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Yohimbine increases submaxillary kallikrein release into the saliva in dogs: evidence for α_2-adrenoceptor-mediated inhibition of cholinergic pathways

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1 The effects of the α_2-adrenoceptor antagonist, yohimbine (0.5 mg kg^{-1}, i.v.) on basal, sympathetic and parasympathetic stimulation-induced submaxillary kallikrein release were investigated in the anaesthetized dog. Kallikrein was measured by its kininogenase activity before and after trypsin activation which also allowed a study of the proportion of active to total enzyme.

2 Yohimbine induced a rapid, three fold increase in basal kallikrein release correlated with an increase in salivary flow rate which lasted for 60 min following injection.

3 Sectioning the chorda tympani did not affect basal kallikrein release but abolished yohimbine-induced rise in salivary kallikrein secretion.

4 Parasympathetic stimulation alone induced a 3 to 4 fold increase in basal kallikrein release correlated with an increase in salivary flow rate. Yohimbine induced a significant additional increase in parasympathetic-stimulated kallikrein release.

5 When the cervical sympathetic nerve was sectioned the basal kallikrein release decreased by 30 to 40%.

6 Sympathetic stimulation alone also induced a 3 to 4 fold increase in basal kallikrein. This was not correlated with the salivary flow and unaffected by yohimbine.

7 The results indicate that yohimbine increases submaxillary kallikrein release into the saliva by inhibition of presynaptic α_2-adrenoceptors located on the chorda tympani nerve endings.

Introduction

One of the major actions of glandular kallikrein is to release bradykinin, a potent vasodilator peptide. The kallikrein-kinin system has been localized in several glands such as pancreas, kidney and salivary glands and various biological activities for it have been proposed: e.g. in the regulation of both local blood flow and in the secretion of electrolytes and water secretion from these glands (Fuller & Funder, 1986; Carretero & Scicli, 1989). In particular, large amounts of glandular kallikrein have been detected in the salivary glands (Orstavik, 1978); salivary secretion of kallikrein may be mediated by β-adrenoceptors in cat (Gautvik *et al.*, 1974) and in rat (Orstavik & Gautvik, 1977). Moreover, in the rat, the release of submandibular kallikrein into blood paralleled that into saliva and its secretion was largely enhanced by α-adrenoceptor stimulation (Rabito *et al.*, 1983; Berg *et al.*, 1985). These results suggest a possible independent regulation of salivary flow and endocrine enzyme secretion.

We recently demonstrated that the α_2-adrenoceptor antagonist, yohimbine, increases submaxillary salivation in dogs (Montastruc *et al.*, 1989) and in man (Chatelut *et al.*, 1989). It has been proposed that the release of kallikrein by production of an associated kinin may regulate the ionic composition of saliva (Carretero & Scicli, 1989; Fuller & Funder, 1986). Thus, the aim of the present study was to evaluate the effect of yohimbine on the secretion of kallikrein into saliva during parasympathetic or sympathetic stimulation.

Methods

Animals and general procedure

Dogs of either sex (7 to 15 kg) were prepared as recently described (Montastruc *et al.*, 1989). Animals were anaesthetized with a mixture of chloralose (40 mg kg^{-1}, i.v.) plus urethane (500 mg kg^{-1}, i.v.). To avoid the potential α_2-adrenoceptor antagonist properties of high doses of urethane (Moore *et al.*, 1984), low sub-anaesthetic concentrations were used. Body temperature was kept constant at around 38.5°C, arterial pH was 7.42. Blood pressure and heart rate were monitored with a Statham P23 Id pressure transducer connected to a Honeywell Bull recorder and a tachocardiometer triggered by electrocardiogram (lead 2) linked to a chart recorder.

Protocol for salivary secretion study

Following anaesthesia, Wharton's duct was catheterized by means of a polyethylene tube (Montastruc *et al.*, 1989), three separate experimental protocols were conducted.

Experiment 1: effects of yohimbine on spontaneous kallikrein release Yohimbine (0.5 mg kg^{-1}, i.v. 3 ml, $n = 6$) or saline vehicle (0.9% NaCl 3 ml, in sham experiments, $n = 6$) were injected and, after a resting period of at least 15 min, salivary secretion from both submaxillary glands was collected every 15 min for 90 min.

Experiment 2: effects of yohimbine on parasympathetic kallikrein release In the first experimental sub-group, the left chorda tympani nerve was dissected (Montastruc *et al.*, 1989) and sectioned before starting the experiment. The effect of yohimbine (0.5 mg kg^{-1}, i.v.) was investigated on the spontaneous release of kallikrein from the intact (right) side and compared simultaneously with the release observed from the sectioned (left) side. In the second sub-group of experiments, the effect of yohimbine was investigated during electrical stimulation of the peripheral cut end of the chorda tympani nerve. The parameters of stimulation used (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine or saline (3 ml) administration were

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selected to mimic the salivary secretion found with an unstimulated intact nerve (Montastruc *et al.*, 1989). Kallikrein was measured in samples collected during these 15 s periods of stimulation and during the 15 min period following the end of stimulation.

Experiment 3: effects of yohimbine on sympathetic kallikrein release The left cervical sympathetic nerve was isolated and cut in the vagal sympathetic trunk: the effects of yohimbine (0.5 mg kg^{-1}, i.v.) on kallikrein release on the (right) intact nerve were compared with the values obtained from the sectioned stimulated (left) side. The parameters of stimulation were 15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine or saline (3 ml) administration.

Kallikrein measurement

The level of kallikrein in the saliva was measured by the kininogenase activity by use of a kinin radioimmunoassay (Alhenc-Gelas *et al.*, 1981), before and after trypsin activation, with the nonapeptide bradykinin as standard and heat-treated citrated dog plasma as substrate. This crude substrate preparation was devoid of spontaneous kininogenase and kininase activity. The incubation buffer consisted of 0.1 M Tris HCl, pH 8.5, containing 0.3 mM ortho-phenanthroline and 0.3 mM EDTA as kininase inhibitors. Total activatable kallikrein was measured after trypsinization of the samples which activates prekallikrein by liberating an inactive heptapeptide profragment. The proportion of active over inactive kallikrein, expressed as a percentage of the active form, gives complementary information regarding the secretion and activation mechanisms of kallikrein. Samples were incubated for 30 min with 5 μg trypsin in 0.2 M Tris pH 7.8 at 37°C. The action of trypsin was stopped by adding 20 μg of soybean trypsin inhibitor (SBTI). The results are expressed in ng of bradykinin generated per minute in a 15 min salivary volume (ng BK per 15 min).

The possible degradation of kallikrein or the presence of kallikrein inhibitors in the incubation medium was also checked by adding a fixed amount of standard kallikrein purified from dog urine (personal data) and measuring the recovery of activity. In these test experiments, the recovery of purified dog kallikrein was 86 ± 9%.

Statistical analysis

Results are expressed as mean ± s.e.mean ($n = 6$ in each group). Significance was estimated by Student's t test (after one-way analysis of variance). Intact and sectioned sides were compared by use of Student's t test for paired comparison. P values less than 0.05 were considered significant. Correlation studies were carried out with the results of the salivary volume investigation performed in the same dogs (Montastruc *et al.*, 1989).

Results

Yohimbine administration (0.5 mg kg^{-1} i.v.) did not induce any significant variation of blood pressure or heart rate as previously reported (Montastruc *et al.*, 1989).

Experiment 1: effects of yohimbine on spontaneous kallikrein release (Figure 1)

Compared with saline, yohimbine induced a 3 fold increase in the basal kallikrein secretion which reached maximal values 45 to 60 min after administration. The proportion of active to total kallikrein remained unchanged with an average value of 54 ± 9%. The increase in kallikrein secretion was significantly correlated with the rise in salivary volume ($r = 0.88$, $P < 0.001$; $n = 36$).

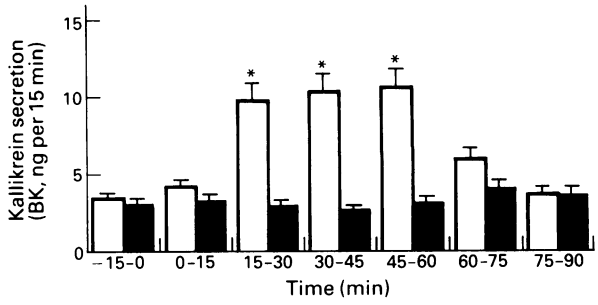

Figure 1 Comparison of the effects of yohimbine (0.5 mg kg^{-1} i.v. injected at time 0: open columns) and i.v. saline vehicle (solid columns) on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min from both submaxillary ducts (with intact nerves). * $P < 0.01$. Values are means with s.e.mean shown by vertical bars; $n = 6$.

Experiment 2: effects of yohimbine on parasympathetic kallikrein release

Sectioning of the chorda tympani had no effect on basal kallikrein secretion but abolished the stimulating effect of yohimbine (Figure 2). Stimulation of the parasympathetic chorda tympani nerve induced a 3 to 4 fold rise in kallikrein release which was significantly correlated with the increase in salivary volume ($r = 0.79$, $P < 0.001$; $n = 28$). In fact, the concentration of kallikrein did not change significantly throughout the different periods of stimulation (1.27 ± 0.38 versus 1.1 ± 0.25 ng BK per ml saliva). The increase in kallikrein was due to the enhancement of salivary volume. Yohimbine administration during parasympathetic stimulation induced a further significant increase in kallikrein release (Figure 3).

Experiment 3: effects of yohimbine on sympathetic kallikrein release (Figure 4)

Sectioning of the cervical sympathetic nerve induced a 50 to 70% decrease in basal kallikrein secretion (1.1 ± 0.2 versus 3.4 ± 0.3 ng BK per 15 min, $P < 0.05$). Sympathetic stimulation induced an increase in kallikrein secretion, but this was due to a 3 to 6 fold rise in kallikrein concentration (5.2 ± 0.62 versus 0.91 ± 0.32 ng BK per ml saliva). Furthermore, the increase in kallikrein secretion was not correlated with

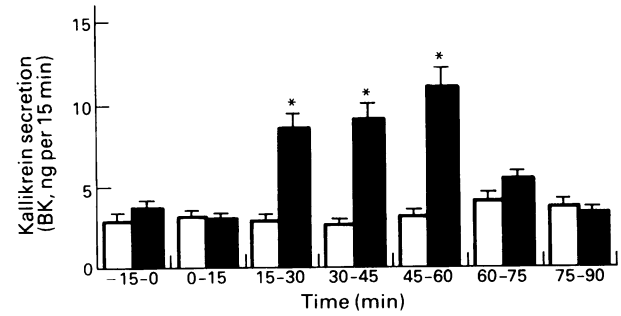

Figure 2 Effect of sectioning the chorda tympani nerve on the effect of yohimbine (0.5 mg kg^{-1} i.v. injected at time 0) on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. * $P < 0.01$. Values are means with s.e.mean shown by vertical bars, $n = 6$; open columns: sectioned side, the nerve was sectioned before starting the experiment; solid columns: intact side.

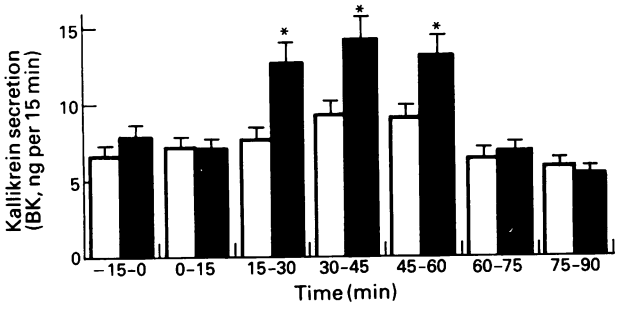

Figure 3 Effect of yohimbine (0.5 mg kg^{-1}, i.v. injected at time 0: solid columns) and i.v. saline (open columns) during parasympathetic stimulation of the peripheral end of the chorda tympani nerve on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. Electrical stimulation began at time −15 min * $P < 0.01$. Values are means with s.e.mean shown by vertical bars. $n = 6$.

changes in salivary volume. The magnitude of kallikrein release after yohimbine administration was similar on the intact and sectioned sides.

In the three experiments, the ratio of active to total kallikrein did not change, indicating that the variations in kallikrein secretion resulted from a proportionate secretion of both the active and inactive forms (data not shown).

Discussion

It has been reported previously that submaxillary kallikrein secretion is mainly under sympathetic control both in cat (Gautvik *et al.*, 1974; Garret *et al.*, 1987) and in rat (Orstavik & Gautvik, 1977; Orstavik *et al.*, 1982a,b; Berg *et al.*, 1985; Rabito *et al.*, 1986). However, a mild parasympathetic stimulating effect was also described (Orstavik & Gautvik, 1977), although its involvement remains controversial (Schachter, 1979). Nevertheless, it should be noted that the release of submaxillary kallikrein has been essentially investigated in blood whereas that in saliva has received less attention. Our data demonstrate that kallikrein release into saliva is also greatly increased by parasympathetic nerve stimulation. Furthermore, the fact that yohimbine, an α_2-adrenoceptor antagonist, induces a significant increase in basal kallikrein secretion, clearly suggests the involvement of an α_2-adrenoceptor in this

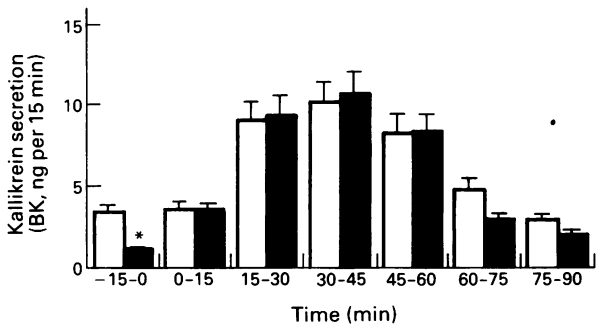

Figure 4 Effect of yohimbine (0.5 mg kg^{-1}, i.v. injected at time 0) during stimulation of the superior cervical nerve on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. Sectioning the superior cervical nerve induced a decrease in kallikrein secretion: period −15–0 min, * $P < 0.05$ when the intact (right) side (solid columns) was compared with the sectioned (left) side (open columns). Electrical stimulation began at time 0 and restored the basal secretion of kallikrein during the period 0–15 min. The effects of yohimbine on the intact (right) side (solid columns) were compared with those obtained from stimulation of the sectioned (left) side (open columns). The magnitude of kallikrein release after yohimbine administration (periods 15–30, 30–45 and 45–60 min) was similar on the intact and sectioned sides. Values are means with s.e.mean shown by vertical bars. $n = 6$.

effect. The present study demonstrates that yohimbine not only increases salivary water content (Montastruc *et al.*, 1989) but also maintains the levels of kallikrein, a physiological factor normally present in the saliva. Several reports have already demonstrated the involvement of α_2-adrenoceptors in the control of salivation in the rat (Bylund & Martinez, 1980; 1981) and in the dog (Montastruc *et al.*, 1989). The stimulating effect of yohimbine on kallikrein release is also dependent on the integrity of parasympathetic innervation: it disappears after sectioning the chorda tympani but is restored during stimulation of the distal end of the nerve. Thus, the present data demonstrate the existence of α_2-adrenoceptors located on the chorda tympani nerve endings which inhibit the basal secretion of kallikrein.

The present data also emphasize the importance of α_2-adrenoceptors in the secretory control of salivary glands especially in the regulation of cholinergic (but not sympathetic) transmission during submaxillary salivation as has already been suggested (Green *et al.*, 1979). The reasons why previous reports have failed to demonstrate such an important activating effect of parasympathetic stimulation may be related to the anaesthetics used since we observed that under chloralose, urethane or sodium thiopentane anaesthesia, yohimbine remained ineffective on salivary secretion (Montastruc *et al.*, 1989). Anaesthetics interfere with α_2-adrenoceptors (Moore *et al.*, 1984). Parasympathetic nerve impulses are however required for normal synthesis of submaxillary kallikrein (Garrett *et al.*, 1987); the submaxillary glands, 3 weeks after chorda tympani excision, showed a very weak light staining due to kallikrein-like activity as revealed by cryostat sections using histochemical methods.

In addition to the indirect sympathetically-induced cholinergic inhibition of kallikrein secretion mediated by α_2-adrenoceptors, the influence of sympathetic nerves *per se* was also confirmed. Sectioning of the superior cervical sympathetic nerve reduced the spontaneous secretion of kallikrein. This result probably indicates that basal kallikrein secretion is under tonic sympathetic influence. Sympathetic stimulation also induced a significant increase in kallikrein above the basal levels. However, this increase is not linked to a rise in salivary secretion and results from an increased concentration of kallikrein.

It appears likely that in the salivary gland, kallikrein release into saliva is under the control of both sympathetic and parasympathetic nerves through two different mechanisms. The main result of the present study is to demonstrate that parasympathetically-induced kallikrein secretion in saliva is under a tonic α_2-adrenoceptor inhibitory control. Therefore, α_2-adrenoceptor blockade induces a stimulation of kallikrein secretion in saliva. The stimulation reflects a true increase in kallikrein secretion since the proportion of active to total kallikrein remained unchanged. It is worth pointing out that this increase in kallikrein secretion is significantly correlated with an enhancement of the salivation rate recently described during parasympathetic stimulation (Montastruc *et al.*, 1989). In contrast to the parasympathetic control of kallikrein secretion, which parallels that of salivation, the sympathetic innervation exerts a tonic effect: its stimulation results in the release of high concentrations of kallikrein independently of salivary secretion. Finally it seems that α_2-adrenoceptors may control in an opposite way blood and salivary release of submandibular kallikrein: stimulation of α_2-adrenoceptors increased the secretion of kallikrein into blood (Rabito *et al.*, 1983) whereas α_2-adrenoceptor blockade enhanced the release of the enzyme into the saliva.

The physiological significance of kallikrein in the submaxillary gland is still questionable. However, the release of kallikrein into the circulation may play a role in the regulation of blood flow and in vasodilatation after treatment with converting enzyme inhibitors or after sympathetic stimulation (Orstavik *et al.*, 1982a,b; Berg *et al.*, 1989). Whether the release of kallikrein into the saliva is involved in the increase of salivary secretion remains to be determined.

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