644 was used in an attempt to modify the inhibitory effect of some powerful oligopeptide on the $^{45}Ca^{2+}$ influx. It has been established that Bay K8644 increased $^{45}Ca^{2+}$ uptake by pituitary cells in a dose-dependent fashion, and its stimulatory action was additive to the inhibitory action of Z-Ala-Leu-Phenylalaninal (data not shown).

GH and PRL release by pituitary cells in culture.

In previous papers we have shown that some tripeptide aldehydes interfered with both basal and stimulated release of hormones by anterior pituitary cells [9, 17–19]. In the present work we studied the effect on hormone secretion of some oligopeptides which proved to be potent calcium channel antagonists. One typical response by 7-day-old pituitary cells in culture to an oligopeptide, Z-Phe-His-Leu-Valinal, has been demonstrated in Fig. 5. Both GH and PRL release were inhibited by the peptide during a 2-hr incubation period in a dose-dependent manner.

The oligopeptides which showed some calcium antagonistic property have been systematically tested for their hormone release inhibiting activity. As demonstrated in Fig. 6, most of the substances were

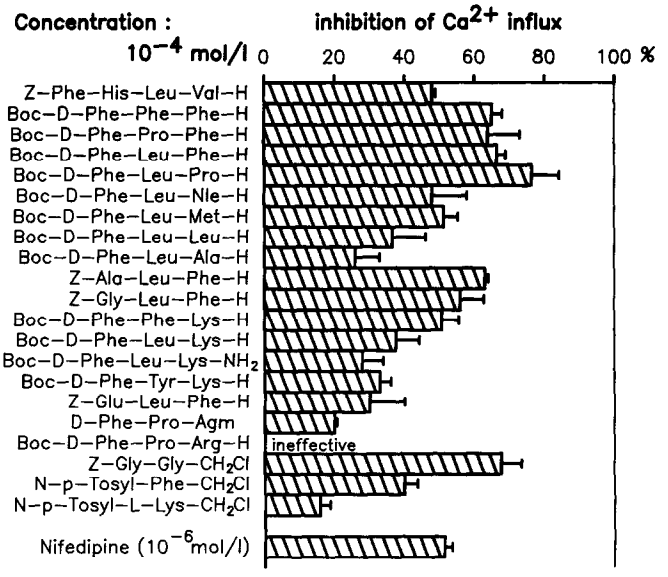

Fig. 2. Comprehensive data on the inhibition of $^{45}Ca^{2+}$ influx into K^+-stimulated 6-day-old pituitary cells in culture when they were incubated in the presence of oligopeptides and other drugs in 10^{-4} mol/L concentration. Each bar represents the mean of at least two independent experiments.

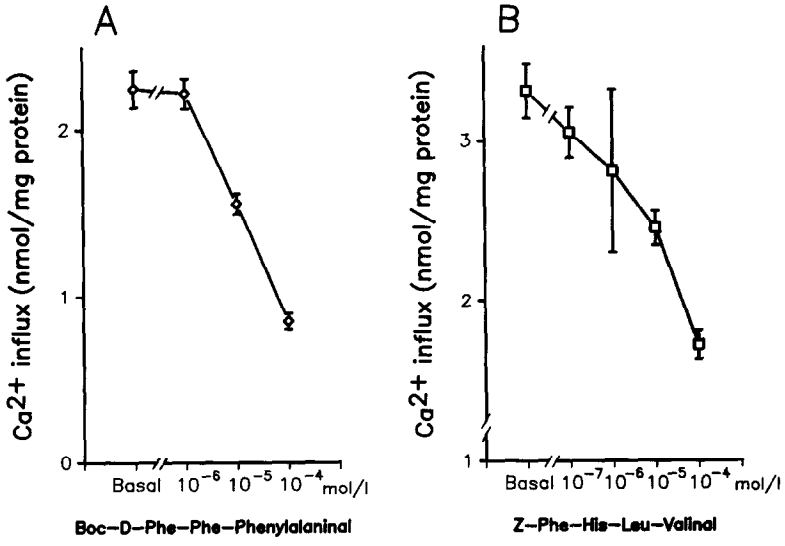

Fig. 3. (A) Dose-dependent inhibitory effect of BOC-D-Phe-Phe-Phenylalaninal on $^{45}Ca^{2+}$ uptake by potassium-induced 6-day-old pituitary cells in culture. The 50% inhibitory concentration (IC_{50}) is 3×10^{-5} mol/L. (B) Z-Phe-His-Leu-Valinal inhibits $^{45}Ca^{2+}$ uptake by pituitary cells with an IC_{50} of 7.4×10^{-6} mol/L. Each value represents the mean ± SE (N = 3).

inhibitory to PRL release by cultured pituitary cells at 10^{-4} mol/L for 2 hr. The effect of the same inhibitory peptides on GH release was generally less pronounced (data not shown).

When the C terminal function of the tripeptide D-Phe-Phe-Lysine remained unchanged, i.e. acidic (Lys-OH), or was modified to an alcohol (Lys-ol), amide (Lys-NH_2) or aldehyde (Lys-H) derivative, there was a sharp contrast in their effects on both $^{45}Ca^{2+}$ uptake and PRL release. As depicted in Fig. 7, the free acid had practically no effects, whereas the alcohol and the amide exerted but a slight inhibition of both events. Solely the aldehyde derivative caused a remarkable inhibition of $^{45}Ca^{2+}$ influx into and PRL release by pituitary cells. There is a good correlation between the inhibition of $^{45}Ca^{2+}$ uptake and the inhibition of PRL release. This is illustrated in Fig. 8, where the data from Figs 2 and 6 are plotted against each other.

DISCUSSION

In the present studies we have demonstrated that

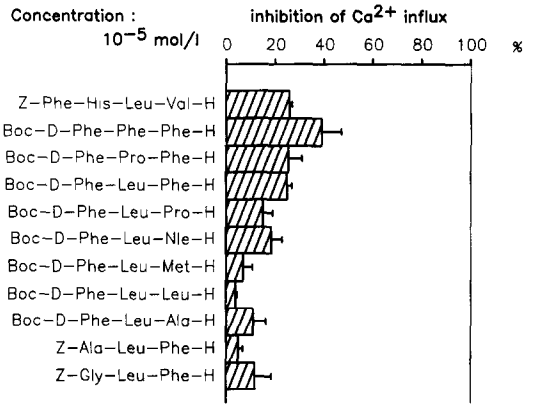

Fig. 4. $^{45}Ca^{2+}$ influx into stimulated 6-day-old pituitary cells in culture in the presence of some tripeptide aldehydes at 10^{-5} mol/L. Each bar represents the means of at least two independent experiments.

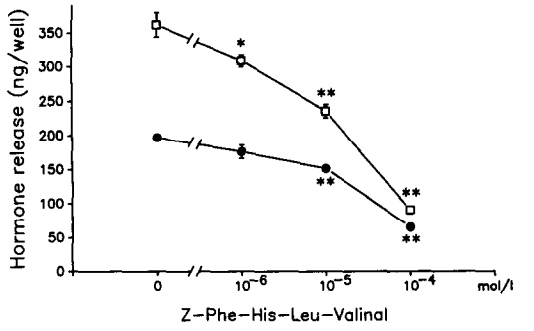

Fig. 5. Effect of a tetrapeptide aldehyde (Z-Phe-His-Leu-Valinal) on the growth hormone (GH, □) and the prolactin (PRL, ●) release by 7-day-old pituitary cells in culture. The cells were incubated for 2 hr. Each value is the mean ± SE (N = 6).

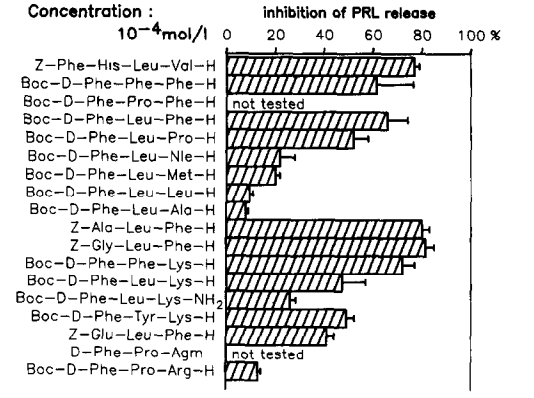

Fig. 6. Comprehensive demonstration of the prolactin (PRL) release inhibition of 7-day-old pituitary cells in culture upon the incubation for 2 hr with oligopeptides in concentrations of 10^{-4} mol/L. Each value represents the means of at least two independent experiments.

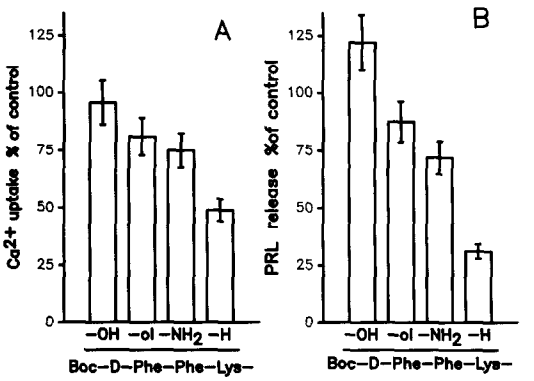

Fig. 7. Effects of various derivatives of BOC-D-Phe-Phe-Lysine on pituitary cells in culture. Each bar represents the means of at least two independent experiments.

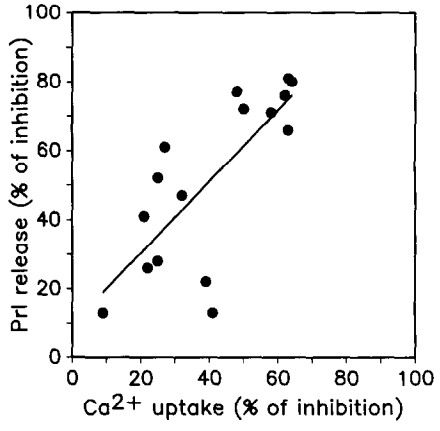

Fig. 8. Correlation of inhibition of calcium uptake (horizontal axis) and inhibition of PRL secretion (vertical axis) calculated from data on Figs 2 and 6. Each point corresponds to results with one compound. Correlation coefficient $r = 0.7596$.

oligopeptides, protected at their N termini and possessing an aldehyde residue at their C terminal amino acid, are able to inhibit $^{45}Ca^{2+}$ influx into cultured anterior pituitary cells, depolarized with high extracellular potassium concentration. In addition, the same oligopeptides interfere with hormone release, especially by lactotrophs. Our findings imply that these and similar oligopeptides may represent a new class of calcium channel ligands, and the pituitary cells are sensitive targets for them.

Previous observations like the calcium uptake during *in vitro* release of hormones from the rat adenohypophysis [20], the existence of ion channels in its cells [21], the calcium antagonist dihydropyridine interference with the hormone secretion [16], and the existence of voltage-activated currents through calcium channels in lactotrophs [22, 23] establish our experiments with the inhibitory peptides and support our conclusions.

Several types of synthetic calcium channel antagonists are known so far: benzothiazepines (cf. diltiazem), phenylalkylamines (cf. verapamil), 1,4-

dihydropyridines (cf. nifedipine) as reviewed by Triggle and Janis [24], and a novel calcium antagonist family of benzothiazoline type has been described by Yamamoto *et al.* [25] recently. We used nifedipine to compare its effect with that of the peptides listed in Fig. 2. On the basis of these comparative studies we may conclude that some peptides are eligible candidates to be calcium channel ligands of synthetic nature. The member of the 1,4-dihydropyridine family, Bay K8644, stimulates rather than inhibits calcium influx into pituitary cells [26]. The same holds true in the case of BOC-Gln-Leu-Lysinal which has an ionophoric property [27] unlike the other oligopeptides. Cellular voltage-operated calcium channels may be influenced by a number of endogeneous substances like neurotransmitters and neuropeptides including somatostatin and opiates [28, 29] or the recently described endogenous peptide modulator [6–8]. All this indicates that peptides may also interact with calcium channels in the plasma membrane.

The fact that the peptide aldehydes are inhibitors of certain serine/cysteine proteinases [14] may suggest that their mode of action includes interference with some proteolytic process(es) interconnected with calcium channel function. To our knowledge there are but scanty hints for such a suggestion [28, 30]. The channel function could be modulated by a peptide aldehyde inhibitor of trypsin and plasmin, i.e. Leupeptin [30].

According to recent suggestions the calcium channel is composed of four homologous protein domains with a unit size of approximately 30 kDa forming a special infrastructure [4]. It has been suggested that the 1,4-dihydropyridine binding site resides within the channel proper modulating calcium influx [5]. It is also conceivable that oligopeptides, especially those consisting of apolar amino acids with C terminal aldehyde residues, may interact at or near the channel with the cell membrane, without affecting any proteolytic reaction, like those oligopeptides that specifically inhibit membrane fusion by paramyxoviruses [31]. To test this possibility photoaffinity labelling of peptide aldehydes would be necessary. If an intracellular peptide, similar to that isolated from brain tissue (6–8) would also modulate voltage dependent calcium channels in the pituitary gland, then the tripeptide aldehydes might act by interfering with such a peptide modulator.

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