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EVALUATION OF PARAMETERS FOR CENTRAL NEURONAL ACTIVITY IN CEREBROSPINAL FLUID OF RABBITS FOLLOWING YOHIMBINE

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Abstract—Rabbits were treated intravenously with yohimbine at a dose of 5 mg/kg. The concomitant increase in noradrenergic activity was followed in function of time by measuring dopamine-β-hydroxylase activity and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) and 3-methoxy-4-hydroxymandelic acid (VMA) levels in cerebrospinal fluid. In addition, the effect of yohimbine on the dopaminergic, serotonergic and enkephalinergic neurotransmission was also determined. For this purpose, the dopamine metabolite, 3-methoxy-4-hydroxyphenylacetic acid (HVA), the serotonin metabolite, 5-hydroxyindole acetic acid (5-HIAA) and methionine-enkephalin (Met-Enk) were quantified. The DβH activity in control experiments, in which physiological saline was administered, increased up to 200% whereas in the yohimbine experiments a rise to 500–600% was observed. VMA and MHPG levels increased to 290%, and 209% respectively. HVA levels reached a value of 233% versus the concentration before drug injection, whereas 5-HIAA concentrations initially slightly increased and thereafter decreased. In the corresponding control experiments metabolite concentrations were virtually stable.

Following yohimbine injection, methionine-enkephalin concentrations did not show significant variations compared with the control experiments. We conclude that noradrenergic and dopaminergic neurotransmission are increased following administration of the α_2-antagonist yohimbine whereas serotonergic neurotransmission is slightly decreased and enkephalinergic neurotransmission is unaltered. The value of the different parameters for measuring neuronal activity in cerebrospinal fluid is discussed.

The alkaloid yohimbine, which effectively blocks the α_2-adrenoceptors, enhances the stimulation-evoked release of noradrenaline at sympathetic nerve terminals and within the central nervous system [1–3]. Administration of yohimbine results in a rise in brain and CSF concentrations of 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (MHPG-SO_4) [4, 5]. The effects of yohimbine are, however, not only restricted to the noradrenaline system. Yohimbine also produces a significant increase in dopamine turnover by blocking dopamine receptors [6, 7]. The interactions of yohimbine on the central serotonergic neurotransmission are not equivocal though some investigators found a decreased serotonin turnover [4, 8].

Measurement of parameters in the cerebrospinal fluid (CSF) may give an indication about the central neuronal activity. The activity of the enzyme, dopamine-β-hydroxylase (DβH; EC 1.14.17.1) in CSF has been demonstrated to correlate with the DβH activity in the brain; and in the brain, DβH levels have been found to correlate significantly with the noradrenaline levels [9]. Drugs, which alter the central noradrenergic activity, induce changes in DβH activity. Clonidine and phenoxybenzamine for example change the DβH activity in the predicted directions based on their known α-agonist and antagonist properties [10]. Phenoxybenzamine and yohimbine treatment increases DβH activity in CSF of rabbits [9]. Monoamine oxidase inhibitors have been demonstrated to cause a significant decrease in DβH activities in CSF of depressed patients [11]. In addition to DβH, monoamine metabolites in CSF can also be used as indices for central neuronal activity [12, 13].

The objectives of the present study were to compare different parameters for central neuronal activity, i.e. 3-methoxy-4-hydroxyphenylmandelic acid (VMA), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA); the enzyme dopamine-β-hydroxylase (DβH) and methionine-enkephalin (Met-Enk) in cerebrospinal fluid of rabbits after intravenous yohimbine administration. The neuropeptide Met-Enk has also been included because co-storage and co-release with noradrenaline has been demonstrated for peripheral nervous tissues [14, 15].

MATERIALS AND METHODS

Animals and treatment. Experiments were performed with rabbits of either sex, weighing between 2 and 3 kg. CSF samples were collected from the cisterna magna following anaesthesia with urethane (25% w/v in 0.9% NaCl) via the marginal ear vein as described by De Potter *et al.* [16]. A control CSF sample was taken just before the injection of yohimbine. Yohimbine was administered slowly by injection of 5 mg/kg in the marginal ear vein. In control experiments physiological saline was injected. Aliquots of 600 μl CSF were sampled at 60 min intervals up to 4 hr after yohimbine injection.

DβH measurements. DβH activity in CSF was

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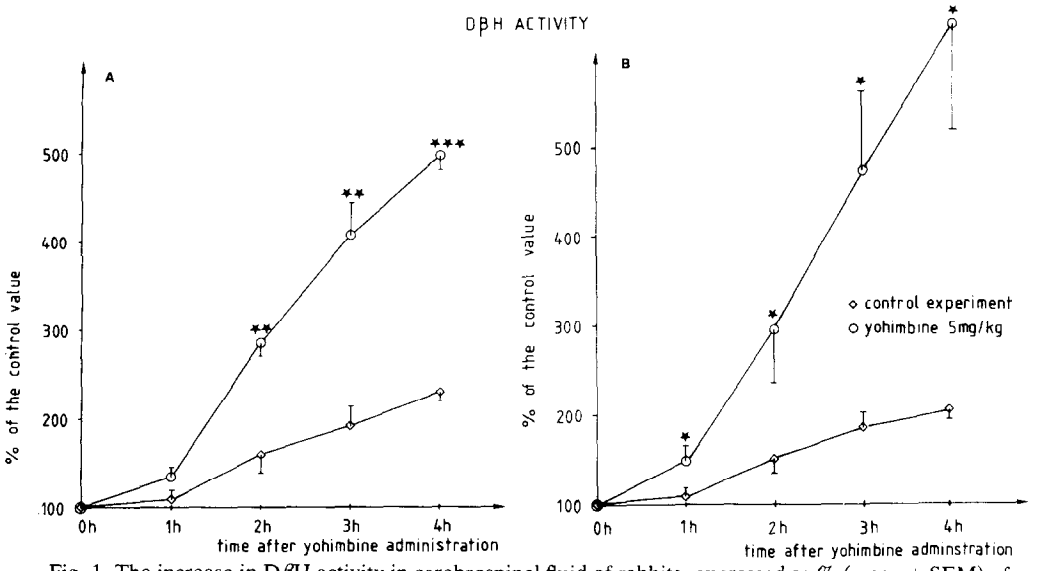

Fig. 1. The increase in DβH activity in cerebrospinal fluid of rabbits, expressed as % (mean ± SEM) of DβH levels preceding yohimbine administration (5 mg/kg intravenously), is shown in function of time following drug administration (○) (N = 4). In control experiments (◇) rabbits received an intravenous saline injection after sampling of control CSF (N = 4): (A) metabolite measurements; (B) Met-enkephalin measurements. Statistical analysis, unpaired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

determined using an HPLC-EC method [17, 18]. Briefly, a sample of 300 μl CSF was incubated at 37° in the presence of the substrate dopamine (20 mM). After stopping the enzymatic reaction, the internal standard was added and the reaction product, noradrenaline, was isolated from the excess amounts of dopamine and quantified by HPLC-EC. Quantitative calculations were based on the construction of a standard curve. The enzymatic activity is expressed as U/ml; 1 U being equivalent to 1 nmol NA formed in 1 hr.

Metabolite analyses. Gas chromatography-mass spectrometery (GC/MS) was used to measure the monoamine metabolite levels. For the mass fragmentographic analyses of VMA, MHPG, HVA and 5-HIAA, the procedure described by Swahn *et al.* [19] was applied with minor modifications. After

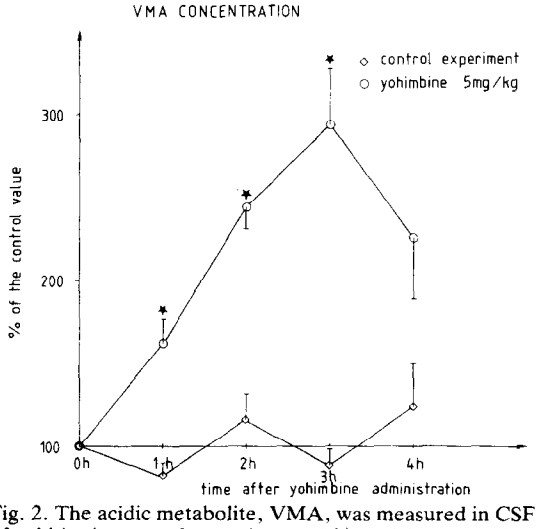

Fig. 2. The acidic metabolite, VMA, was measured in CSF of rabbits in control experiments (◇) and in yohimbine experiments (○). CSF was taken as described in the Materials and Methods section. In the yohimbine experiments (N = 3) a predrug sample was taken, thereafter yohimbine was administered intravenously (5 mg/kg) and every hour CSF was sampled to measure DβH and monoamine metabolites. In control experiments (N = 4) saline was administered. The results are expressed as % (mean ± SEM) of the predrug level. Statistical analysis, unpaired Student's *t*-test: * $P < 0.01$.

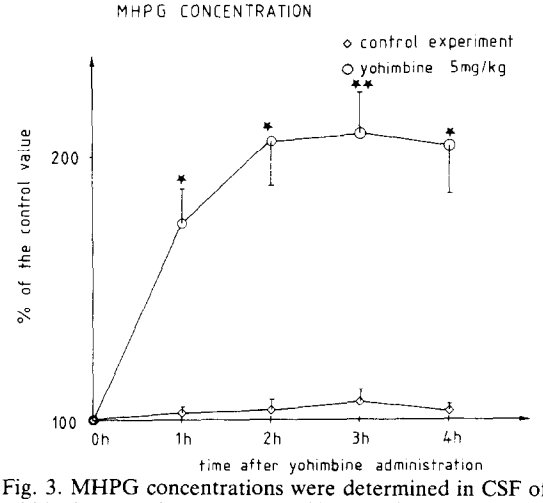

Fig. 3. MHPG concentrations were determined in CSF of rabbits in control experiments (◇) (N = 4) and in yohimbine experiments (○) (N = 4). A small aliquot CSF (600 μl) was sampled before yohimbine administration (intravenously, 5 mg/kg) and then every hour following the drug injection to determine DβH and monoamine metabolites. Results are expressed as % (mean ± SEM) of the predrug level. Control experiments, in which saline was administered, were carried out as well. Statistical analysis, unpaired Student's *t*-test: * $P < 0.01$; ** $P < 0.001$.

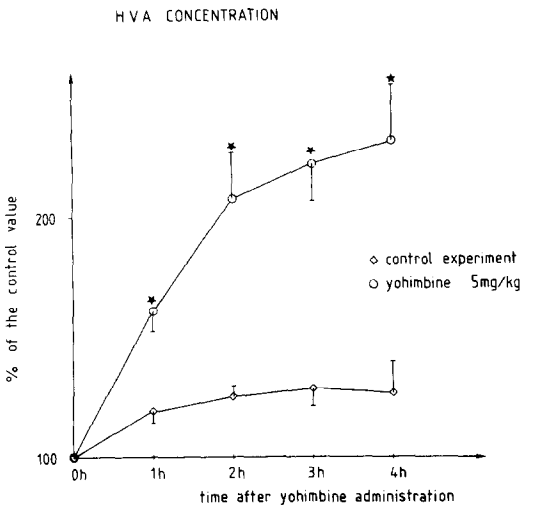

Fig. 4. HVA concentrations in CSF of rabbits were determined in control experiments (◇) (N = 4) and in yohimbine experiments (○) (N = 4). A predrug CSF sample was taken. Following intravenous yohimbine administration (5 mg/kg) CSF was sampled every hour to determine DβH and monoamine metabolites. Results are expressed as % (mean ± SEM) of the predrug HVA level. In control experiments saline was administered. Statistical analysis, unpaired Student's *t*-test: * $P < 0.01$.

adding 20 μl 5% aqueous ascorbic acid solution and deuterated internal standards (±3 ng $^2H_3$-VMA, ±9 ng $^2H_3$-MHPG, ±25 ng $^2H_5$-HVA, ±25 ng $^2H_2$-5-HIAA) the samples were extracted twice with 3 ml ethyl acetate. The combined extracts were evaporated under nitrogen and the residues were reacted with 100 μl pentafluoropropionic anhydride (PFPA) and 25 μl pentafluoropropanol (PFPOH) for 15 min at 75°. A second derivatization step was carried out

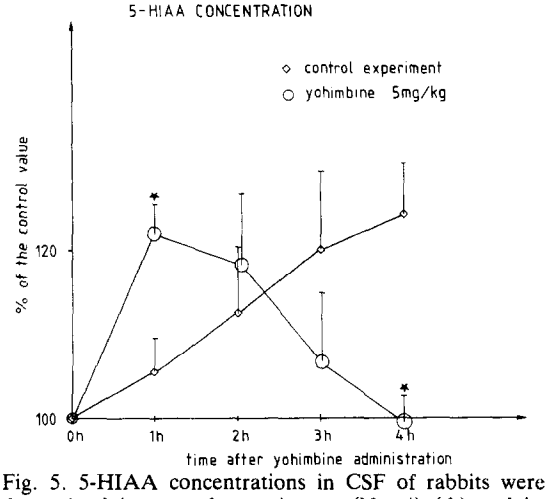

Fig. 5. 5-HIAA concentrations in CSF of rabbits were determined in control experiments (N = 4) (◇) and in yohimbine experiments (N = 4) (○). A predrug CSF sample was taken. Following the intravenous yohimbine administration (5 mg/kg) CSF was sampled every hour. Results are expressed as % (mean ± SEM) of the predrug 5-HIAA levels. In control experiments saline was administered. Statistical analysis, unpaired Student's *t*-test: * $P < 0.05$.

with 50 μl PFPA for 40 min in order to enhance the derivative yield of 5-HIAA. Immediately before GC/MS analysis, the derivatizing reagent was evaporated and the residues redissolved into 15 μl ethyl acetate containing 1% PFPA. The molecular ions were selected for quantitative analysis. These ions are at a *m/z* value of 622 (625) for VMA and MHPG; for HVA at 460 (465) and for 5-HIAA at 615 (617). The *m/z* value of the corresponding deuterated internal standard is given between brackets. Quantitative calculations were based on the construction of a standard curve, covering the concentration range of interest. The $^2H_3$-VMA was a gift from Dr S. P. Markey (NIMH, Bethesda) whereas the other deuterated internal standards were purchased from Merck, Sharp & Dohme, Pointe Claire-Dorval, Canada.

Methionine-enkephalin assay. After purification on Sep-pak C_{18} columns (Waters Inc., MA), the CSF samples were assayed for Met-enkephalin by a radioimmunoassay [15] using an anti-Met-enkephalin serum (Amersham International) with a cross-reactivity of 7% for Leu-enkephalin and less than 0.1% for other peptides at a 1/10,000 dilution. The radioimmunoassay mixture contained RIA buffer (125 mM physiological phosphate buffer containing 40 mM NaCl, 0.05% sodium acetate and 0.125% bovine serum albumin pH 7.5), the antibody, 10,000 cpm of ^{125}I-methionine-enkephalin (Amersham International S.A.; 2000 Ci/mmol) and the sample or methionine-enkephalin standard. After incubation at 4° for 18–24 hr, the assay was stopped by precipitating the [^{125}I]-Met-enkephalin-antibody complexes with 1 ml of a 20% polyethylene glycol solution and 0.1 ml horse serum as carriers and the radioactivity in the samples measured by gamma-spectrometry.

RESULTS

Two series of experiments were performed; in one series 600 μl CSF was used to determine DβH activity and metabolite concentrations; in the other series the DβH activity and Met-enkephalin levels were assayed.

DβH measurements

Yohimbine increased about a factor 5–6 (500% and 641%) the DβH activity in rabbit CSF vs the control value determined before injection of yohimbine. The control levels ranged between 0.5 and 1 U/ml. In control experiments rabbits received a physiological saline injection; the DβH levels also slightly augmented vs the control level determined before saline injection (232%, 205%). Figures 1A and B show the increase in DβH activity as a function of time. An unpaired Student's *t*-test was applied for comparing the DβH levels after yohimbine with the corresponding DβH levels of the control experiments. $P < 0.05$ was taken as the level of significance.

Metabolite measurements

Yohimbine administration results in a maximum rise of VMA, MHPG and HVA concentrations to 293%, 209% and 233% respectively vs the concentration found prior to injection of yohimbine

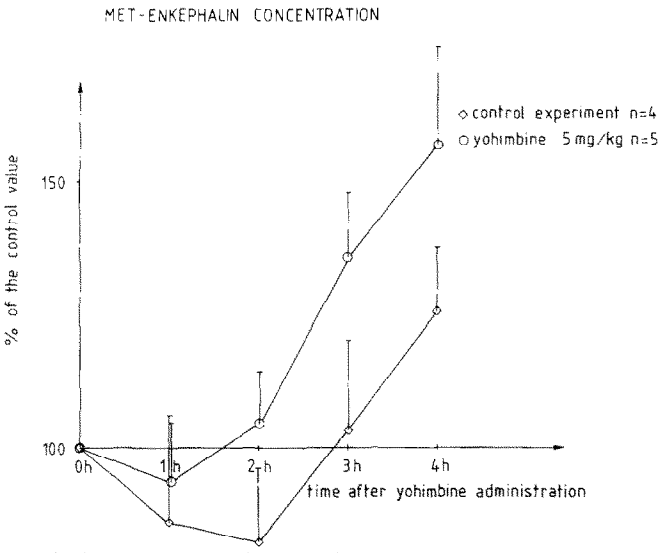

Fig. 6. The Met-enkephalin concentration in CSF of rabbits was determined in control experiments (N = 4) (◇) and in yohimbine experiments (N = 5) (○). A predrug CSF sample was taken. Following intravenous yohimbine administration (5 mg/kg) CSF was sampled every hour. Results are expressed as % (mean ± SEM) of the predrug Met-enkephalin concentration. In control experiments saline was administered. Compared with the controls, no significant increases or decreases are observed.

(Figs 2–4). 5-HIAA levels are increased 1 hr but do not change 2 hr following drug administration; thereafter, a decrease which is significant at 4 hr after yohimbine injection is observed vs the control values (Fig. 5). In the control experiments metabolite levels are virtually stable; the maximum increase is observed for the HVA concentration 3 hr after saline injection (128%) whereas MHPG concentrations for instance only show a maximum increase of hardly 108% compared with the control levels. The control levels in the different experiments ranged between not measurable—2 ng/ml CSF (detection limit = 100 pg/200 μl) for VMA, 16–30 ng/ml for MHPG; 100–210 ng/ml for HVA and 60–149 ng/ml for 5-HIAA.

Met-enkephalin concentrations

Figure 6 shows the changes in Met-enkephalin levels as a function of time for the yohimbine and saline experiments. Yohimbine administration results in a maximum increase vs the control value to 157%. In the control experiments a rise to 126% was observed. The initial values determined in samples collected prior to drug or saline injection varied between 79.6 and 263 pg/ml. The control and yohimbine experiments do not yield significantly different results. In most of the experiments the control level of Met-enkephalin was higher than the level found in the sample collected during the following hour for the control as well as for the yohimbine experiments (Fig. 6).

DISCUSSION

The present experiments indicate that the α_2-antagonist yohimbine causes a considerable increase in DβH levels in CSF. As in earlier experiments on rabbits [9], a slow onset of the increase in DβH activity, which persisted for several hours, was found. In these earlier studies (as well as in our study), the necessary control experiments were performed in order to exclude that the observed changes in DβH activity were artefacts due to changes in CSF volume. These investigators also found a much higher specific activity for DβH in the CSF as compared to the specific activity of the enzyme in the blood. They concluded that DβH activity in the CSF did not originate from the blood but was derived from central noradrenergic neurons. Furthermore, protein measurements indicated that changes in DβH activity were not paralleled by changes in protein concentration, which favours the idea of exocytotic release from central neurons [10]. In the control experiments with rabbits receiving a saline injection carried out in the present study, DβH activities increase up to 200% of the control value. A possible explanation for this apparent increase may be that the surgical procedure necessary to obtain clear CSF induces a stress situation causing increased neurotransmission. While the increase in DβH activity appears to be a valuable parameter for measuring central noradrenergic activity the relationship of the monoamine metabolites to monoaminergic neuronal activity seems to be controversial. On the one hand Palfreyman *et al.* [12], found close linear correlations between CSF and brain metabolites for dihydroxyphenylacetic acid (DOPAC), HVA and 5-HIAA, whereas Commissiong [20] pointed out that metabolite studies alone cannot be employed as the sole criterion of neuronal activity; not only the release but also transport out of the brain and enzymes of the metabolic pathway may be involved. Indeed, although metabolite levels in the brain are reflected in the CSF this on its own provides no justification for their use as indices for brain monoamine turnover. Simultaneous measurement of a more specific parameter for the release, i.e. DβH, together with the monoamine metabolites, as is carried out in our

experiments, therefore can support metabolite results indicating changes in neuronal activity.

DβH activity augments to 5 times the pre-drug level whereas VMA and MHPG concentrations are 2–3 times the control level following yohimbine administration. The increase in metabolite concentrations is rapid in onset and sometimes reaches a maximum after 1–2 hr, whereas the onset of the DβH activity increase is slow as described by De Potter *et al.* [9] (see figures). These results suggest that DβH may be a more sensitive index than VMA and MHPG for measuring noradrenergic neuronal activity in the central nervous system, as already is apparent from the increases in DβH activity in the control experiments, probably due to the surgical procedure.

Up to 4 hr after administration DβH levels as well as MHPG and VMA levels are still high. The mechanism and rate of loss of DβH activity from CSF are unknown [21]. On the other hand, acid metabolites (VMA, HVA, 5-HIAA) are removed from the central system by a probenecid-sensitive transport mechanism whereas the likely mechanism of clearance of free MHPG from the CSF is by diffusion to capillary blood [22]. Kopin *et al.* [23], stated that MHPG concentrations in lumbar CSF can provide a valid index for central MHPG production but only when a correction is made for the contribution of plasma MHPG, which by diffusion contributes to the CSF value. Our results seem to indicate that the elimination of metabolites proceeds much more slowly than the increase induced by administration of yohimbine. Indeed, almost no decline in metabolite levels can be observed 4 hr following drug injection, whereas the increase is already significant 1 hr following yohimbine administration. With regard to the increases of VMA observed in CSF following yohimbine, the possibility exists that they are reflections from rises in the peripheral plasma concentration. Support for the central origin of VMA, however, is that previous studies employing isotope labelling techniques and large intravenous doses have indicated that only insignificant amounts of the other acidic metabolites (HVA, 5-HIAA) are recovered in CSF and brain tissue [24].

After yohimbine administration, concentrations of VMA, MHPG and HVA in CSF increased significantly, whereas 3 hr following injection, 5-HIAA levels show a slight decrease which is significant at 4 hr. The concentrations of free MHPG, HVA and 5-HIAA in rabbit CSF are in agreement with previous results obtained by Van Bockstaele *et al.* [25].

Our results on the metabolite increases are also comparable with data obtained for the rat, where following intraventricular administration of yohimbine, CSF MHPG reached 9 times the pre-drug level. However, in this study 5-HIAA levels were not significantly altered by yohimbine [5]. Intraperitoneal yohimbine and clonidine administration changed rat brain DHPG and MHPG levels in the predicted directions (increase and decrease respectively) [26].

The effect of yohimbine on the dopaminergic, serotonergic and enkephalinergic systems was determined by measuring HVA, 5-HIAA and Met-enkephalin levels respectively. Our data, showing an increased dopaminergic and slightly decreased serotonergic neurotransmission, are in agreement with literature data [6, 8].

While the present experiments suggest an increased dopamine release no conclusions can be drawn whether yohimbine directly interacts with receptors on dopaminergic neurons or indirectly via an interaction between the noradrenergic and dopaminergic system [6, 7, 27].

In the periphery evidence accumulates for a co-storage and co-release of Met-enkephalin and noradrenaline [15]. Also in the cat brain, more specifically in the locus coeruleus, noradrenaline coexists together with Met-enkephalin in the same neurons [28]. Hence if Met-enkephalin is also released together with noradrenaline from central noradrenergic neurons one might expect that increases in DβH activity are paralleled by increases in Met-enkephalin levels. Our results, however, indicating no changes in CSF Met-enkephalin concentration following yohimbine cannot provide support for co-release of noradrenaline and Met-enkephalin. The most likely explanation for this discrepancy may be that the amounts of Met-enkephalin released following stimulation of central noradrenergic neurons are too low to be detected. Most of the Met-enkephalin immunoreactivity was found in the globus pallidus, the substantia nigra, the caudate and putamen whereas lower concentrations were present in the locus coeruleus [29, 30]. Another possible explanation may be that stimulation by α_2-antagonists is not the most appropriate stimulus for co-release of noradrenaline and Met-enkephalin. In adrenal glands for example, it has been shown that physiological stimulation by activation of the splanchnic nerve or more intense stimulation by K^+-depolarization results in different co-release patterns of noradrenaline and Met-enkephalin [14]. A similar phenomenon may well occur for noradrenergic neurons in the central nervous system. The enkephalin concentration in CSF initially decreases vs the control level as well in the treated as in the control animals. It is possible that the surgical procedure to obtain CSF initially causes pain resulting in higher control levels. Furthermore, release of enkephalin peptides in the central nervous system is followed by a rapid degradation by peptidases [31]. The value of the measurement of enkephalin levels in CSF as indices for enkephalinergic activity therefore remains to be investigated.

CONCLUSION

Different parameters for monoaminergic and enkephalinergic activity have been measured in CSF of rabbits following intravenous yohimbine administration. Increased noradrenergic and dopaminergic activity has been observed. The importance of an enzyme specifically reflecting the release, i.e. DβH, has been discussed. After an initial increase the serotonergic neurotransmission is slightly decreased. No changes have been observed in enkephalinergic neurotransmission as reflected by the concentrations of Met-enkephalin in CSF.

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