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Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: Functional significance of coexisting transmitters for vasodilation and secretion

(acetylcholinesterase/atropine-resistant vasodilation)

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ABSTRACT **By a combination of the indirect immunofluorescence technique with acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) staining, it was shown that vasoactive intestinal polypeptide (VIP) is present in cholinergic (acetylcholinesterase-rich) neurons involved in control of secretion and vasodilation in exocrine glands of cat. The submandibular salivary gland was used as a functional model. Preganglionic nerve stimulation induced an atropine-resistant, hexamethonium-sensitive vasodilation and release of VIP into the venous outflow from the gland and an atropine- and hexamethonium-sensitive secretion. Infusion of VIP antiserum reduced both the vasodilation and secretion. Infusion of VIP caused vasodilation only, whereas acetylcholine caused both vasodilation and secretion. Simultaneous infusion of VIP and acetylcholine in low doses resulted in a marked potentiation of both vasodilation and secretion. The present morphological and functional data support the following hypothesis for regulation of vasodilation and secretion in exocrine glands. Preganglionic cholinergic nerves activate, via nicotinic receptors, postganglionic neurons, causing concomitant release from the same nerve endings of two coexisting putative transmitters, acetylcholine and VIP. Acetylcholine produces mainly secretion by a muscarinic action and VIP causes mainly vasodilation, but the two substances seem to cooperate directly or indirectly in both types of response. Thus, the coexistence of two putative neurotransmitters, VIP and acetylcholine, in one neuron may explain the dual effector response (i.e., the cholinergic secretion and the atropine-resistant vasodilation) caused by nerve stimulation in exocrine glands.**

The vasodilation induced by nerve stimulation in exocrine glands—e.g., submandibular salivary gland (1), tongue (2), sweat glands (3), and nasal mucosa (4)—is largely atropine resistant, whereas secretion easily can be blocked by muscarinic antagonists. The occurrence of a subpopulation of vasodilatory nerves was first suggested, but later a kinin mechanism was suggested to be responsible for this vasodilation (5–9). The importance of a kallikrein–kinin activation has, however, been questioned (see refs. 10 and 11). Another possibility has recently been proposed: Immunohistochemical studies indicate that vasoactive intestinal polypeptide (VIP), a potent vasodilatory agent (12, 13) with a putative transmitter function (14, 15), is present in nerves in exocrine glands (16, 17). Furthermore in cats, VIP seems to be present in cholinergic neurons in several exocrine glands (18–20). It was therefore suggested that these nerves release both acetylcholine (AcCho) and VIP, causing secretion and vasodilation, respectively (18).

In the present paper we report further evidence for this hypothesis by demonstrating the presence of a VIP-like peptide in acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) (AcChoE)-rich neurons innervating several exocrine glands. Furthermore, pharmacological effects of VIP and AcCho as well as of various blocking agents, including VIP antiserum, on submandibular salivary secretion and vasodilation have been studied. Release of immunoreactive VIP into the venous outflow from the submandibular gland after nerve stimulation was also demonstrated.

MATERIALS AND METHODS

Ten cats (body weight 2–4 kg) of both sexes were used for immunohistochemical studies. In two of these cats the superior cervical ganglion was excised unilaterally 14 days before they were killed. In four other cats vinblastine sulfate (Sigma) (1% in 0.9% NaCl) was locally applied to the submandibular and sphenopalatine ganglia and to pancreas 24 hr before perfusion with ice-cold 10% (vol/vol) formalin (21). These ganglia and parts of the submandibular gland, the nasal mucosa, tongue, lung, and pancreas were then processed for indirect immunohistochemical studies according to Coons (22) as described (see ref. 18) by use of a VIP antibody (5603-5; for detail about this antiserum, see refs. 23 and 24) and AcChoE staining (18, 25, 26). In some cases, immunohistochemical analysis for VIP and AcChoE staining were performed on the same section. Alternatively, adjacent sections were processed for the respective procedure. The latter approach was used because Triton X-100 (added to VIP antiserum to enhance penetration of antibodies) inactivated AcChoE (18, 27). For details and validity of procedures and for controls, see ref. 18.

Physiological experiments were done in 15 cats anesthetized with chloralose (50 mg/kg) plus urethane (100 mg/kg) intravenously. The trachea was cannulated. The blood pressure was recorded via a femoral artery. Rectal temperature was kept constant at 38°C. The submandibular gland, its duct, and nerve supply were exposed. The duct was cannulated with a polyethylene tube (PE 50) and salivation was measured with a drop recorder. The chorda-lingual nerve was cut, and the peripheral cut end was mounted to a bipolar platinum electrode. Preganglionic stimulation was performed with square wave pulses of either 2 or 15 Hz, 8 V, and a duration of 1 msec. To measure the venous outflow from the gland, we introduced a polyethylene catheter (PE 160) into the external jugular vein after all branches, except the one draining the submandibular gland, had been tied. Blood flow was measured by a drop recorder and blood was reinfused into the cephalic vein with a peristaltic pump. Heparin (1000 IU/kg) was given intravenously. The

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Abbreviations: VIP, vasoactive intestinal polypeptide; AcCho, acetylcholine; AcChoE, acetylcholinesterase.

facial artery was cannulated for close intra-arterial infusions into the carotid artery. All branches of the carotid artery were ligated except the one supplying the submandibular gland. VIP (purified from porcine intestine by V. Mutt) and AcCho chloride (Sigma) were dissolved in 50 mM phosphate-buffered saline (pH 7.4) containing 0.5% bovine serum albumin and infused intra-arterially at a constant rate of 50 μl/min for 2 min. Atropine (0.5 mg/kg) and hexamethonium (20 mg/kg) were given intravenously 20 min prior to the various experiments. Finally, undiluted VIP antiserum was infused intra-arterially at a rate of 50 μl/min starting 10 min prior to and during low-frequency (2 Hz) stimulation. Normal rabbit serum infused under similar conditions served as control. As a further control for the specificity of the VIP antibody effects, the ipsilateral cervical symphatic trunk was isolated, transected, and stimulated in a cranial direction during infusion of VIP antiserum and control serum. The concentration of VIP in plasma (1.2 ml/sample) from the submandibular venous outflow was measured by radioimmunoassay (23, 24). To compensate for blood loss during sampling for radioimmunoassay, we infused Macrodex (Pharmacia) intravenously.

RESULTS

The morphological studies revealed that most neurons in local submandibular ganglia and almost all neurons in the sphenopalatine ganglion of normal and vinblastine-treated cats were both immunoreactive for VIP (Fig. 1 *A* and *B*) and rich in AcChoE (Fig. 1 *D* and *E*). This was also observed for local intramural ganglia of the tongue, bronchi, and pancreas. In untreated cats, the VIP-like immunoreactivity was found within small rounded structures in the perinuclear region, probably corresponding to the Golgi apparatus (Fig. 1*A*). Vinblastine pretreatment caused a marked increase in the amount of fluorescent material, which occupied the entire cytoplasm and extended into the cell processes (Fig. 1*B*). AcChoE activity was localized diffusely in the cytoplasm. Furthermore, both VIP-positive (Fig. 1*C*) and AcChoE-rich (Fig. 1*F*) nerve terminals with a similar distribution pattern were seen around the gland acini and ducts. Both VIP-immunoreactive and AcChoE-rich nerve endings were also seen around small blood vessels, mainly arteries and arterioles, in the gland tissues and in the skeletal muscle of the tongue. No definite effect on VIP-immunoreactive or AcChoE-rich nerve endings was seen after sympathectomy. None of the fluorescent structures described above was seen after incubation with VIP control serum; i.e., VIP antiserum pretreated with an excess of VIP (15 nmol per ml antiserum diluted 1:10). No AcChoE activity was observed in ganglion cells or nerve fibers after incubation with medium containing the specific AcChoE inhibitor BW284C51.

Supramaximal stimulation (15 Hz) of preganglionic fibers in the chorda-lingual nerve resulted in a more than 5-fold increase in blood flow from the submandibular gland accompanied by a marked salivary secretion (Fig. 2 *C* and *B*). Simultaneously, a marked increase in plasma VIP levels in the venous effluent from the gland was observed ($n = 4$). A typical example is seen in Fig. 2*E*, in which VIP increased from 2 pmol/liter to a peak of 60 pmol/liter after approximately 1.5 min of stimulation. Secretion was completely blocked by atropine, whereas vasodilation and VIP release were unaffected. Hexamethonium abolished all three responses (Fig. 2 *G*, *H*, and *J*) ($n = 3$). The plasma VIP levels and the vasodilation returned to normal within 5 min after cessation of the stimulation (Fig. 2 *E* and *C*).

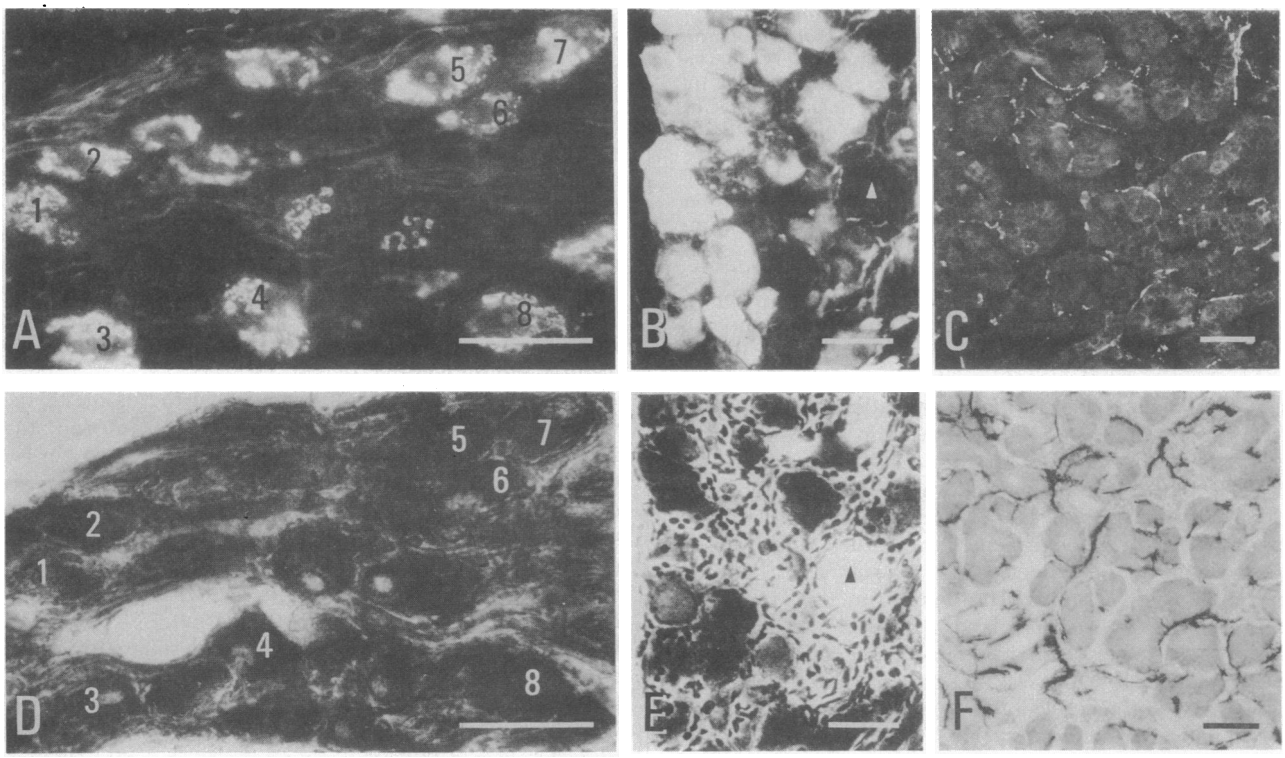

FIG. 1. Immunofluorescence and light micrographs of a local submandibular ganglion (*A* and *D*), the sphenopalatine ganglion (*B* and *E*), and the submandibular salivary gland (*C* and *F*) of the cat after incubation with VIP antiserum (*A–C*) and AcChoE staining (*D–F*). Incubation time for AcChoE staining was 20 min (*D*), 60 min (*E*), and 90 min (*F*). (*A* and *D*) Consecutive sections of an untreated submandibular ganglion; (*B* and *E*) same section of a vinblastine-treated sphenopalatine ganglion (section exposed to Triton X-100 during immunohistochemical incubation); (*C* and *F*) semiconsecutive section of a sympathectomized gland. In both ganglia (*A*, *D*, *B*, and *E*), the same cells are both VIP immunoreactive and rich in AcChoE (see areas 1–8 in *A* and *D*). Triangles in *B* and *E* indicate the same blood vessel. In *D* and *F*, numerous VIP-immunoreactive and AcChoE-rich nerve fibers with a similar distribution can be seen. Bars, 50 μm.

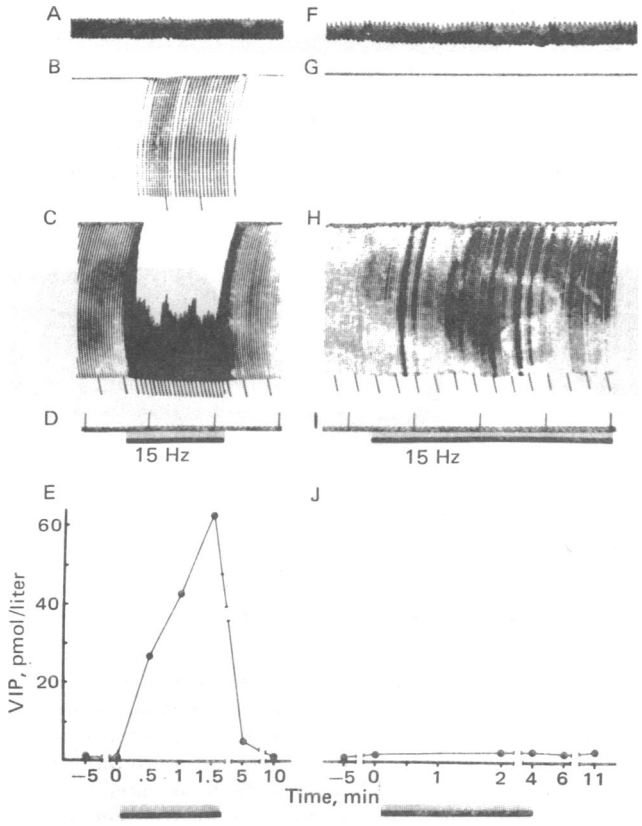

FIG. 2. Effect of supramaximal (15 Hz) chorda-lingual nerve stimulation on submandibular salivary secretion (*B*), blood flow (*C*), and VIP plasma levels measured in the venous outflow (*E*). *G*, *H*, and *J* show the effect of nerve stimulation (15 Hz) on the respective responses after hexamethonium pretreatment. In the recordings of secretion and blood flow, every 10th drop is separately marked. In *D* and *I*, the time scale unit is 1 min. Stimulation periods are marked under the time scales. The systemic blood pressure (*A* and *F*) was 130 and 110 mm Hg, respectively.

Low-frequency stimulation at 2 Hz induced a clearcut increase in blood flow and a salivary response. The blood flow showed a rapid initial transient rise followed by a more modest and maintained increase, which lasted the rest of the stimulation period (Fig. 3 *B* and *C*). Close intra-arterial infusion of undiluted VIP antiserum markedly reduced both phases of the vasodilation as well as secretion (Fig. 3 *G* and *F*) ($n = 3$). Close intra-arterial infusion of normal rabbit antiserum, on the other hand, did not affect the responses (Fig. 3 *B* and *C*).

The vasoconstrictory effect of sympathetic stimulation (6 Hz, 1 msec, 6 V for 1 min) in the gland was unaffected by infusion of VIP antiserum, indicating its selective effects on the chorda-lingual induced responses.

Local intra-arterial infusion of AcCho caused a dose-dependent vasodilation (25 pmol/min–25 nmol/min) and secretion (1.25–25 nmol/min). Both effects were blocked by atropine. VIP infusion in doses of 0.5 pmol/min–5 nmol/min caused marked atropine-resistant vasodilation but not salivary secretion. On a molar basis, VIP was at least 50 times more potent than AcCho as vasodilator.

Combined intra-arterial infusion of AcCho and VIP in low doses caused a marked potential of both secretion and vasodilation (Fig. 4), as compared to each substance alone. Atropine completely blocked the potentiation.

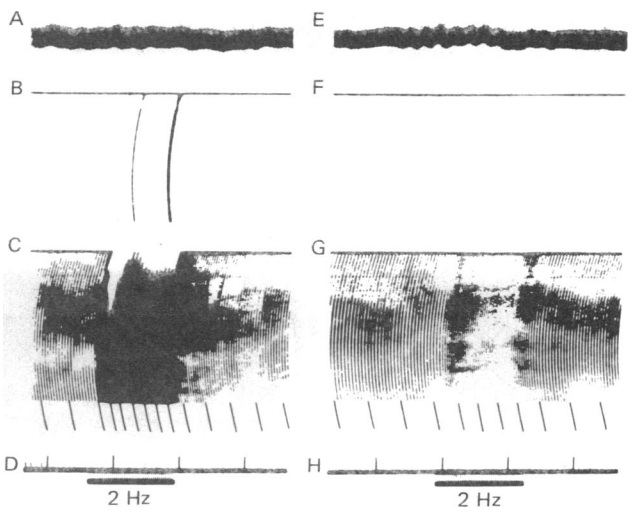

FIG. 3. Effect of low-frequency (2 Hz) chorda-lingual nerve stimulation on secretion (*B* and *F*) and blood flow (*C* and *G*) after infusion of normal rabbit serum (*B* and *C*) and of VIP antiserum for 10 min before and during stimulation (*F* and *G*). Recordings are as in Fig. 2. Time scales (*D* and *H*) denote every minute. Systemic blood pressure (*A* and *E*) was 140 mm Hg.

DISCUSSION

The present morphological analysis indicates that VIP is present in postganglionic cholinergic neurons innervating exocrine gland tissues, such as the submandibular salivary gland, nasal mucosa, tongue, and bronchi. Unfortunately there exists no reliable histochemical method for identification of cholinergic neurons. One approach is staining for AcChoE according to the classical principles of Koelle and Friedenwald (28), and in previous (18–20) and the present studies, VIP-like immunoreactivity and high AcChoE activity were present in the same cells. Although the validity of using AcChoE activity as a marker for cholinergic neurons can be questioned, there is evidence that this technique, when used on cat tissues and with short incubation times (27), preferentially visualizes cholinergic structures. This is supported by the good correlation between the proportion of AcChoE-rich and choline-acetyltransferase-containing cells in the L7 sympathetic ganglion of cat (29, 30). Furthermore, the fact that VIP-like immunoreactivity is present in practically all principal ganglion cells of a classically cholinergic ganglion, such as the sphenopalatine ganglion, further supports the hypothesis that VIP, in fact, is present in cholinergic neurons. (For a more detailed discussion of this topic, see ref. 18.)

The occurrence of VIP-like immunoreactivity has previously been demonstrated in nerve fibers of salivary glands (16), in the pancreas (17, 31, 32), and in nasal mucosa (33), as well as in cells and fibers in the tracheobronchial wall (33). Our findings clearly demonstrate VIP-immunoreactive ganglion cells in the cat pancreas, in contrast to a previous study (31).

The ultrastructural features of the presumed "VIP/AcCho" neurons in exocrine glands are uncertain. Larsson (34) and Polak and coworkers (35, 36) have identified VIP-immunoreactive nerve endings characterized by dense-core vesicles with a diameter up to 1600 Å. Such nerve endings were originally described with routine fixation procedures for electron microscopy in the gut by Baumgarten *et al.* (37). They were called "p-type" nerves due to their similarity to presumably peptide-containing fibers in the hypothalamus. However, such fibers have never been described in glandular tissues (38–42). Instead, the common feature of glutaraldehyde/OsO_4-fixed nerves in glands previously identified as cholinergic is the occurence of many small agranular (diameter about 500 Å) and a few large gran-

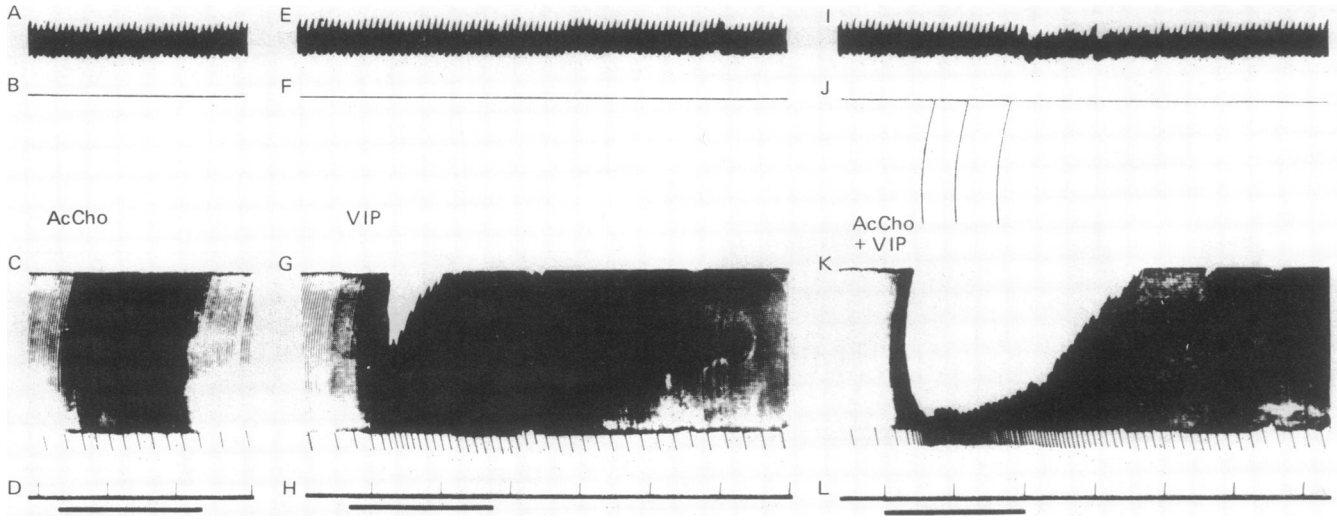

FIG. 4. Effects of close intra-arterial infusion of AcCho (250 pmol/min), VIP (100 pmol/min), and AcCho (250 pmol/min) plus VIP (100 pmol/min) on secretion (*B*, *F*, and *J*, respectively) and blood flow (*C*, *G*, and *K*, respectively). Recordings are as in Fig. 2. Time scales (*D*, *H*, and *L*) denote every minute. Systemic blood pressure (*A*, *E*, and *I*) was 130 mm Hg. Peak blood flow after onset of response was 1.8 ml/min (*C*), 3.0 ml/min (*G*), and 9.3 ml/min (*K*). Note the long duration of effect when VIP and AcCho were applied together (*K*).

ular vesicles (diameter 700–1000 Å) (39, 40). The presence of VIP-like immunoreactivity in large granular vesicles (34–36) suggests that the latter vesicles store the peptide, whereas AcCho is present in the small agranular vesicles (see refs. 43 and 44). It is interesting that Kidd and Garret (45) recently described nerve fibers in the submandibular gland particularly rich in dense-core vesicles with a size of about 1000 Å and speculated that they may be peptide storage sites.

The present physiological results demonstrate a pronounced release of immunoreactive VIP into the venous effluent from the submandibular gland upon preganglionic stimulation of the chorda-lingual nerve. The amount of VIP released is probably even larger than shown in this study because part of the VIP released may be inactivated postsynaptically by peptidases or removed by the lymph (46) and because dilution effects by the increase in blood flow were not accounted for. Nerve-stimulation-induced VIP release into venous outflow and increase in local blood flow have previously been demonstrated by Fahrenkrug *et al.* (15) in the cat colon during pelvic nerve stimulation.

VIP is most likely released from postganglionic neurons because the ganglionic blocking agent hexamethonium prevented the increase of VIP concentration in the venous outflow in addition to blocking secretion and vasodilation. Furthermore, the experiments with hexamethonium showed that the preganglionic neurons are cholinergic. These findings agree with data on VIP neurons in the gastrointestinal tract (47).

A matter of interest is the mode of release of the VIP-like peptide and AcCho from the same nerve endings. Are the substances released together or separately? The experiments of Beilenson *et al.* (48) and Darke and Smaje (49) show that salivation and vasodilation are simultaneously activated by nerve stimulation. This observation has also been made in the nasal mucosa (4), thus indicating a concomitant release of VIP and AcCho. This is further supported by the findings that botulinum toxin, thought to be a selective inhibitor of AcCho release (50, 51), inhibited both nerve-stimulation-induced secretion and vasodilation from the submandibular gland (6).

In the present study, a marked increase in blood flow and salivary secretion occurred concomitantly with VIP release. Whereas salivary secretion was completely blocked by atropine, vasodilation and VIP release were unaffected, indicating the importance of VIP as a regulator in the control of local blood flow. This is further supported by the marked vasodilation induced by intra-arterial infusion of VIP, an effect that could not be blocked by atropine. Furthermore, intra-arterial infusion of VIP antiserum markedly reduced both the initial rapid and the sustained vasodilation induced by low-frequency nerve stimulation. Although VIP may be of major importance for inducing a vasodilator response, other agents may also contribute. A kallikrein–kinin mechanism has been implicated (see ref. 9), and a role for AcCho in the rapid onset of vasodilation has also been proposed because atropine reduces this phase (49, 52). The importance of VIP for vasodilation in salivary glands has recently been pointed out in a parallel study on dogs (53).

Our findings and those of Shimizu and Taira (53) do not support a secretagogue effect of VIP by itself, not even when administered in very high doses. On the other hand, AcCho by itself can cause secretion. However, the intimate cooperation of these two agents is suggested by the marked potentiation of both the secretory and circulatory response observed when both substances are administered together. The reduction in nerve-stimulation-induced secretion observed after infusion of VIP antibodies is not fully understood. Several explanations are possible. Thus a proper blood flow may be a prerequisite for secretion. Alternatively, the antibody may interfere with a suggested concomitant release of VIP and AcCho. In conclusion, our experiments suggest that secretovasomotor responses are intimately coupled whereby VIP seems to be more important for vasodilation and AcCho for secretion. However, the exact mechanism by which AcCho and VIP interact, as well as their postsynaptic intracellular mediators, remain to be elucidated.

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