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Release of secretoneurin and noradrenaline from hypothalamic slices and its differential inhibition by calcium channel blockers

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Abstract. Secretoneurin is a newly discovered peptide found in high concentrations in brain. We have studied the release of secretoneurin and noradrenaline from superfused hypothalamic slices from rat brain. Both electrical stimulation and potassium induced depolarisation released secretoneurin and noradrenaline from these slices in a calcium-dependent manner. Electrical stimulation caused a preferential release of noradrenaline when compared to the secretion elicited by high potassium. The time course of secretoneurin release was more protracted than that of noradrenaline. The calcium channel blocker ω-conotoxin inhibited only the electrically induced release of noradrenaline, whereas nifedipine inhibited only that of secretoneurin. These results establish that secretoneurin is secreted from neurons. Inhibition of this release by nifedipine is consistent with the concept that secretion from large dense core vesicles occurs at sites different from that of small vesicles and depends on calcium influx via L-type calcium channels.

Key words: Secretoneurin – Noradrenaline – Large dense core vesicles – Calcium channel blockers – Secretion

Introduction

Secretoneurin is a newly characterized peptide of 33-amino acid length (Kirchmair et al. 1993; Marksteiner et al. 1993). It is formed in vivo by proteolytic processing of secretogranin II which belongs to the group of proteins called chromogranins (Blaschko et al. 1967). Secretogranin II (Rosa and Zanini 1981; Rosa et al. 1985; Fischer-Colbrie et al. 1986) like chromogranin A and B (Winkler and Fischer-Colbrie 1992) has a widespread distribution in the neuroendocrine system. Secretoneurin is found in brain especially in the hypothalamus in high concentrations comparable to those of classical neuropeptides (Kirchmair et al. 1993) and is co-stored with them in large dense core vesicles (Mahata et al. 1993). However in order to qualify as a typical neuropeptide, its release from brain tissue has to be demonstrated.

In the present paper we establish that secretoneurin is released from hypothalamic slices by electrical stimulation and potassium induced depolarisation and compare its release with that of noradrenaline. The release of both transmitters is differentially influenced by two types of calcium channel blockers which is consistent with a secretion of these two substances from large and small dense core vesicles, respectively.

Materials and methods

Perfusion of brain slices. Female Sprague-Dawley rats weighing 190–210 g at random stages of the estrous cycle were used. In a few experiments also male rats were ultilized. Rats were killed by decapitation, their brains rapidly removed and the hypothalamic region dissected out. Hypothalamic slices (400 µm) were cut on a vibratome. Six slices were placed in a polyacrylamide chamber connected to a Grass S48 stimulator and superfused at a rate of 0.5 ml/min at 37 °C with gassed (95% O_2/5% CO_2) Krebs-bicarbonate buffer containing 118.0 mM NaCl, 4.60 mM KCl, 1.17 mM $MgSO_4 \cdot 7H_2O$, 2.50 mM $CaCl_2 \cdot 2H_2O$, 1.17 mM $NaH_2PO_4 \cdot H_2O$, 25.0 mM HEPES pH 7.4, 25.0 mM $NaHCO_3$, 10.0 mM glucose and 0.015 mM bovine serum albumin. After an initial wash period of one hour, 13 fractions of 1 ml each were collected every 2 min. During collection of fraction 2 an electrical field stimulation (150 V, 15 Hz, 2 ms) was performed for 30 sec and during collection of fraction 7 the standard Krebs-bicarbonate buffer was substituted with a buffer containing 60 mM K^+ (see Fig. 1). In the buffer containing high K^+ the Na^+ concentration was decreased in equimolar amounts to maintain osmolarity. In certain experiments drugs (0.5 µM ω-conotoxin or 10 µM nifedipine) were added to the Krebs-bicarbonate solution 15 min prior to collection and remained throughout the experimental procedure.

Aliquots of the fractions were analysed for secretoneurin and noradrenaline. For secretoneurin determination the samples were immediately boiled for 5 min and centrifuged for 20 min at 14,000 g. The supernatants were used for the RIA (see below). Stimulation evoked overflow was obtained by subtracting basal outflow from total overflow. As basal outflow the fraction prior to stimulation was taken. Statistical signifi-

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cance was calculated with the unpaired Student's *t*-test by comparing the fractions containing stimulation-evoked release with that of the basal outflow.

HPLC. To characterize the released secretoneurin-immunoreactive material the boiled samples were separated by reversed phase high performance liquid chromatography (μ-Bondapak C_{18} column, Waters) as already described (Kirchmair et al. 1993). Eluted fractions were analysed by RIA (see below).

Noradrenaline assay. Noradrenaline was measured by HPLC with electrochemical detection using a reversed phase column (C_{18} μ-Bondapak, Waters). The mobile phase consisted of 50 mM trichloroacetic acid, 0.27 mM EDTA, 1.4 mM NaCl, 1.8 mM 1-heptanesulfonic acid adjusted to pH 4 by sodium acetate. Noradrenaline eluted about 10 min after injection.

Radioimmunoassay. A rabbit antiserum, raised against rat secretoneurin (rat secretogranin II 154-186: Gerdes et al. 1988) synthesized by standard t-BOC chemistry was used for the RIA (for details see: Kirchmair et al. 1993). The RIA did not recognize other peptides co-stored in large dense core vesicles, i.e. peptides derived from chromogranin A, calcitonin gene-related peptide, substance P, neurotensin, angiotensin II and neuropeptide Y or two peptides corresponding to the N- and C-termini of secretogranin II. The sensitivity of the radioimmunoassay was 2 fmol per assay tube. In rat brain tissue the free peptide secretoneurin represents the predominant form of the immunoreactive material (Marksteiner et al. 1993).

Drugs. Nifedipine and ω-conotoxin were purchased from Sigma (Deisenhofen, Germany). Nifedipine was dissolved in dimethylsulfoxide (0.3% final concentration) and diluted with Krebs-bicarbonate buffer.

Results

Release of secretoneurin and noradrenaline

Both electrical stimulation and high potassium (60 mM) elicited the secretion of secretoneurin immunoreactivity and of noradrenaline from hypothalamic slices (Fig. 1). The immunoreactive material was characterized by HPLC chromatography followed by RIA. The main immunoreactive component eluted exactly at the position of synthetic secretoneurin peptide (Fig. 2).

In the absence of calcium the secretion of both secretoneurin and noradrenaline was totally blocked irrespective of the type of stimulation (Fig. 1).

The time course of the secretion of secretoneurin differed from that of noradrenaline. Depending on the stimulus noradrenaline release was concentrated in one to three fractions whereas secretoneurin secretion was more protracted. Furthermore, the relative amounts of the two compounds released by the two types of stimulation differed. As shown by the ratios of the amounts released (potassium induced depolarisation versus electrical stimulation) high potassium elicited a relatively more pronounced secretion of secretoneurin (Fig. 1).

Effect of calcium channel blockers on electrically stimulated release

As shown in Table 1 the two calcium channel blockers, i.e. nifedipine and ω-conotoxin, influenced the electrically stimulated release of secretoneurin and of noradrenaline differentially. Nifedipine inhibited secretoneurin release by 71.8% whereas noradrenaline secretion was not affected. On the other hand, noradrenaline secretion was specifically blocked by ω-conotoxin (by 70.0%).

Discussion

Secretoneurin is a peptide derived by endogenous proteolytic processing from secretogranin II, a member of the

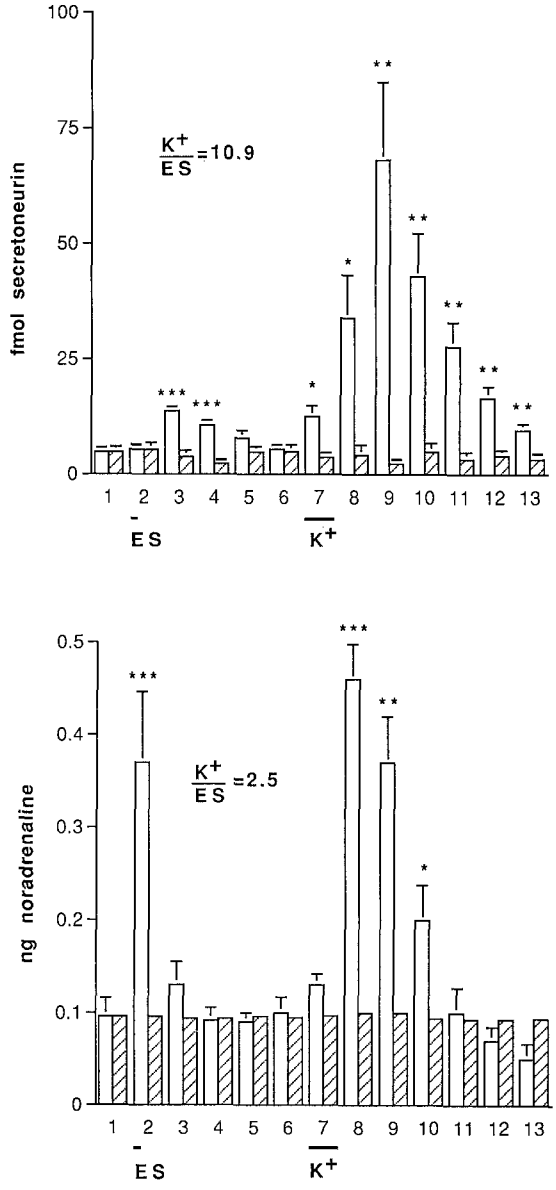

Fig. 1. Release of secretoneurin and noradrenaline from hypothalamic slices. Rat hypothalamic slices were perfused at a flow rate of 0.5 ml/min. Aliquots of the outflow (fraction in *numbers* indicated below the abscissae) were analysed for secretoneurin (*top*) and noradrenaline (*bottom*). Electrical field stimulation for 30 s (ES: 150 V, 15 Hz, 2 ms-rectangle pulses) and stimulation with 60 mM potassium (2 min) were performed as indicated by the *bars*. In the histograms the *open columns* represent perfusion experiments with standard Krebs-bicarbonate buffer and the *hatched ones* those with Ca^{2+}-free medium. Values represent means ± SEM of 6 experiments, in the case of the calcium-free experiments for noradrenaline $n = 2$. For statistical analysis of differences between basal outflow and stimulation-evoked release the Student's *t*-test was used (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The ratios of potassium evoked depolarisation versus electrical stimulation (K^+/ES) were calculated by dividing the total amounts of the two components released above background by the respective stimulation

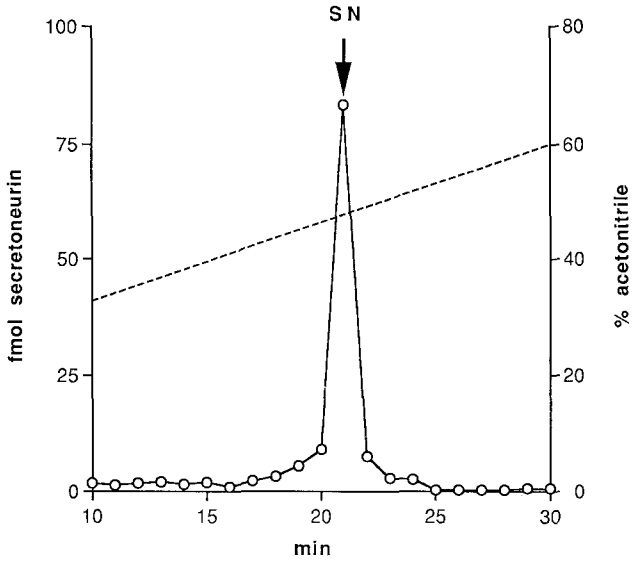

Fig. 2. Characterization of secretoneurin-immunoreactivity in perfusates. Perfusates from slices stimulated with 60 mM potassium were characterized by HPLC chromatography followed by RIA. The immunoreactive material eluted exactly in the same position (indicated by an *arrow*) as the synthetic secretoneurin (SN) peptide

Table 1. Effect of calcium channel blockers on electrically stimulated release

	Secretoneurin (fmol/chamber)	Noradrenaline (ng/chamber)
Control	19.3 ± 3.9	0.30 ± 0.06
ω-Conotoxin	24.5 ± 11.3	0.09 ± 0.03*
Control	23.9 ± 5.9	0.24 ± 0.05
Nifedipine	5.5 ± 2.6*	0.36 ± 0.06

Rat hypothalamic slices were superfused with standard Krebs-bicarbonate buffer (control), or with the same solution containing 0.5 μM ω-conotoxin or 10 μM nifedipine. Nifedipine experiments and controls contained 0.3% dimethylsulfoxide as solvent. Neurotransmitter release was evoked by electrical field stimulation for 30 s (150 V, 15 Hz, 2 ms rectangle pulse). Values give the stimulation-evoked overflow and represent means of 7 experiments ± SEM (*$P < 0.05$)

chromogranin peptides. By immunelectron microscopy these peptides have been localized in the large dense core vesicles of brain (chromogranin A: Somogyi et al. 1984; secretogranin II: Kagotani et al. 1991; Mahata et al. 1992; secretoneurin: Mahata et al. 1993) whereas small synaptic vesicles or adrenergic small dense core vesicles do not contain these peptides (Somogyi et al. 1984; Kagotani et al. 1991; Neuman et al. 1984) or classical neuropeptides (Hökfelt 1991; Klein and Thureson-Klein 1990). As the content of large dense core vesicles is secreted by exocytosis one can predict that secretoneurin is released during stimulation of neurons. The present study confirms this concept. Thus, the novel peptide secretoneurin fulfills essential criteria to qualify as a true neuropeptide: it is stored in large dense core vesicles of brain, it is secreted from this tissue and, as recently shown, has defined functions (stimulation of release of dopamine from striatal slices: Saria et al. 1993).

Stimulation of the hypothalamic slices electrically or with high potassium elicited the release of both noradrenaline and secretoneurin. Previously concomitant secretion has been reported for various other classical transmitters and neuropeptides (Dooley et al. 1988; Herdon and Nahorski 1989; Lundy et al. 1991; Verhage et al. 1991 b; Martin and Magistretti 1989; Peng and Zucker 1993; Perney et al. 1986). In the present study two significant differences between the release of noradrenaline and secretoneurin were seen. First, the time course of secretoneurin release was more protracted with a relatively broad peak in several fractions. This can be readily explained by a slower diffusion of molecules of the larger peptide through the tissue slices. Secondly, electrical stimulation caused a preferential release of noradrenaline over that of secretoneurin when compared to the release elicited by potassium. This result has to be discussed in relation to the exact subcellular localization of these two components. In principle, this difference might also result from their secretion from different neurons, since we have not established that noradrenaline and secretoneurin are co-stored within the same neurons. However, it is more likely that the different subneuronal localization of these two components is the cause of a differential release. Noradrenaline is found both in small and large dense core vesicles. However, due to the fact that in rat brain the small ones predominate in the synapse the bulk of the noradrenaline release is likely to be derived from these vesicles (Klein and Thureson-Klein 1990). Since the peptide secretoneurin is confined to large dense core vesicles (see above), a preferential release of noradrenaline during electrical stimulation would indicate that this kind of stimulation preferentially affects small dense core vesicles. Synaptic vesicles release at the active zone of synapses, whereas large dense core vesicles have no such preferential release site (Zhu et al. 1986; Klein and Thureson-Klein 1990; Verhage et al. 1991 a). Release of small vesicles requires a sharp rise of the presynaptic calcium level close to these active zones (Llinas et al. 1992), release from large dense core vesicles on the other hand depends on a significant rise in the bulk of presynaptic calcium throughout the terminal (Verhage et al. 1991 b). Thus, in isolated synaptosomes significant release of a peptide depends on a net calcium influx as caused by an ionophore (Verhage et al. 1991 b), which in our system was apparently induced by the long exposure (2 min) to a high concentration of potassium (60 mM). The shorter electrical pulse apparently led to a sufficient calcium increase at the active site but to a less sufficient one throughout the terminal thus causing relatively less release of large dense core vesicles. It has been shown previously that peptide release relative to neurotransmitter release rises with the frequency of stimulation (Peng and Horn 1991; Bean and Roth 1991; for reviews see: Bartfai et al. 1988). These findings are also consistent with the above hypothesis since repetitive action potentials are likely to increase bulk calcium level causing the release of large dense core vesicles.

Further support for the concept of differences in the release mechanisms can be seen in the effects of calcium channel blockers. Several previous studies have estab-

lished that secretion of noradrenaline and other classical neurotransmitters cannot be blocked by calcium channel blockers of the dihydropyridine type, whereas ω-conotoxin is effective in this respect (Reynolds et al. 1986; Dooley et al. 1988; Maggi et al. 1988; Clasbrummel et al. 1989; Edmonds et al. 1990; Herdon and Nahorski 1989; Hirning et al. 1988). Our results confirm these data on the apparent involvement of N-type calcium channels in noradrenaline release at the active presynaptic site (Lundy et al. 1991; Verhage et al. 1991 b). In addition P-type calcium channels which, as recently shown can be blocked by a toxin from the funnel-web spider venom, might be involved (Uchitel et al. 1992; Llinas et al. 1989). For release of neuropeptides data on the influence of calcium channel blockers are limited. In isolated neurosecretosomes nifedipine blocked the release of vasopressin (Cazalis et al. 1987) and in cultured sympathetic neurons that of substance P (Perney et al. 1986). Our results now establish for the first time that L-type channel blockers can inhibit the release of a neuropeptide, i.e. secretoneurin, from intact brain slices. These results are in accordance with the concept (Miller 1987; Verhage et al. 1991 b) that L-type channels outside the active presynaptic zone mediate a rise in bulk calcium eliciting exocytosis from large dense core vesicles.

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