Role of the Autonomic Nervous System in the Release of Rat Submandibular Gland Kallikrein into the Circulation

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SUMMARY. We have previously demonstrated that the rat submandibular gland releases immunoreactive kallikrein into the circulation. To study the role of the autonomic nervous system in this release, submandibular gland blood flow and kallikrein concentration in peripheral arterial and venous blood from the gland were measured and secretion rates calculated before and after parasympathetic and sympathetic nerve stimulation (8V, 2 msec, 10 Hz) for 1 minute. Immunoreactive kallikrein in plasma was measured by radioimmunoassay, and timed collections of venous outflow were used to measure blood flow. During basal conditions, the unstimulated submandibular gland of the rat released immunoreactive kallikrein into blood at the rate of 0.92 ± 0.07 ng/min. Parasympathetic stimulation increased blood flow 4-fold (before, 68.5 ± 8.3 µl/min; after, 253.5 ± 76.2; P < 0.05) without significantly changing immunoreactive kallikrein secretion rate. Sympathetic stimulation produced an 11-fold increase in blood flow (before, 64.9 ± 9.3 µl/min; after, 709.6 ± 97.5; P < 0.05) and a 57-fold increase in immunoreactive kallikrein secretion rate from the gland (before, 1.05 ± 0.25 ng/min; after, 59.8 ± 18.6; P < 0.05). Sympathetic stimulation also produced a 4-fold increase in the concentration of immunoreactive glandular kallikrein in arterial plasma (before, 15.2 ± 1.1 ng/ml; after, 56.2 ± 12.9; P < 0.05). Pretreatment with phentolamine (1 mg/kg) or prazosin (0.2 mg/kg) blocked the increase in kallikrein secretion rate produced by sympathetic stimulation. These results indicate that the sympathetic nervous system, through activation of α-adrenoreceptors, controls kallikrein secretion from the submandibular gland into the circulation. Released kallikrein may be responsible for the reactive vasodilation observed in the rat submandibular gland after sympathetic stimulation. (Circ Res 52: 635-641, 1983)

GLANDULAR kallikreins are serine proteases which generate kinins, potent vasodilator peptides, from plasma substrates called kininogens. Although glandular kallikreins have been implicated in the regulation of local blood flow through the formation of kinins (Hilton and Lewis, 1956; Carretero and Scicli, 1981), their physiological role still remains controversial.

The submandibular gland of the rat is an organ very rich in glandular kallikrein. Here, kallikrein is localized in the ductal system where it is secreted into saliva (Beilenson et al., 1968; Garrett and Kidd, 1975; Ørstavik et al., 1975). It has been reported that in the rat the salivary secretion of kallikrein is regulated through the activity of the sympathetic and parasympathetic nervous systems, although the stimulation of the former produces a much higher secretion rate of kallikrein than the stimulation of the latter system (Beilenson et al., 1968; Ørstavik and Gautvik, 1977).

Recent studies in our laboratory have demonstrated that, under resting conditions, the rat submandibular gland also releases immunoreactive kallikrein into the vascular compartment (Rabito et al., 1982). Glandular kallikrein that reaches the interstitial and vascular spaces of the submandibular gland may release kinins from substrates before kallikrein is inactivated by plasma protease inhibitors. These kinins could directly and/or indirectly regulate local vascular resistance and glandular function.

Thus, to define more clearly the physiological role played by submandibular gland kallikrein, it is important to determine the mechanisms that regulate its release into blood. The present investigation was designed to evaluate the role of the autonomic nervous system in the control of kallikrein release into the circulation by the rat submandibular gland. For this, the secretion rate of immunoreactive kallikrein into blood was studied before and after parasympathetic and sympathetic nervous stimulation. Since, during sympathetic stimulation, a marked vasoconstriction develops in the gland which may alter kallikrein release, we have also studied the effect of glandular ischemia on kallikrein secretion rate into blood and compared its effect with that of sympathetic stimulation. Furthermore, we also studied the effect of α- and β-blockers on the increase in immunoreactive kallikrein into blood produced by sympathetic stimulation.

Methods

The following drugs were obtained commercially: pentobarbital sodium (Nembutal, Abbott Laboratories), heparin (Elkins-Sinn, Inc.), propranolol (Ayerst Laboratories, Inc.), phentolamine (Regitine, Ciba Pharmaceutical Company),
clonidine (Catapres, Boehringer Ingelheim, Ltd.), yohimbine (Sigma Chemical Co.), and prazosin (Pfizer, Inc.).

**Experimental Procedure**

Male Sprague-Dawley rats, 400–500 g, were anesthetized with sodium pentobarbital (50 mg/kg, ip) and tracheotomized. The right submandibular gland was exposed and the animals were heparinized (1000 U/kg). All tributaries joining the submandibular gland vein were tied off and a catheter was positioned to collect only submandibular gland venous blood, as previously described (Görstavik et al., 1980). Timed collections of glandular venous outflow, approximately 500 μl, were made into preweighed plastic tubes for measurement of blood flow and immunoreactive glandular kallikrein concentration. An equivalent volume of blood obtained from a donor rat was returned to the rat after each blood withdrawal. Cannulas were placed in the left femoral vein for intravenous injections and blood replacement, in the right femoral artery for collection of arterial blood samples, and in the left femoral artery for blood pressure monitoring. Uncollected submandibular venous outflow was returned to the rat via a catheter placed into the right femoral vein. Mean arterial blood pressure was monitored with a Statham P23Gb pressure transducer connected to the arterial catheter and to a Brush 440 carrier preamplifier and recorder. The main excretory duct of the right submandibular gland was exposed and cannulated. The appearance of saliva was used as criterion for successful stimulation.

The carotid sheath was dissected, and the cervical sympathetic trunk was isolated from the common carotid artery and vagus nerve. Electrical stimulation of the gland was done for 1 minute at 8V, 2 msec duration, and 10 Hz with silver wire electrodes connected to a Grass SD 9 stimulator. Parasympathetic stimulation was obtained by placing the tip of the electrodes around the ductal nerve plexus near the gland hilus. Sympathetic stimulation was done by placing the tip of the electrodes under and around the sympathetic cervical trunk.

Submandibular gland blood flow was estimated gravimetrically and transformed into volume units by using 1.054 as specific gravity for blood (Altman and Dittmer, 1971). Submandibular gland plasma flow was calculated from the values of blood flow and hematocrit. Glandular kallikrein in arterial and submandibular venous plasma was measured with a recently developed radioimmunoassay for immunoreactive glandular kallikrein in plasma (Rabito et al., 1982). Glandular kallikrein secretion rate was calculated using the following equation:

\[
\text{kallikrein secretion rate} = \frac{(V - A) \times PF}{t}
\]

where V is the concentration of immunoreactive kallikrein in submandibular gland venous plasma, A is the concentration of immunoreactive kallikrein in arterial plasma, and PF is the submandibular gland plasma flow.

The following experimental groups were studied:

1. Basal release of immunoreactive glandular kallikrein into blood by the submandibular gland (n = 35).
2. Parasympathetic nervous stimulation (n = 5): Submandibular gland blood flow and the secretion rate of immunoreactive glandular kallikrein into blood were determined 10 minutes before, and 1 and 10 minutes after parasympathetic stimulation.
3. Sympathetic nervous stimulation (n = 13): In this group, submandibular gland blood flow and immunoreactive glandular kallikrein secretion rate were measured 10 minutes before, and 1, 10, and 70 minutes after sympathetic nerve stimulation.
4. Carotid artery occlusion (n = 7): Glandular blood flow and immunoreactive kallikrein secretion rate were determined 10 minutes before, and 1 and 10 minutes after carotid artery occlusion for 1 minute. At the end of the experiment, 25–30 minutes after carotid artery occlusion, the cervical sympathetic trunk was stimulated for 1 minute, as described above, and gland blood flow and immunoreactive kallikrein secretion rate were measured 1 and 10 minutes after.
5. Sympathetic nervous stimulation in rats pretreated with propranolol (n = 7): Submandibular gland blood flow and immunoreactive glandular kallikrein secretion rate were measured 10 minutes before, 30 minutes after propranolol treatment (2 mg/kg, iv), and 1 and 10 minutes after sympathetic nervous stimulation (40 minutes after the propranolol administration).
6. Sympathetic nervous stimulation in rats pretreated with phentolamine (n = 6): Protocol similar to group 5 using phentolamine (1 mg/kg, iv).
7. Sympathetic nervous stimulation in rats pretreated with clonidine (n = 5): Protocol similar to group 5 using clonidine (10 μg/kg, iv).
8. Sympathetic nervous stimulation in rats pretreated with yohimbine (n = 6): Protocol similar to group 5 using yohimbine (10 mg/kg).
9. Sympathetic nervous stimulation in rats pretreated with prazosin (n = 7): Protocol similar to group 5 using prazosin (0.2 mg/kg).

**Statistical Analysis**

To analyze the significance within and between the groups, we used the logarithmic transformation of the data. To determine the within-group significance, we used a paired t-test with an experimentwise α-error of 5%, using the Bonferroni method (Neter and Wasserman, 1974). Comparison between groups was done by Student’s t-test (Snedecor and Cochran, 1973). Data are expressed as the arithmetic mean plus or minus the standard error of the mean (mean ± sem).

**Results**

During resting conditions, the submandibular gland’s average basal release of immunoreactive glandular kallikrein into blood was 0.92 ± 0.07 ng/min, ranging from 0.35 to 1.87 ng/min.

Electric stimulation of the ductal parasympathetic nervous plexus for 1 minute produced a rise in the ipsilateral submandibular gland blood flow from 68.5 ± 8.3 μl/min before stimulation, to 253.5 ± 76.2 μl/min, 1 minute after stimulation (Fig. 1, middle panel). Stimulation of the parasympathetic innervation of the submandibular gland produced no significant changes in the basal release of immunoreactive kallikrein into the blood (0.69 ± 0.09 ng/min before stimulation, and 0.51 ± 0.18 and 0.59 ± 0.06 ng/min at 1 and 10 minutes after stimulation, respectively; Figure 1, lower panel).

Stimulation of the cervical sympathetic trunk for 1 minute produced a marked increase in the release of immunoreactive kallikrein into blood from 1.05 ± 0.25 ng/min to a peak of 59.8 ± 18.6 ng/min 1 minute after stimulation (Fig. 2). During sympathetic stimulation, an intense vasoconstriction was observed in the gland. However, 1 minute after the stimulation was discontinued, an abrupt increase in glandular blood flow from 64.9 ± 9.3 to 709.6 ± 97.5 μl/min.
developed. The gland blood flow then decreased, and 10 and 70 minutes after the stimulation it was 149.4 ± 26.8 and 64.3 ± 5.7 μl/min, respectively. Sympathetic stimulation also increased the concentration of kallikrein in arterial blood flow from 14.9 ± 2.1 ng/ml before to 56.2 ± 12.9 and 56.8 ± 12.7 ng/ml at 10 and 70 minutes after stimulation, respectively (Fig. 2, upper panel). No significant changes in mean arterial blood pressure were observed after sympathetic stimulation.

Figure 3 shows the effects of carotid artery occlusion on immunoreactive kallikrein release into blood, and on glandular blood flow. Circulatory arrest in the gland for 1 minute did not affect postocclusion kallikrein release or glandular blood flow. Sympathetic stimulation performed 25–30 minutes after the occlusion of the carotid artery induced a 150-fold increase in immunoreactive kallikrein release (from 1.24 ± 0.2 ng/min before, to 175.5 ± 51.5 1 minute after stimulation), and a 7-fold increase in blood flow (from 86.5 ± 12.0 μl/min before, to 685.6 ± 127.8 1 minute after stimulation) (Fig. 3).

The type of adrenoceptor involved in the release of kallikrein in response to stimulation of the sympathetic cervical trunk was investigated in rats pretreated with propranolol or phentolamine. Figure 4 and Table 1 show that the changes in immunoreactive kallikrein secretion rate in glandular blood flow in response to sympathetic stimulation were almost completely blocked by phentolamine and not affected by propranolol. Neither propranolol nor phentolamine affected the basal release of kallikrein into the circulation (Table 1).

The subclass of α-adrenoreceptor which mediate the kallikrein response to adrenergic stimulation was studied in rats pretreated with the α2-agonist, clonidine, the α2-blocker, yohimbine, or the α1-blocker, prazosin. Pretreatment of the animals with clonidine or yohimbine had no effect on the increase in kallikrein released in response to adrenergic stimulation (Fig. 5 and Table 1). In contrast, pretreatment of the animals with prazosin blocked more than 90% of the increase in kallikrein secretion rate into blood in response to sympathetic stimulation. Prazosin did not significantly affect the increase in glandular blood flow induced by sympathetic stimulation.

Discussion

By using an RIA for immunoreactive glandular kallikrein in plasma, we previously found that venous blood from the submandibular gland has a higher concentration of kallikrein than arterial blood, and that the removal of the submandibular and sublingual glands significantly decreases the concentration of the
immunoreactive glandular kallikrein in peripheral blood (Rabito et al., 1982). These results indicate that, during resting conditions, glandular kallikrein is secreted into blood by the rat submandibular gland.

Confirming these findings, the present results show that, at rest, the submandibular gland of the anesthetized rat releases kallikrein into blood at the rate of 0.92 ± 0.07 ng/min. Previously it has also been reported that small amounts of kinin-forming enzymes escape from the submandibular gland into the perfusate when the gland is at rest (Hilton and Lewis, 1956). These authors thought that kallikrein was not normally released from the resting gland, but was detectable in the perfusate due to the artificial perfusion circumstances. Both our current results and our previous findings that removal of the salivary glands decreases circulating glandular kallikrein (Rabito et al., 1982), indicate that the submandibular gland normally releases kallikrein into the circulation at a basal rate.

Several investigators have proposed that the atropine-resistant vasodilation which develops in the submandibular gland in response to parasympathetic stimulation can be accounted for by release of kallikrein into the interstitial space (Hilton and Lewis, 1955, 1956; Gautvik, 1970). However, this hypothesis has been questioned because, while sympathetic stimulation almost completely depletes the submandibular gland of its kallikrein, parasympathetic stimulation has little or no effect on the kallikrein content of the gland in rats (Ørstavik and Gautvik, 1977) and cats (Barton et al., 1975). In the present study, we found that stimulation of the parasympathetic innervation of the submandibular gland does not affect the basal release of kallikrein into the circulation while it promotes a marked increase in blood flow to the gland. Thus, the results of the present experiment provide further evidence that kallikrein does not play a significant role in the mediation of the vasodilation which develops in the rat submandibular gland after parasympathetic stimulation. It has been suggested that acetylcholine (Garrett, 1966), vasoactive intestinal polypeptide (VIP) (Bloom and Edwards, 1980; Lundberg et al., 1981), and substance P (Robinson et al., 1980) are mediators of the parasympathetic control of submandibular gland blood flow.

Immediately after stimulation of the cervical sympathetic trunk, there was a 57-fold increase in the release of immunoreactive kallikrein into blood, a response which was accompanied by an 11-fold increase in blood flow to the gland (Fig. 2). It is known that sympathetic stimulation of the submandibular gland leads to the secretion of a small amount of saliva accompanied by vasoconstriction (Bernard, 1858; Hilton and Lewis, 1955), and that, after the stimulation, a vasodilation develops (Carlson, 1907). Since kallikrein release did not change after occlusion of the ipsilateral carotid artery for 1 minute (Fig. 3), it is unlikely that the increase in kallikrein release after sympathetic stimulation is due to the circulatory vasodilation.
TABLE 1
Immunoreactive Glandular Kallikrein Secretion Rate into Blood by the Submandibular Gland of the Rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control period</th>
<th>Before sympathetic stimulation</th>
<th>After sympathetic stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Minute</td>
</tr>
<tr>
<td>Saline (n = 13)</td>
<td>1.05 ± 0.25</td>
<td>1.07 ± 0.25</td>
<td>59.8 ± 18.6†</td>
</tr>
<tr>
<td>Propranolol (n = 7)</td>
<td>0.86 ± 0.19</td>
<td>0.73 ± 0.14</td>
<td>83.0 ± 32.5†</td>
</tr>
<tr>
<td>Phentolamine (n = 5)</td>
<td>1.31 ± 0.20</td>
<td>1.02 ± 0.30</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>Clonidine (n = 5)</td>
<td>0.74 ± 0.10</td>
<td>1.48 ± 0.70</td>
<td>124.8 ± 55.1†</td>
</tr>
<tr>
<td>Yohimbine (n = 6)</td>
<td>1.04 ± 0.17</td>
<td>0.98 ± 0.15</td>
<td>65.6 ± 25.4†</td>
</tr>
<tr>
<td>Prazosin (n = 7)</td>
<td>0.88 ± 0.20</td>
<td>0.78 ± 0.16</td>
<td>4.7 ± 0.76*†</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± se. n = number of rats in each group.
* P < 0.05 compared to saline group
† P < 0.05 compared to before sympathetic stimulation period

Arrest in the gland during stimulation. When sympathetic stimulation was performed 25-30 minutes after circulatory arrest, kallikrein release increased 150-fold over the basal rate. We do not know the reason for this potentiation of the sympathetically induced increase in kallikrein release. However, it has been reported that exocytosis in the submandibular gland may be related to increased intracellular Ca++ (Bhoola et al., 1979, Quissel et al., 1981). It could be speculated that, because of the glandular ischemia, the intracellular sources of ATP and other energy-donor compounds may have been depleted. As a consequence, the capability of the tubular cells to extrude the increase in intracellular Ca++ induced by sympathetic stimulation may have been reduced. This could result in a further rise in the intracellular Ca++ and potentiation of the effect of sympathetic stimulation on kallikrein release.

In the present study, we have also observed that pretreatment with phentolamine or prazosin almost completely blocked the release of kallikrein into blood observed after sympathetic stimulation. This indicates that the sympathetic control of kallikrein release into blood by the submandibular gland operates through activation of α1-adrenoreceptors.

The increase in blood flow that developed after sympathetic stimulation was discontinued must have been derived entirely from vasodilation of the arterioles in the gland, since no significant changes in mean arterial blood pressure were observed. This post-stimulation vasodilation can be attributed to the release of kallikrein into blood. The kinin output from the submandibular gland of the rat increases about 700-fold, 1 minute after the gland has been sympathetically stimulated (Scicli et al., 1983). This indicates that active kallikrein is released into the interstitial and vascular compartments of the submandibular gland upon adrenergic stimulation. The released kinins may, in turn, contribute to the vasodi-
latter response observed in the gland after sympathetic stimulation. Ten minutes after we discontinued stimulation of the sympathetic innervation, kallikrein released into the blood was 39-fold over the basal level. However, at this time, the blood flow to the gland decreased to values close to those observed before stimulation. This dissociation between kallikrein release and blood flow does not exclude the possibility that the kallikrein-kinin system contributes to the vasodilator response of the submandibular gland vasculature observed 1 minute after sympathetic stimulation. It is possible that the large amount of kallikrein released into the vascular compartment after stimulation rapidly consumed the kininogen available in the interstitial space. Another possibility is the desensitization of the kinin receptors. The massive release of kinins from kininogen would result in maximal receptor binding, prompting an alteration in the receptor so that it could no longer bind kinins. Such a desensitization has already been observed in the submandibular gland of the cat, following close arterial injection of large doses of bradykinin (Bhoola et al., 1965). A third possibility is that a vasopressor substance is released with kallikrein simultaneously after sympathetic stimulation, and that at any given time the glandular blood flow depends on the balance between these two substances with opposite effects. It is known that the submandibular gland of the rat is very rich in tonin, an enzyme which releases angiotensin II directly from angiotensinogen (Boucher et al., 1974; Garcia et al., 1976). In the submandibular gland, kallikrein and tonin are located within the same granular tubular cell (Ørstavik et al., 1982). Furthermore, it has also been reported that tonin is released into the vascular compartment upon sympathetic stimulation (Boucher et al., 1977).

Pretreatment with phentolamine blocked both the increase in the kallikrein release and the increase in glandular blood flow which develops after sympathetic stimulation. Prazosin, on the other hand, did not significantly affect the increase in blood flow in response to adrenergic stimulation, despite the fact that it did block 90% of the increase in kallikrein release. Sympathetic stimulation of the submandibular gland in animals pretreated with prazosin induced a 6-fold increase in kallikrein release into the vascular compartment (Table 1). This amount of kallikrein could have been enough to promote the increase in blood flow to the gland observed after sympathetic stimulation in rats pretreated with prazosin.

Ten minutes after stimulation of the sympathetic cervical trunk, the concentration of glandular kalli-

krene in arterial plasma increased 4-fold (Fig. 2, upper panel). This increase in glandular kallikrein in peripheral arterial blood occurred without significant changes in mean arterial blood pressure. Kallikrein which reaches the vascular compartment may be, at least in part, inactivated by protease inhibitors present in rat plasma (Hojima et al., 1977). Furthermore, kininases in the vascular bed of the lung and in plasma may break down the kinins which are being formed before they reach their receptors on the arterioles. We have recently reported that sympathetic stimulation of the submandibular gland does not affect the kinin concentration in arterial blood. However, if the kininase II inhibitor, captopril, is administered after the gland has been sympathetically stimulated, a pronounced hypotension develops which is accompanied by a marked increase in the concentration of kinins in arterial blood (Scicli et al., 1983).

In summary, our data indicate that, although parasympathetic stimulation produced an increase in glandular blood flow, it did not cause changes in kallikrein released into the blood. Sympathetic stimulation of the submandibular gland results in both increases in immunoreactive kallikrein released into the circulation, and in increased blood flow to the gland. The sympathetically stimulated release of immunoreactive kallikrein is mediated through stimulation of α1-adrenoceptors. Stimulation of the sympathetic innervation of the submandibular gland also increases the concentration of immunoreactive kallikrein in arterial plasma.

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