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AUTACOIDS IN THE GASTROINTESTINAL TRACT - PROTEASES, KININS, ACETYLCHOLINE AND VASOACTIVE INTESTINAL POLYPEPTIDE

by

DAVID J. LONGRIDGE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Date 16th Sept 85

DEDICATION

This thesis is dedicated to the memory of my father

ABSTRACT

Kallikrein, a serine protease, has been successfully localized in the gastrointestinal tract of the cat and the rat. Employing two immunocytochemical techniques, namely the peroxidase-antiperoxidase and the avidin-biotin complex techniques, specific staining was confined to the goblet cells and in some cases the brush border. Several related enzymes were used to raise a series of antibodies. Antibodies raised against cat salivary kallikrein, rat intestinal kallikrein and rat pancreatic kallikrein yielded specific staining. Employing antibodies raised against the related proteases, acrosin and coagulating gland kallikrein failed to yield a specific staining pattern. Parasympathetic stimulation (pilocarpine) caused movement of kallikrein and the associated mucus out of crypt goblet cells of the intestine. Goblet cells in the intestinal villi were unaffected by pilocarpine.

The effects of acetylcholine, bradykinin and vasoactive intestinal polypeptide (VIP) and various muscarinic antagonists were examined on atropine-resistant vasodilatation in the cat submandibular gland. VIP is a powerful vasodilator in the submandibular gland of the cat and its effects can be reduced by avian pancreatic polypeptide (APP), or by desensitization of the gland's blood vessels to VIP. However, the vasodilatation caused by parasympathetic nerve stimulation is not reduced by either of these means. Despite atropine-resistance, the vasodilatation induced by parasympathetic nerve stimulation could be reversibly inhibited by the muscarinic antagonists, 4-diphenyl acetoxy-N-methyl piperidine (4-DAMP) and pentamethylene-bis-4-diphenyl acetoxy-N-methylpiperidine (bis 4-DAMP), both of which act on M_2 muscarinic receptors. The muscarinic antagonists, secoverine and pirenzepine, were ineffective in reducing parasympathetic nerve-induced vasodilatation. Experiments with a potentiator of acetylcholine, eserine, and with a depleter, hemicholinium, suggest that acetylcholine may indeed play a major role in parasympathetic nerve-mediated vasodilatation. Substance P and ATP were neither potent nor consistent vasodilators, and thus appear to be unlikely mediators.

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SECTION A:

KALLIKREINS IN INTESTINE AND COLON

1. INTRODUCTION

1.1 Historical Background

The kallikreins (EC 3.4.21.34 and EC 3.4.21.35) are a group of serine proteases present in an active and/or inactive but activatable form in various glandular organs as well as in blood, lymph and urine. The kallikreins in glands are called tissue or glandular kallikreins. Both plasma and tissue kallikreins release a peptide, kinin, by limited proteolysis from a substrate, kininogen, that is present in lymph, plasma, interstitial fluid and urine. Kinins have marked actions on blood vessels and smooth muscles *in vivo* and *in vitro*. In recent years, it has been suggested that some of the physiological significant actions of these enzymes may be independent of their ability to release kinin (see Schachter, 1980).

The discovery of kallikrein can be regarded as having begun between 1926-1928 when Frey and his coworkers demonstrated that human urine contains a substance that produces a prolonged fall of arterial blood pressure of the dog after intravenous injection. This substance was characterized as being of high molecular weight, nondialyzable and thermolabile (Frey, 1926; Kraut *et al.*, 1928).

The early workers, seeking the source of this vasodepressor substance in urine, found a similar substance in blood and pancreas (Frey and Kraut, 1928; Kraut *et al.*, 1930). They assumed that the substance in urine and blood originated from the pancreas, and named it kallikrein, from "kallikreas", a Greek word for pancreas (Kraut *et al.*, 1930).

During the 1930's, Werle and his colleagues described most of the known components of the kallikrein-kinin system, and they characterized the kallikreins in pancreas, urine, blood and salivary glands (Frey *et al.*, 1950; Werle, 1960).

In 1937, Werle *et al.* demonstrated that kallikreins released a substance enzymatically from an inactive precursor in plasma and that the released substance contracted various isolated smooth muscle preparations. They named this substance DK, from "darmkontrahierende Substanz". In 1948, Werle and Berek recommended that substance DK be renamed kallidin, and

that its precursor, the substrate for kallikrein, be called kallidinogen.

In 1949, Rocha e Silva *et al.* described the release of an active peptide by trypsin and certain snake venoms from a substrate in plasma. They named the active peptide bradykinin. It is now apparent that the trypsin- or snake venom-bradykininogen-bradykinin system described by Rocha e Silva is very similar to the kallikrein-kallidinogen-kallidin system described earlier by Werle.

In the early 1960's, Webster and Pierce (1960, 1961, 1963) demonstrated that kallikrein of plasma differs significantly from the other kallikreins of tissue origin with respect to proteolytic action and behaviour towards inhibitors.

Subsequently, Habermann and Klett (1966) determined a much higher molecular weight of 97,000 for plasma kallikrein compared to reported molecular weights of between 33,000 and 36,000 for kallikreins from pancreas, urine and the submandibular gland (Fritz *et al.*, 1967).

Plasma and tissue kallikreins were given the Enzyme Commision Number EC 3.4.21.8, signifying that they are closely related serine proteases. The results of many studies on plasma and tissue kallikreins have demonstrated that whilst they are both kininogenases, they have in fact, markedly different physical, enzymological and immunological properties. In 1981, the Enzyme Commission distinguished these two groups of kallikreins by renumbering them 3.4.21.34 and 3.4.21.35 for plasma and tissue kallikreins respectively.

1.2 Tissue, or Glandular Kallikreins — Distribution and Overview

1.2.1 Salivary Gland Kallikrein

Kallikrein was first demonstrated in the salivary glands of mammals by Werle and Roden (1936, 1939). Kininogenase activity of the various salivary glands of different species was measured using a kininogen substrate prepared from dog plasma. The concentrations of kallikrein were much higher in the submandibular than in the parotid or sublingual gland.

Werle (1960) described a wide species variation of kallikrein concentration in the submandibular gland, the greatest concentration being present in the rat. Bhoola and Dorey (1971) subsequently confirmed the highest kininogenase activity in the rat and mouse submandibular gland, moderate levels in the guinea-pig, rat and hamster, and the least in the dog and rabbit (Bhoola and Cogdell, 1974). Very low concentrations of kallikrein were found in the submandibular glands of lamb and sheep using sheep kininogen as substrate, and less in the parotid gland (Beilenson, 1967).

Since the early work of Werle, salivary kallikreins from cat, mouse, rat, pig, guinea-pig and man have been isolated and characterized (Moriwaki *et al.*, 1976; Fukuoka *et al.*, 1979; Porcelli *et al.*, 1976; Lemon *et al.*, 1979; Fiedler *et al.*, 1982; Hofmann *et al.*, 1983; Gautvik *et al.*, 1980). Kallikreins purified from salivary glands or saliva, in general, resemble (both biochemically and in inhibition profile) other tissue kallikreins purified from the same species.

Evidence for the ductal localization of salivary kallikrein was independently provided in 1975 for the cat and rat (Barton *et al.*, 1975; Ørstavik *et al.*, 1975). In a series of experiments, Schachter *et al.* (1977) demonstrated a 90-95% depletion of kallikrein from the cat submandibular gland following sympathetic nerve stimulation. They also correlated the decrease in kallikrein content with a depletion of granules in the cells of the striated ducts (Schachter *et al.*, 1977).

Ørstavik *et al.* (1975, 1980, 1980a) and Simson *et al.* (1979) have localized kallikrein in the apical region of the striated duct cells of the submandibular and parotid salivary glands of man and within the granular tubule cells of the submandibular gland of the rat, employing both immunofluorescent and immunoperoxidase techniques. Kallikrein was not found in the acinar cells and/or in the cells of the intercalated ducts. The same location has been confirmed in salivary glands of the cat, guinea-pig, mouse and dog (Hojima *et al.*, 1977; Maranda *et al.*, 1978; Schachter *et al.*, 1980). Subsequently, Schachter *et al.* (1983), using an immunoperoxidase method adapted for electron microscopy, showed that kallikrein is specifically located in the apical ductal granules of the cat submandibular gland.

More recently, Kimura and Moriya (1984) employed both enzyme (histochemical) and immunohistochemical techniques, demonstrating that the enzymatic and antigenic activities of kallikrein were in the same locations, viz., in the apical region of the ductal cells of the human parotid gland. No kallikrein activity, enzymatic or antigenic, was detected either in acini or in intercalated ducts, which is in agreement with the earlier work of both Schachter's and Ørstavik's groups. However, Kimura and Moriya also reported kallikrein-like activity in the basement membrane region of acini and of the ductal system.

Employing an enzyme histochemical technique by which the synthetic substrate D-Val-Leu-Arg-4-methoxy-2-naphthylamide (MNA) is preferentially cleaved by glandular kallikrein, Garrett *et al.* (1982, 1984) localized active kallikrein in granular tubule cells of the rat and hamster submandibular gland and in the striated duct cells of the same gland in man, guinea-pig, cat and dog. The parotid gland showed less reactivity and none was detectable in this gland of the hamster and guinea-pig.

1.2.2 Pancreas Kallikrein

Kallikrein in the pancreas was first characterized by Werle *et al.* in 1937. It was found to occur in an inactive form (Werle *et al.*, 1937; Fielder and Werle, 1967) and was secreted into the duodenum mainly as the proenzyme, where it was activated by enterokinase (Werle and Eckey, 1934; Werle and Urhahn, 1940; Werle *et al.*, 1955). Studies on freeze-dried pancreas revealed that a significant amount of kallikrein was present in an inactive form. The amount of active kallikrein was small and probably arose from the readily activatable nature of the pro-enzyme.

Porcine pancreatic kallikrein is the most extensively characterized of the pancreatic kallikreins (Fiedler, 1979) and has been found to exist in three molecular forms, kallikrein A, B and C. The difference in the molecular forms is due to differences in the carbohydrate component, kallikrein B containing the most, kallikrein C the least (Fritz *et al.*, 1977). Fritz *et al.* (1977) also state that porcine pancreatic kallikreins A, B and C are immunologically identical

and possess molecular weights in the range 27,000 to 29,000.

Pancreatic kallikrein has also been purified and characterized from the rat (Hojima *et al.*, 1975, 1977). From amino acid composition data, Hojima *et al* (1975, 1977) postulated a molecular weight of 31,000. Recently, Swift *et al.* (1982) demonstrated that the cloned mRNA sequences for pancreatic kallikrein encode a precursor kallikrein containing a predicted secretory prepeptide, an activation peptide and an active form of kallikrein. The polypeptide molecular weights of the predicted prepro-, pro-(zymogen) and active forms of the enzyme are 29,200, 27,300 and 26,100 respectively.

Numerous studies have been performed to localize pancreatic kallikrein. In 1978, Dietz *et al.* and Ørstavik and Glenner independently demonstrated via immunofluorescent techniques the presence of kallikrein in granule-containing luminal portion of pancreatic acinar cells of the pig and rat respectively. Subsequently, Ørstavik and coworkers (1980a, 1981) reported the acinar localization of kallikrein in the human pancreas, which agreed with the previous reports on the rat and pig. Lechene de la Porte *et al.* (1981) also reported the acinar localization of kallikrein in the human pancreas at the electron microscopic level employing an immunoferritin method. None of these studies has provided evidence for the presence of kallikrein in endocrine cells. By contrast, ole-Moi Yoi *et al.* (1979) and Pinkus *et al.* (1983) reported that kallikrein was present in both the β cells of the islets as well as the acinar cells. Employing methods of differential cell separation within the pancreas, Schachter and Fujimori (unpublished observations) were unable to detect kallikrein-like activity within the islet cell population.

1.2.3 Renal/Urinary Kallikrein

In 1926, Frey found a nondialyzable substance in urine that had a hypotensive effect on intravenous injection in the dog. He later named this material kallikrein and suggested that this enzyme might be produced by the pancreas (Frey *et al.*, 1930). It has since been assumed that the kininogenase activity in urine originates in the kidney rather than the pancreas (Fiedler, 1979). However, recently within our laboratory, Fujimori *et al.* (1985) have been able to purify

a kininogenase in cat urine, but have been unsuccessful in purifying a related enzyme from the kidney. Perhaps the cat is an exception.

The kallikreins of kidney have been purified from dog and rat kidney and similar ones from the urine of horse, rabbit, rat, pig, man (Fiedler, 1979; Fritz *et al.*, 1982) and cat (Fujimori *et al.*, 1985). These enzymes are all acidic glycoproteins with isoelectric points near 4.0 and derived molecular weights of 25,000 to 48,000. Urinary kallikrein also seems very similar to the kallikrein found in salivary glands, pancreas and gastrointestinal tract of the same species (Fujimori *et al.*, 1985). Differences in the forms of urinary kallikrein are due, in part at least, to differences in the charged carbohydrate moieties (Chao and Margolius, 1979).

The localization of renal kallikrein is of much interest and a variety of biochemical and immunocytochemical techniques have been employed. Renal kallikrein is confined almost entirely to the cortex with very small amounts in the medulla. Isolated glomeruli contain a very small proportion of the total kallikrein present in the cortex. Most of the kallikrein has been localized to the luminal surface of the distal convoluted tubule of the rat kidney, beginning at a point distal to the juxtaglomerular apparatus and ending where the tubule ends in the collecting duct (Ørstavik *et al.*, 1976; Ørstavik and Inagami, 1982). Simson *et al.* (1979) reported the presence of kallikrein in the distal convoluted tubule and in portions of the duct systems near the tips of the renal medulla. In the microdissected nephron of the rabbit, kininogenase activity has been localized in the connecting tubule (Omata *et al.*, 1982), whereas immunoreactive kallikrein has been reported in broader distribution in the microdissected rat distal nephron (Proud *et al.*, 1983).

Recently, Figueroa *et al.* (1984) localized kallikrein in the connecting tubule cells of the rat at the subcellular level. They reported that kallikrein was concentrated mainly in the upper one-third of the cell and at both sides of the nuclei, and to a lesser extent is associated with the plasma membranes and basolateral infoldings. The immunoreactive material was related to free polyribosomes, the rough endoplasmic reticulum and the Golgi complex, suggesting that kallikrein is actively synthesized in this particular type of cell.

1.2.4 Stomach Kallikrein

In 1980, a kallikrein-like enzyme was first reported to be present in the rat stomach (Uchida *et al.*, 1980). Uchida *et al.* purified an enzyme with a calculated molecular weight of 29,000 that was inhibited by both diisopropylfluorophosphate and Trasylol, but not by trypsin inhibitors from soybean, lima bean and ovomucoid. However, Uchida *et al.* reported a pH optimum at pH 11, which is significantly higher than the often quoted pH optima of rat tissue kallikreins of pH 8-9. This enzyme also displayed weak kininogenase activity on heat-treated rat plasma.

Later, Uetsuji *et al.* (1982) reported the purification of human stomach kallikrein with a molecular weight of 38,000, and a similar pH optimum and inhibitor spectrum as the afore-mentioned rat stomach kallikrein.

In a series of papers, Kobayashi and coworkers (Kobayashi *et al.*, 1979; Kobayashi and Ohata, 1981, 1982, 1983, 1984) have described a kinin-forming enzyme in rat stomach mucosa. However, this enzyme exhibited a pH optimum of 4.5 to 5.5 and was regarded as being similar to cathepsin D, an acidic protease. In an independent study, Muto *et al.* (1983) also purified and characterized a cathepsin D-like enzyme from the rat stomach mucosa.

1.2.5 Small Intestine Kallikrein

Werle (1960) reported that the small intestine of mammals contained a trypsin-activatable hypotensive substance. This observation was later confirmed by Amundsen and Nustad (1965), who demonstrated the presence of a small but distinct kinin-forming capacity in isotonic saline extracts of rat intestinal mucosal cells. The presence of kinin-forming activity in hypotonic and hypertonic aqueous extracts of rat intestinal tissue has also been reported by Zeitlin (1970). Subsequently, Zeitlin (1971, 1972) demonstrated that the kallikrein from auto-lyzed rat small intestine resembles a glandular kallikrein with respect to substrate specificity and inhibitor spectrum. Later, Zeitlin *et al.* (1976) reported a molecular weight for rat intestinal kallikrein of 33,000, which was later confirmed by Moriwaki *et al.* (1980). Moriwaki *et al.*

demonstrated that rat intestinal kallikrein exists in two molecular forms, RIK-A and RIK-B, which may differ in their carbohydrate content. Moriwaki *et al.* concluded that both molecular forms of rat intestinal kallikrein exhibit properties of other tissue kallikreins.

1.2.6 Colon Kallikrein

In the same report in which Werle (1960) described a kallikrein in the small intestine, he also described a similar enzyme in the colon of man, rat, dog and cat which was activatable by trypsin. Subsequently, Seki *et al.* (1972) reported that a kallikrein extracted from the colon of rat, pig, monkey, dog and man was similar to plasma kallikrein. They reported that this enzyme existed in a pre-form, was readily activated by trypsin, and released bradykinin from human kininogen. The molecular weight of human colon kallikrein was 71,000 and it exhibited an inhibition profile characteristic of plasma kallikrein. However, Zimmermann *et al.* (1979) found that human colon kallikrein resembled human urinary kallikrein in its chemical and immunological properties, suggesting human colonic kallikrein is similar to other tissue kallikreins.

Recently, Schachter *et al.* (1983) localized kallikrein in the goblet cells of cat and man using immunocytochemical techniques. Subsequently, Fujimori *et al.* (1985) reported the purification and characterization of cat colon kallikrein. Cat colon kallikrein has an estimated molecular weight of 40,000, is a potent kininogenase, and exhibits an inhibition profile similar to other tissue kallikreins purified from the submandibular gland, pancreas and urine of the cat, but is quite different from cat pancreatic trypsin.

1.2.7 Accessory Reproductive Organ Kallikrein

In 1962, Bhoola *et al.* demonstrated the existence of a potent kininogenase in the accessory sex glands of the guinea-pig. The highest concentration of this enzyme was found in the coagulating gland, about half this concentration in the prostate, and one-fiftieth in the seminal vesicles. Later, Moriwaki and Schachter (1971) partially purified and characterized this

enzyme, which they named coagulating gland kininogenase (CGK). This kininogenase was not inhibited by the usual plasma and glandular kallikrein inhibitors. Subsequently Barton *et al.* (1973) showed that CGK was present almost exclusively in the nonparticulate, cytoplasmic fraction of homogenates of the gland based on differential centrifugation. Using antibodies raised to purified CGK, Schachter *et al.* (1978) localized kallikrein diffusely in the cells lining the crypts of the coagulating gland.

Recently, Lazure *et al.* (1984) described a partial amino acid sequence of a protease in the dog prostate which they suggest belongs to the kallikrein family of enzymes. This protease was found to be an Arg-esterase that was inhibited by diisopropylfluorophosphate, which is a potent inhibitor of all serine proteases. However, Bhoola *et al.* (1962) previously demonstrated that the dog prostate does not contain a kininogenase-like enzyme, in that extracts failed to release kinin.

1.2.8 Brain Kallikrein

The existence of kallikrein in the brain remains uncertain. However, some kinin-generating activity has been detected in homogenates of rabbit (Hori *et al.*, 1969; Hori, 1968) and rat brain (Shikima *et al.*, 1973). Highest activities were noted in cerebral cortex, with less in the brain stem and cerebellum. Recently, Chao *et al.* (1983), using monoclonal and poly-clonal antibodies against rat urinary kallikrein, established that the brain contains and is capable of synthesizing a kallikrein indistinguishable from purified tissue kallikrein. Simson *et al.* (1984) reported diffuse low level immunocytochemical staining within the anterior pituitary and the hypothalamus of the rat.

1.2.9 Pituitary Kallikrein

Rat pituitary has been shown to contain kininogenase activity which is highly concentrated in the intermediate lobe. This kininogenase was shown to release bradykinin from kininogen substrate and cleaved chromogenic substrates which are more specific for tissue

kallikrein (Powers and Nasjletti, 1983). In a later study, Powers and Nasjletti (1984) showed that the female anterior pituitary of the rat contains over eighteen times more kininogenase activity than the male anterior pituitary, but that kininogenase activity within the intermediate lobe showed no major sex difference.

In an earlier study, the same authors describe a kininogenase in the porcine anterior pituitary (Powers and Nasjletti, 1982). These findings have been confirmed by other workers who demonstrated that the kallikrein-like enzyme in the porcine pituitary exhibited similar properties to those kallikreins which had previously been purified from the submandibular gland, pancreas and urine of the pig (Polivka *et al.*, 1982).

1.2.10 Blood Vessel Kallikrein

Recently it has been shown in the rat, ox and man that an acidic protease present in cardiac tissue is able to release kinin from kininogen, and in some ways appears to be similar to tissue or glandular kallikrein but quite distinct from plasma kallikrein (Nolly *et al.*, 1982; Britos and Nolly, 1981).

In two later studies, the same authors demonstrated the presence of at least two enzymes in blood vessels, one of which was an acidic protease and the other a neutral protease with physicochemical characteristics quite similar to tissue kallikrein (Nolly and Lama, 1982, Nolly *et al.*, 1982). These authors termed this latter enzyme 'vascular kallikrein'.

Subsequently, Nolly *et al.* (1984) suggested that the kallikrein-like enzyme which they detected in the mesenteric arteries may be due to contamination from the pancreas. They also describe the presence of a kininogenase within rat tail arteries and veins which is distinct from plasma kallikrein but similar, if not identical to, glandular kallikrein.

1.2.11 Tissue or Glandular Kallikrein in Plasma

The existence of immunoreactive tissue kallikrein in rat plasma was first demonstrated by Nustad *et al.* (1978). Subsequently, these findings have been confirmed in the rat and

extended to man, and it was demonstrated that the rat salivary glands may be a major source of the immunoreactive enzyme in the rat circulation (Lawton *et al.*, 1981; Rabito *et al.*, 1982, 1983; Shimamoto *et al.*, 1984). Ørstavik *et al.* (1980b) demonstrated the transport of radio-labelled rat submandibular gland kallikrein into the venous circulation. They concluded that kallikrein present in the duct lumen or in the interstitium is able to reach the circulation, thereby making possible the local generation of kinins by kallikrein.

Lawton *et al.* (1981) demonstrated that following bilateral submandibular/sublingual gland excision in rats, the amount of tissue kallikrein in the circulation was reduced significantly, suggesting that the salivary glands may be a major source of immunoreactive tissue kallikrein in plasma. They also suggested that the pancreas is unlikely to be a major source of this immunoreactive tissue kallikrein in plasma. Following acute nephrectomy, immunoreactive tissue kallikrein in the circulation increased six-fold, suggesting that the kidney plays an important role in the clearance and/or metabolism of glandular kallikrein from plasma.

1.2.12 Other Tissue Kallikrein-Like Enzymes

Lokshina *et al.* (1976) were the first to characterize in bovine spleen, a kininogenase with an optimum pH in the neutral range. Subsequently, Chao *et al.* (1984), using monoclonal antibody-affinity chromatography, have successfully identified and purified a kallikrein-like enzyme in the rat spleen. They demonstrated that rat splenic tissue kallikrein was indistinguishable from rat urinary kallikrein in its physicochemical and immunological properties and in its subceptibility to inhibitors.

The presence of a kallikrein-like enzyme in cat nasal secretion has been demonstrated (Eccles and Wilson, 1973). They concluded that the incubation of cat nasal secretion and plasma releases a kinin-like substance. More recently, Proud *et al.* (1983) have demonstrated the *in vivo* generation of kinins (lys-BK and BK) within the nasal airways following allergen challenge to allergic human subjects. However, contamination from saliva cannot be discounted.

Recently, Uchida *et al.* (1982) reported the purification and characterization of rat thyroid kallikrein. They showed that this kallikrein had kininogenase activity, a molecular weight estimated at 30,000, and an inhibitor profile similar to other rat tissue kallikreins, although it exhibited a pH optimum at pH 11. However, Levison and Schachter (unpublished observations) were unable to demonstrate a kininogenase in the rat thyroid.

Fox and Hilton (1958) reported that human sweat releases a kinin from dog plasma and also that kinins appear in a subcutaneous perfusate if the body temperature is raised. Werle (1960) also reported the presence of a kininogenase in sweat. However, Schachter (unpublished observations) demonstrated that the ability of human sweat to release kinin is slight and inconsistent.

The presence of a kallikrein-like substance in lung tissue was first mentioned by Werle and Maier (1955). Subsequently, Havez (1966) and Havez *et al.* (1966) also concluded that kallikrein is present in bronchial mucosa.

Earlier work by Malofiejew (1973) suggested that although reproductive tissue contains no free kinins, kininogenase activity could be detected within human placenta, myometrial tissues and amniotic fluid. Subsequently, Marin-Grez *et al.* (1982) described a tissue kallikrein-like and also a trypsin-like enzyme in rat uterus.

1.3 Plasma Kallikrein-Kinin System

As described previously (1.1), plasma kallikrein is distinct from tissue kallikreins. In plasma, kallikrein exists as a precursor, prekallikrein. That such an inactive precursor exists in plasma is evident from the fact that high molecular weight kininogen, a substrate of plasma kallikrein, exists in high concentrations in plasma, whereas only minute quantities, if any, of free kinins, can be detected. Once kallikrein is activated in plasma, it converts a large fraction of the kininogen to bradykinin in a matter of minutes (Colman and Bagdasarian, 1976).

Human plasma kallikrein has been purified and characterized (Colman and Bagdasarian, 1976; Heber *et al.*, 1978). For the hydrolysis of synthetic substrates and

generation of bradykinin from high molecular weight kininogen, human plasma kallikrein exhibits a pH optimum of 7.6, which is significantly lower than that for tissue kallikreins (usually around pH 9.0). Molecular weight estimations yield a value of 99,800 for human plasma kallikrein, which is much higher than the reported values for human tissue kallikreins (Colman *et al.*, 1969).

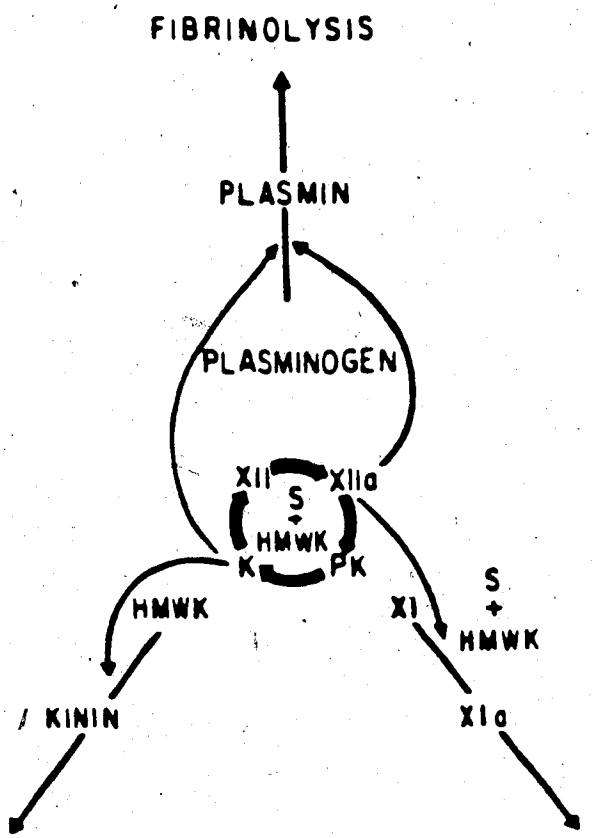
Several reports have suggested that both human plasma kallikrein and high molecular weight kininogen play a central role in the initiation of the intrinsic-coagulation pathway by activating Factor XII (Hageman factor). Plasma kallikrein also mediates fibrinolysis via activation of plasminogen, either directly or indirectly.

Some of the probable relationships between the kallikrein-kinin system in plasma and the blood coagulation system are illustrated in Figure 1 (Cochrane *et al.*, 1970, 1973; Colman and Bagdasarian, 1976; Ogston and Bennett, 1978).

In 1973, Wuepper discovered an inherited deficiency of plasma prekallikrein, which has been named Fletcher factor after the patient in whom the condition was first described by Hathaway *et al.* (1965). Individuals with Fletcher factor deficiency have *in vitro* defects in coagulation (intrinsic system), in fibrinolysis, in kinin generation, and in chemotactic properties of their plasmas; however, these individuals do not have either coagulation or other hemostatic problems (Hathaway *et al.*, 1965; Wuepper, 1973). All these defects are corrected by the addition of prekallikrein which activates preHageman factor.

In addition to prekallikrein deficiency, there are now known inherited deficiencies of HMW kininogen, known as Flaujeac, Williams or Fitzgerald trait (Saito *et al.*, 1975; Wuepper *et al.*, 1975; Colman *et al.*, 1975). These patients also have little or no hemostatic problems *in vivo.*, but their plasmas show abnormalities in surface-mediated coagulation, fibrinolytic and kinin-generating pathways.

Figure 1. Diagram showing surface-mediated reactions in the blood coagulation system in man and possible sites of participtation of plasma prekallikrein, kallikrein, and high molecular weight kininogen. (Modified from Ogston and Bennett, 1978)



XII:	Hageman factor (factor XII)	K:	Kallikrein
XIIa:	Activated Hageman factor	PK:	Prekallikrein
XI:	Factor XI	S:	Surface
XIa:	Activated factor XI	HMWK:	High molecular weight kininogen

1.4 Kininogens

Kininogens are plasma proteins which contain the peptide sequence of the kinin polypeptides, bradykinin and lysyl-bradykinin. These peptides are released from their precursors by limited proteolysis by both plasma and tissue kallikreins. For the measurement of kallikrein or kininogen, it is important to prepare kininogen free of prekallikrein, circulating inhibitors of kallikrein and of kininases. Werle *et al.* (1937) achieved this by heat-treating plasma at 56°C to 60°C for up to 3 hours. Subsequently Diniz and De Carvalho (1963) developed a simple method for the estimation of kininogen in plasma that has been used for many years.

In 1966, Jacobsen reported that mammalian plasma contains at least two kininogens which differ in molecular weight. The high molecular weight (HMW) form of kininogen is the substrate for plasma kallikrein, while the low molecular weight (LMW) form of kininogen is the preferential substrate for tissue kallikrein. Limited proteolysis of LMW kininogen by tissue kallikrein liberates the kinin moiety and the residual protein consists of two disulphide-linked chains, a heavy chain of about 62,000 molecular weight and a light chain of about 4,000 (Muller-Esterl *et al.*, 1982). The heavy chain corresponds to the amino-terminal part of the original kininogen molecule and the light chain corresponds to its carboxyl-terminal part.

High molecular weight kininogen (MW 120,000) plays an important role in the initiation of Hageman factor-dependent (Factor XII) pathways of blood coagulation and fibrinolysis (see Schachter, 1980) (Figure 1).

The biological function of low molecular weight kininogen, apart from acting as a substrate for tissue kallikrein, is not well understood, but it may well play a role in blood pressure regulation (Overlack *et al.*, 1979) and in the control of cellular glucose uptake (Wicklmayr *et al.*, 1979). Recently, Ohkubo *et al.* (1984) demonstrated that LMW kininogen can act as an inhibitor of thiol proteases (cathepsins and papain). Since the release and regulation of thiol proteases is of significance in mediating inflammatory responses, it is interesting to speculate as to whether LMW kininogen is involved in such inflammatory responses.

Kininogen has been known for some time to be present in lymph, urine and interstitial fluid as well as in plasma (Schachter, 1980). Pisano *et al.* (1978) demonstrated that under neutral conditions, kinin could be generated in urine by human urinary kallikrein. These results indicated the presence of a kinin-containing protein, which was named urokininogen. Human kininogen has subsequently been localized in the cytoplasm of the distal tubule cells and collecting tubule cells as well as in the glomerular basement membranes by an indirect immunofluorescence technique (Proud *et al.*, 1981).

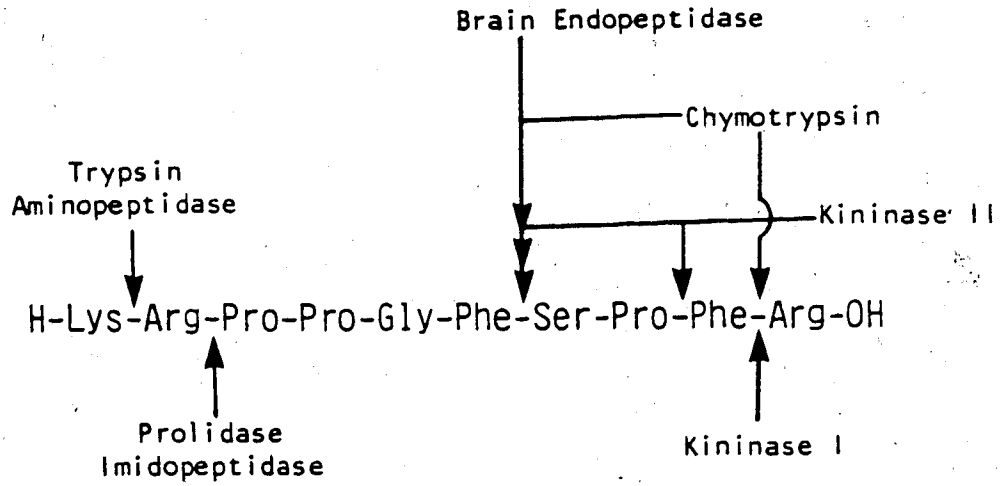
1.5 Kinins

Kinins are peptides very widely distributed in the animal kingdom. They are present in many species including reptiles, amphibia, insects (wasp venom), avian plasma and mammalian plasma (Schachter and Thain, 1954; Bertacini, 1976). Kinins belong to a group of vasoactive peptides that can be generated in blood and in various tissues by the action of proteolytic enzymes such as kallikrein. They exist in nature, therefore, in free or peptide form, or as a large precursor released by specific proteolysis.

As described in a recent review (Regoli and Barabe, 1980), all known kinins have structures which are very closely related, e.g. the three mammalian kinins are bradykinin, lys-bradykinin (or kallidin) and met-lys-bradykinin. In all cases, the common bradykinin backbone (see Figure 2) is present. Non-mammalian kinins exhibit more pronounced variation such as in Thr-bradykinin (Dunn and Perks, 1970; Ishikawa *et al.*, 1974), and Val^1, Thr^6-bradykinin (Nakajima, 1968), both of which are common to frogs. Polisteskinin-R and ranakinin-R possess the sequence Ser^6-bradykinin (Ishikawa *et al.*, 1974) and are common to insects and frogs respectively.

Werle *et al.* (1937) were the first to observe that kallikrein released a substance from serum that caused contraction of isolated intestinal smooth muscle. Roche e Silva *et al.* (1949) observed the same phenomenon for trypsin and snake venoms.

Figure 2. Primary structure of kallidin and of bradykinin. Site of action of proteolytic enzymes.



1.5.1 Bioassay

Kinins have a number of pharmacological properties in common. For over three decades, kinins were tested for their stimulant effects on isolated intestinal smooth muscle preparations, such as the guinea-pig ileum (Roche e Silva, 1952) and cat ileum (Erspamer and Erspamer, 1962), where they induce a rapidly reversible change in tension. The guinea-pig ileum is as sensitive to kinins as the cat ileum but is not as selective (Ferreira and Vane, 1967). The guinea-pig ileum appears to contain large amounts of active kininase since the effect of bradykinin is potentiated by inhibitors of kininase II (Rubin *et al.*, 1975, 1978). The major advantage of the cat ileum is that it appears to be devoid of active kininase II since the effects of bradykinin are not modified by inhibitors of kininase II (Barabe and Regoli, 1980).

The isolated rat uterus in estrus is very sensitive to kinins. The major advantage of the rat uterus to kinins is its high sensitivity compared to the isolated intestines (Erspamer, 1948; Barabe *et al.*, 1975).

Kinins possess marked hypotensive effects when injected *in vivo*, producing a rapidly reversible fall of blood pressure that is due to arteriolar vasodilatation. The vascular effects of kinins (particularly bradykinin) vary markedly depending upon the species studied, the dose of kinin applied, and the interference by other endogenous vasoactive substances (Regoli and Barabe, 1980). Kinins are potent stimulators of both arterial and venous vascular smooth muscle *in vitro* (Elliott *et al.*, 1960). One of the most prominent actions of kinins is on the pre-capillary vessels, where they increase vascular permeability (see review by Haddy *et al.*, 1970).

A number of other isolated smooth muscle preparations have also been used for bio-assay of kinins, namely stomach strips of rat (Barabe *et al.*, 1975) and hamster (Moussatche and Dalo, 1982).

1.5.2 Immunological Assay

Radioimmunoassay systems have been developed to quantitate virtually every peptide hormone or mediator available in pure form. Talamo *et al.* (1969) were the first to describe a

radioimmunoassay procedure for the determination of kinin levels in plasma. In 1976, Carretero *et al.* described a method for measuring urinary kallikrein activity using a radioimmunoassay to kinin. Talamo and Goodfriend (1979), however, concluded that the results of radioimmunoassays for kinins are not necessarily indicative of the *in vivo* state because of the large complications during the collection of the blood samples and during the assay *in vitro*. Among the features of the kinin system that complicate its radioimmunoassay are (1) the weak immunogenicity of kinins, (2) the absence of an easily labelled residue in the native kinins necessitating the use of analogues which contain tyrosine, easily iodinated, but antigenically distinct from other endogenous polypeptides, (3) the immunologic similarity of related kinins, Lys-bradykinin, Met-lys-bradykinin, and (4) the cross-reactivity of bradykinin antibodies with kininogen.

1.5.3 Mode of Action

Kinins are positively charged peptides because of the presence of Arg- or Lys- residues in their sequences and are considered to be too large and too hydrophilic to permeate cell membranes. Therefore, they have been thought to produce their biological effects by interacting with receptors localized at the cell surface of target organs. After the interaction of kinins with their receptor, a sequence of cellular events occurs culminating in a response. Cellular processes that might be part of a sequence triggered by the bradykinin-receptor interaction and that might lead to such responses as muscle contraction include: movement of ions across the cell membrane, prostaglandin synthesis or catabolism, and synthesis or degradation of cyclic nucleotides.

Perris and Whitfield (1969) demonstrated that bradykinin increased the mitotic activity of rat thymocytes and that this response was calcium-dependent. An involvement of prostaglandins in the action of bradykinin upon the longitudinal muscle of the rat isolated ileum was reported by Crocker and Willavoys (1976). According to Malone and Trottier (1973), the contraction of the rat isolated uterus in response to bradykinin is partially mediated by

prostaglandins because the effect is reduced in the presence of indomethacin. McGiff *et al.* (1976) provided convincing evidence that prostaglandins are mediators or modulators of renal excretory functions induced by bradykinin.

Reissmann *et al.* (1977) reported that increases in membrane-bound adenylate cyclase activity may be implicated in response of rat duodenum to bradykinin.

1.5.4 Receptor Subclasses

Kinins appear to act on at least two different receptor types, denoted B_1 and B_2. After the demonstration of the existence of at least two receptor types, the B_1 receptor in the rabbit aorta (Regoli *et al.*, 1977) and the B_2 receptor in the cat terminal ileum and the rat uterus (Barabe *et al.*, 1977), various organs have been found to contain both B_1 and B_2 receptors. The criteria for distinguishing B_1 and B_2 receptors include the relative insensitivity of B_1 receptors to bradykinin, and enhanced sensitivity of B_1 receptors to synthetic des-Arg^9-BK (Regoli and Barabe, 1980).

1.6 Kininases

Many tissue homogenates, biologic fluids, plant and bacterial extracts, and certain proteolytic enzymes can cleave kinins. Bradykinin is hydrolyzed by as many as seven different enzymes, of which kininase I and II are the most extensively studied (see Figure 2).

1.6.1 Distribution of Kininases

The lung has been reported as the major site of bradykinin degradation, where more than 90% of the circulating bradykinin is inactivated during a single passage (Dietze, 1982).

Enzymes present in the vascular endothelium are believed to render the kinins inactive (Ryan *et al.*, 1968). However, it has been demonstrated that if the pulmonary circulation is by-passed, bradykinin is still inactivated by circulating kininases as well as by membrane-bound tissue kininases (Ellison *et al.*, 1980).

Kininase I was first discovered in Cohn fraction IV of human plasma (Erdös and Sloane, 1962). Subsequently, related kininases have been shown to be present in amniotic fluid, liver, spleen and lungs of rat (Rybak *et al.*, 1971; Petakova *et al.*, 1972). Kininase activity in rat and rabbit brain have been extensively studied, with the highest concentration being reported in the cerebellum (Iwata *et al.*, 1969; Oliveira *et al.*, 1976).

A greater number of publications deals with the distribution of kininase II. Kininase II is present in a soluble form in plasma and other body fluids such as urine and lymph (Erdös, 1979). Kininase II also occurs in membrane-bound form in vascular endothelial cells, epithelial cells of proximal, convoluted and straight renal tubules, but not within the distal tubule (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). The glomerular vascular tuft, the juxtaglomerular apparatus and all blood vessels within the kidney are free of kininase II immunoreactivity (Defendini *et al.*, 1983). Kininase II has been purified from the brush border of human and porcine intestine (Ward *et al.*, 1980) and localized to the apical surface of the epithelial cell membrane of the human duodenum, jejunum and ileum (Defendini *et al.*, 1983). Defendini *et al.* (1983) failed to detect kininase II in the stomach and colon.

1.6.2 Site of Action on Kinins

Kininase I, also named arginine carboxypeptidase (EC 3.4.17.3), cleaves the Phe^1-Arg^9 bond to inactivate bradykinin (see Figure 2). Kininase II, also named peptidyl dipeptidase and angiotensin I converting enzyme (EC 3.4.14.1), is known to cleave the peptide angiotensin I to the active angiotensin II as well as acting on bradykinin at the Pro^7-Phe^8 bond to release the Phe^8-Arg^9 dipeptide and thus inactivating bradykinin (see Figures 2 and 3). The angiotensin converting enzyme was first discovered in the mid-1950's, when it was found that horse serum contains an enzyme which converts angiotensin I to angiotensin II (Skeggs *et al.*, 1954, 1956).

Earlier, renin was found to release the decapeptide angiotensin I from angiotensinogen, and the decapeptide is in turn converted to the octapeptide angiotensin II by the removal of the histidyl-leucine dipeptide from the C-Terminal end (Braun-Menendez *et al.*, 1940; Page and

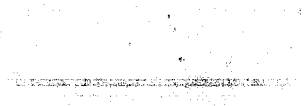
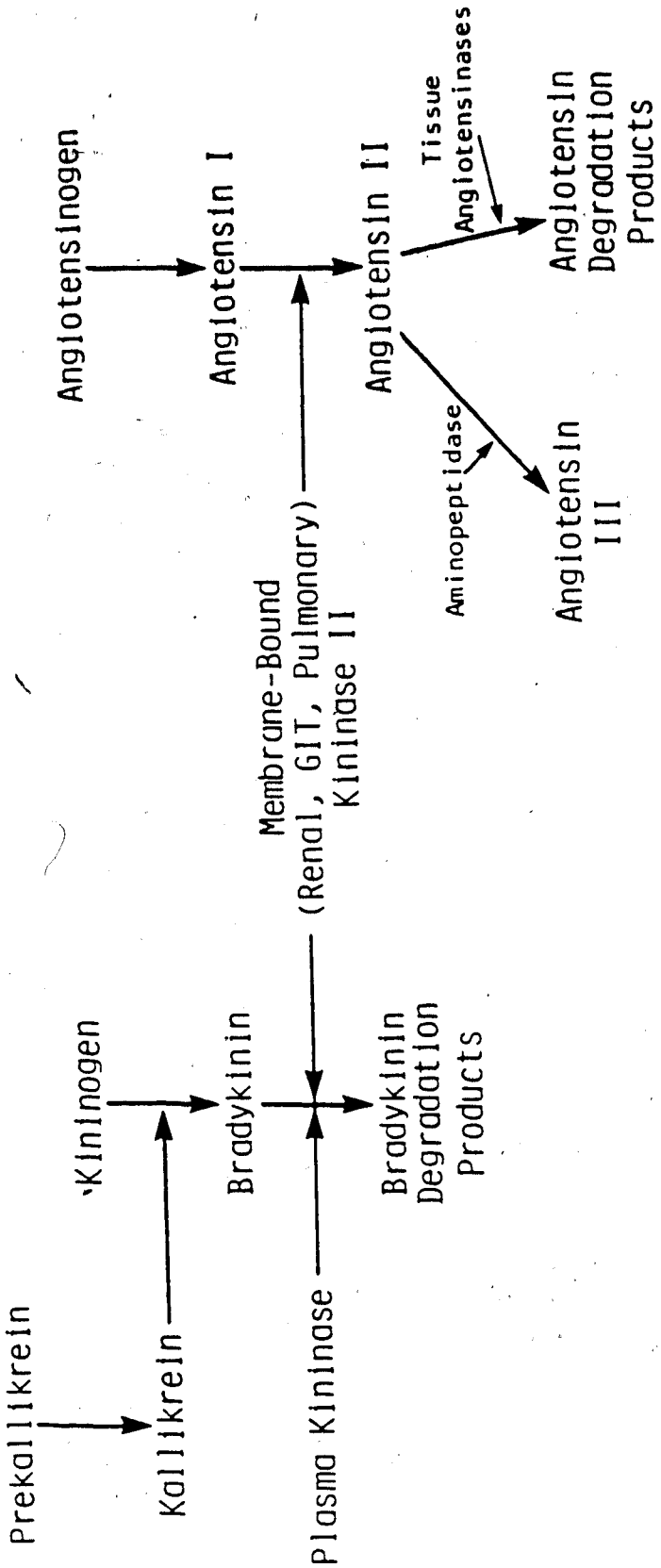


Figure 3. Diagram of two protease pathways that share angiotensin-converting enzyme (kininase II).


Angiotensinogen
Angiotensin I
Angiotensin II
Tissue Angiotensinases
Angiotensin Degradation Products
Aminopeptidase
Angiotensin III
Membrane-Bound (Renal, GIT, Pulmonary) Kininase II
Kininogen
Bradykinin
Bradykinin Degradation Products
Prekallikrein
Kallikrein
Plasma Kininase

Melmon, 1940). Ng and Vane (1967) were the first to demonstrate the conversion of angiotensin I to angiotensin II in the pulmonary circulation. Bradykinin was also shown to be inactivated rapidly in the pulmonary vascular bed (Ferreira and Vane, 1967). Following much confusion, it was finally established that kininase II and angiotensin I converting enzyme were one and the same enzyme (Yang *et al.*, 1970).

It is clear that kininases have a multiplicity of functions, and their ubiquitous distribution in biologic fluids and tissues implies that they affect numerous biologic activities.

1.7 Aim of the Present Study

The aim of the present study was to localize kallikrein immunocytochemically throughout the gastrointestinal tract. As previously mentioned (Sections 1.2.5 and 1.2.6), kallikrein-like enzymatic activity has been described in the mammalian gastrointestinal tract, but the exact location of the enzyme is unknown.

Employing two immunocytochemical techniques, multiple antisera preparations and a number of fixation methods, it was hoped that kallikrein could be localized in the gastrointestinal tract and that its localization would provide insight into its possible roles in gastrointestinal physiology and pathology.

2. MATERIALS AND METHODS

2.1 Animals and Dissection

Adult cats of either sex were starved overnight and allowed water *ad libitum*. Cats were anesthetized with sodium pentobarbitone (30 mg kg^{-1} i.p.) (M.T.C. Pharmaceuticals), or with chloralose (80 mg kg^{-1} i.v.) (Sigma) after induction with chloroform and ether. The abdomen of the cat was shaved and following a midline incision, the duodenum was located in the abdominal cavity. Following blunt dissection to free the duodenum from the surrounding mesentery, the duodenum was clamped and a section removed and cut into small pieces ready for fixation.

Adult Sprague-Dawley rats of either sex were starved overnight in an elevated cage bottom and allowed water *ad libitum*. Rats were anesthetized with sodium pentobarbitone (45 mg kg^{-1} i.p.). The abdomen of the animal was shaved and following a midline abdominal incision, the small intestine and descending colon were located. Following blunt dissection to free the surrounding mesentery, the jejunal section of the rat small intestine (approximately 15 cm distal to the pancreatic ducts) was excised following double clamping and ligation of the mesenteric blood supply. The same procedure was followed for excision of the descending colon. Following its removal, the tissue was cut into small pieces for fixation.

For stimulation experiments in rats, the above procedure was followed except that prior to any surgical procedure the animals were injected intraperitonally with pilocarpine HCl (20-100 mg kg^{-1} in 0.9% saline) (Sigma). After a period of 15-20 minutes, the jejunum located and excised as described previously. Pilocarpine is known to be a potent sialagogue, and this was taken as being a sign that secretion within the small intestine had occurred.

2.2 Tissue Fixation and Embedding

A variety of tissue fixation methods were employed, including freeze-drying and liquid-based fixatives. The major fixatives used will be described here. In some cases, a number of different concentrations of fixative were employed, and thus the fixative concentration range will be given.

2.2.1 Freeze-Drying

Samples of rat descending colon and rat jejunum (15 cm distal to the pancreatic ducts) were removed, cut into small pieces (less than 1 mm^3), blotted with filter paper and quick frozen on a copper block freezing device maintained at the temperature of liquid nitrogen (Coulter and Terracio, 1977).

Tissue pieces (still frozen) were then freeze-dried in an Edwards-Pearse tissue freeze drier at -70°C for 24-48 hours. They were then divided into smaller pieces and embedded directly into vacuum-treated Spurr's resin (Polysciences Inc.) (Spurr, 1969) without vapor fixation stage for 24 hours at 60°C.

Samples of cat duodenum, 4-5 cm distal to the pancreatic duct, were cut into small pieces (less than 1 mm^2), blotted with filter paper and rapidly frozen by the copper block method. Freeze-drying was carried out in an all-glass device designed for low temperature freeze-drying, as previously described by Coulter and Terracio (1978) (Ladd Research Industries). The tissue was freeze-dried for 36-40 hours, then fixed for 3 hours with osmium tetroxide (Polysciences Inc.) vapor. Following fixation, the tissues were embedded directly into vacuum-treated Spurr's resin for 24 hours at 60°C.

2.2.2 Liquid-Based Fixatives

A number of liquid-based fixatives were employed, some of which will be described here.

Samples of rat descending colon and rat jejunum were washed with saline and fixed in (a) 0.5%-2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 1-3 hours at 4°C, (b) 0.5%-1.0% glutaraldehyde (Analychem Corp. Ltd. or J.B. EM Services Inc.) and 1-2% paraformaldehyde (Fisher Scientific Ltd.) in 0.1 M sodium cacodylate buffer, pH 7.2, for 1-3 hours at 4°C, and (c) modified periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) according to Reissig *et al.* (1978), 0.01 M periodate, 0.075 M lysine, 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 3 hours at 4°C.

Samples of rat jejunum, following pilocarpine treatment, were also fixed in the above fixatives. The tissues were then washed for 2-3 hours with at least three changes of the fixative buffer at 4°C. Tissues were then dehydrated in an ascending alcohol series (30%, 50%, 70%, 90%, 95% and 100% twice for 15 minutes) and two changes of propylene oxide (B.D.H. Chemicals) at 4°C. Following the propylene oxide stage of dehydration, a 1:1 ratio of resin:propylene oxide mixture was used for 24 hours to aid infiltration of the resin. Tissues were then embedded in capsules filled with either Araldite or Epon epoxy resins (Analychem Corp. Ltd.) (see Hayat, 1970) and polymerized at 60°C for 48 hours.

2.3 Sectioning and Etching

Semi-thin resin sections were used throughout this study. Sections measuring 1.5 μm thick were cut on a Reichert-Jung ultracut microtome (Sargent-Welch Scientific Co.) using glass knives. Sections were picked up using a pick-up loop and dried on once or twice gelatin-coated glass slides on a hot plate. Gelatin coating ensures that the sections do not float off during the experimental procedure. Spurr's, Araldite and Epon epoxy resins were removed by treating the slides for 45-90 minutes with freshly prepared saturated sodium ethoxide (Aldrich Chemical Co.). In some cases, etching was carried out using NaOH pellets in absolute ethanol as described by Lane and Europa (1965). Following etching, the slides were taken through two changes of absolute ethanol for 15 minutes each, two changes of distilled water for 15 minutes and finally into 0.01 M. Tris buffer at pH 7.4.

For those tissue sections fixed in osmium tetroxide methods are available for removing osmium from semi-thin as well as ultrathin sections. These slides were exposed to a 10% aqueous solution of hydrogen peroxide for 10-15 minutes at room temperature. Exposure to hydrogen peroxide also aids the removal of endogenous pseudoperoxidase activity.

2.4 Antisera and Controls

2.4.1 Pure Antigens

Pure cat salivary kallikrein (CSK), purified according to Fukuoka *et al.* (1979) was kindly donated by the late Dr. C. Moriwaki. Pure CSK showed high esterolytic activity towards tissue kallikrein substrates, high kininogenase activity, and yielded a single band on disc electrophoresis.

Rat pancreatic kallikrein (RPK) and rat intestinal kallikrein (RIK), purified according to Hojima *et al.* (1975, 1977) and Moriwaki *et al.* (1980), were also kindly provided by Dr. Moriwaki.

Two other related enzymes were also donated by Dr. Moriwaki. These were guinea-pig coagulating gland kininogenase (CGK) and boar acrosin, purified according to Moriwaki *et al.* (1974) and Kaneko and Moriwaki (1981), respectively.

2.4.2 Antisera

Antibody to pure cat salivary kallikrein was obtained from serum of New Zealand white rabbits 15 days after three consecutive weekly injections of pure cat salivary kallikrein in 0.9% saline emulsified with complete Freund's adjuvant (Gibco Laboratories) for the first injection and with incomplete adjuvant for the other two (Hojima *et al.*, 1977). Immunodiffusion tests yielded single precipitation arcs between immune serum and pure antigen (Ouchterlony, 1958). Blood obtained from rabbits via ear vein puncture was centrifuged at 3000 rpm for 15-20 minutes and then the sera supernatant was divided into 1.0 ml aliquots and deep frozen

(-20°C) until used.

A similar procedure was followed for the preparation of antisera to purified rat intestinal kallikrein (RIK), rat pancreatic kallikrein (RPK), guinea-pig coagulating gland kininogenase (CGK) and boar acrosin.

2.4.3 Controls

Three types of control antisera were employed in the experiments described: (a) Control normal rabbit serum (NRS) was obtained from the same rabbit prior to immunization with antigen. (b) Normal rabbit serum was obtained from rabbits which were not immunized. (c) The third control antisera involved the use of hyperimmune antiserum raised to related but immunologically distinct antigens, such as coagulating gland kininogenase and boar acrosin. The lack of specific immunocytochemical staining would suggest that the reaction seen with αCSK, αRIK and αRPK antisera was due to specific antigen-antibody binding and not some non-specific attraction between certain cellular components and the specific antibody.

2.5 Immunocytochemistry

Two immunocytochemical techniques were employed to localize kallikrein in the gastrointestinal tract. A modified unlabelled antibody peroxidase-antiperoxidase (PAP) method (Sternberger, 1979) and avidin-biotin complex (ABC) technique originally developed by Hsu *et al.* (1981a).

2.5.1 Peroxidase-Antiperoxidase

Following etching of semi-thin resin-embedded sections, sections were incubated in normal goat serum (Miles Lab Inc.) diluted 1:30 in 0.05 M Tris buffer, pH 7.6, for 30 minutes at 20°C. Sections were then washed in 0.05 M Tris buffer, blotted with filter paper and covered with either immune or normal rabbit serum diluted 1:1000-1:20,000 in 1% goat serum in 0.05 M Tris buffer. Incubations were carried out in humidity chambers for 36-72 hours at 4°C.

Sections were rinsed with buffer, incubated for 30 minutes with goat anti-rabbit immunoglobulin (IgG) Fab fragments, diluted 1:20-1:100 in 0.05 M Tris buffer (Miles Lab Inc.), rinsed with buffer and incubated for 30 minutes with rabbit peroxidase-antiperoxidase complex (PAP) (Cappel Laboratories), diluted 1:100 in 0.05 M Tris buffer. Following a further rinse in buffer, sections were incubated with a freshly prepared and filtered (Millipore Ltd.) solution of 0.025% 3,3'-diaminobenzidine-tetrahydrochloride (Sigma) in distilled water to which 0.01% H_2O_2 was added, until a brown reaction product appeared. Slides were rinsed with distilled water, dried on a hot plate and mounted via Permount (Fisher Scientific Co.) In some cases, counterstains were used after the immunocytochemical procedure (see Section 2.7).

2.5.2 Avidin-Biotin Complex

Avidin is a 68,000 molecular weight glycoprotein which has an extraordinarily high affinity (10^{-15} M^{-1}) for the small molecular weight vitamin, biotin. Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin, unlike antibody-antigen interactions, is essentially irreversible.

The "ABC" procedure for localizing a variety of antigens was recently developed by Hsu *et al.* (1981a, b, c). This technique employs primary antibody, biotinylated secondary antibody and a preformed avidin:biotinylated horseradish peroxidase complex ("ABC"). A similar procedure was employed using a Vectastain ABC kit (rabbit IgG) which is commercially available (Vector Laboratories, Inc.).

Following etching of semi-thin resin-embedded sections, sections were incubated in normal goat serum, washed, blotted and covered with primary antibody as described for the peroxidase-antiperoxidase techniques. Following incubation with primary antibody, sections were rinsed with buffer and incubated with biotinylated goat anti-rabbit IgG, 1:200 in 0.05 M Tris buffer for 30 minutes at 20°C. Avidin (Reagent A) was prepared as a 1:100 dilution in 0.05 M Tris buffer, to which was added biotinylated horseradish peroxidase (Reagent B), also to a final dilution of 1:100. Reagent A and Reagent B were incubated together for 30 minutes

at 20°C. Sections were subsequently washed with buffer and incubated with Reagent AB for 30 minutes at 20°C. Following a further rinse in buffer, sections were incubated with 3,3'-diaminobenzidine, washed with water, dried and mounted via Permount as previously described (Section 2.5.1).

2.6 Microscopy and Photography

A photomicroscope II (Carl Zeiss Oberkochen) was used for light microscopy and tissues were photographed with Panatomic X black and white (Kodak Canada Inc.). Printing of black and white negatives was on Ilfo-speed 4 or 5 paper.

2.7 Histological Staining and Counterstaining

As the background in the peroxidase-antiperoxidase and avidin-biotin complex techniques is often barely visible on semi-thin resin sections stained for light microscopy, counterstaining is of help in establishing the relationship with surrounding tissues or a structure that has been stained brown by diaminobenzidine.

Several counterstaining procedures were employed: Celestin blue B (Matheson, Colman and Bell Manufacturing Chemists) and eosin Y (British Drug Houses (Canada) Ltd.) were prepared according to methods described by Snodgrass *et al.* (1972). Following immunocytochemical staining, sections were stained with Celestin blue B for 5-8 minutes at 20°C and rinsed well with distilled water (three changes of 5 minutes each). Sections were then counterstained with eosin Y for 1-5 minutes, washed with distilled water, dried on a hot plate for 10-15 minutes and cover-slipped via Permount.

Fast green was also used as a counterstain. Following immunocytochemical staining, sections were stained in 0.2-0.3% fast green (Fisher Scientific Co.) in 95% ethanol for 5 minutes at 20°C. Sections were then washed with distilled water (three changes of 5 minutes each) and dried on a hot plate for 10-15 minutes and cover-slipped via Permount.

Periodic acid Schiff's technique (aqueous) was employed as a histological stain for mucus-containing cells in the gastrointestinal tract. Periodic acid was prepared as described by Humason (1979). Schiff's reagent was purchased from Fisher Scientific Company. All sections were etched with sodium ethoxide (see Section 2.3) and oxidized in aqueous 0.6% periodic acid for 5 minutes, washed in running water for 5 minutes, then treated with Schiff's reagent for 10 minutes. Sections were then rinsed in 0.5% sodium metabisulphite (Fisher Scientific Co.) (three changes for 2 minutes each), washed in running water for 10 minutes, dried on a hot plate for 10-15 minutes and cover-slipped via Permount.

3. RESULTS

3.1 Immunocytochemical Localization of Kallikrein in Goblet Cells of Rat Colon

Kallikrein has been localized in goblet cells of the rat descending colon (Figure 4) using two immunocytochemical techniques, namely the peroxidase-antiperoxidase (PAP) and avidin-biotin complex (ABC) techniques (Figures 5 and 6). All goblet cells are known to exist in a number of different phases of maturation (Freeman, 1966). Kallikrein-like immunoreactivity was observed in (a) pre-secretory goblet cells, (b) intermediate goblet cells, (c) mature goblet cells and also during periods of parasympathetic (pilocarpine infusion) stimulation seen to be expelled from (d) evacuated goblet cells.

The stained material within the goblet cells often appeared granular in nature. The organelles are difficult to study at the light microscopic level since in the mature goblet cell, the theca, the apical region distended with mucigen droplets tends to flatten the nucleus and surrounding organelles. In some cases, the brush border showed intense staining (Figure 5a). Secreted material stained strongly and could often be observed in the colonic lumen (Figure 5a). No specific staining was associated with columnar epithelial cells, both on the surface and in the crypts. Specific staining was not present in the lamina propria, muscularis mucosa, submucosa, muscle layers and associated blood vessels. Mast cells, which are known to be interspersed throughout the lamina propria, did not stain.

The colonic crypts appear to be somewhat "compressed". This is due to the fixation procedure (freeze-drying), which requires the mucosal surface of the tissue to strike the cold copper block and thus causes some compression. Employing an aldehyde liquid-based fixative (0.5% glutaraldehyde, 1% paraformaldehyde), an identical staining pattern was observed with regard to the goblet cells and brush border; however, the immunoreactive material within the goblet cells had a smooth homogenous appearance, i.e. non-granular. Of the tissue fixation methods employed in this study, freeze-drying afforded the best compromise, by which antigenicity is intact while maintaining structural integrity.

Figure 4 Immunocytochemical localization of kallikrein in goblet cells (G) of the rat colon. Semi-thin resin sections incubated with αCSK antiserum (1:1000) for 60 hours PAP technique. Tissue was freeze-dried by copper block method (Edwards-Pearse freeze drier). G = goblet cells, L = colonic lumen, C = colonic crypt, b.v. = blood vessel. Magnification X300.

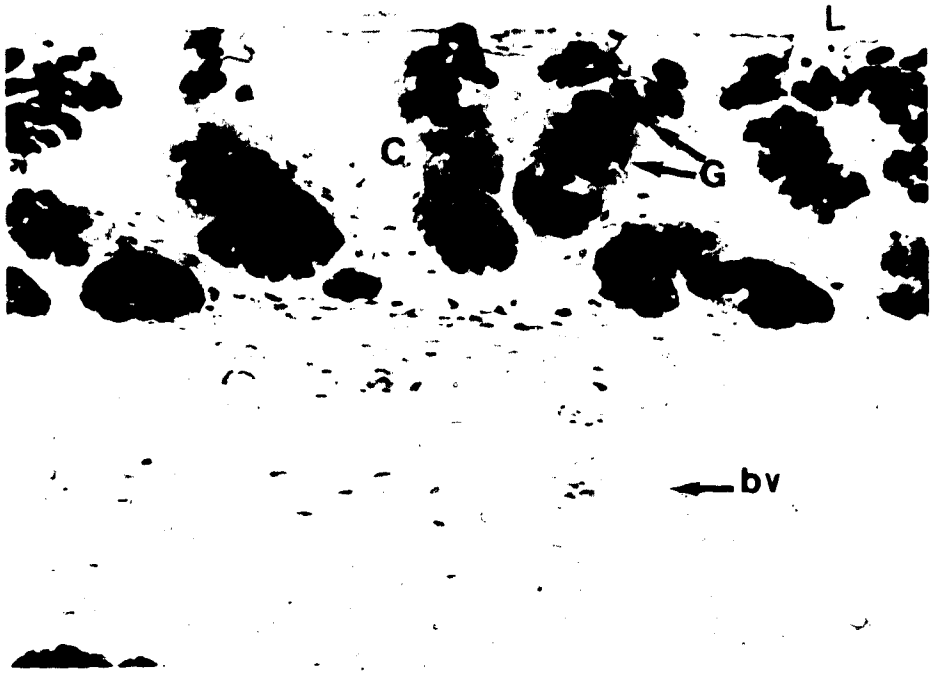


Figure 5a. Immunocytochemical localization of kallikrein in goblet cells of rat colon. As Figure 4. Arrows = goblet cells, L = lumen. Magnification x570.

Figure 5b. Control section for Figure 5a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification x570.

Figure 6a. Immunocytochemical localization of kallikrein in goblet cells of the rat colon. Semi-thin resin sections incubated with αCSK antiserum (1:1000) for 40 hours. Avidin-biotin peroxidase technique. Tissue was freeze-dried by copper block method (Edwards-Pearse freeze-drier). Arrows = goblet cells, L = lumen. Magnification X570.

Figure 6b. Control section for Figure 6a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification x570.

A comparison of two immunocytochemical techniques (Figures 5 and 6) reveals that both the PAP and ABC methods gave similar results. The ABC method provides a quicker method since the primary antisera incubation step was usually 48 hours or less, compared to 60 to 72 hours for the PAP method. Background staining for the ABC method was often higher than that for the PAP method. Primary antisera dilutions for the ABC method were often higher than that for the PAP method, 1:20,000 to 1:10,000. For the PAP method, primary αCSK antisera dilutions in excess of 1:10,000 resulted in a very weak or frequently negative result. However, for the ABC method, primary αCSK antisera dilutions in excess of 1:20,000 resulted in a very weak result. The use of avidin-biotin peroxidase complex not only shortened primary antisera incubation time and afforded greater sensitivity, but also increased the staining intensity both on immune and background staining on control sections.

Using antisera raised against rat intestinal kallikrein and rat pancreatic kallikrein gave similar staining patterns as that observed for cat salivary kallikrein antiserum (Figures 7 and 8). This suggests that,immunogenically at least, the kallikrein which has been localized in rat colon is related to rat intestinal kallikrein, rat pancreatic kallikrein and cat salivary kallikrein. Again, high antisera dilutions (up to 1:20,000) showed specific staining which was gradually reduced upon higher dilution.

Using antisera raised against coagulating gland kallikrein (CGK) and acrosin failed to yield specific staining, but gave a background non-specific staining intensity similar to control sections using normal rabbit serum (Figures 9 and 10). These findings suggest that rat colon kallikrein is immunogenically distinct from CGK and acrosin. However, enzymatically and chemically, they may show some similarities, since it is known that both CGK and acrosin are kininogenases, which is also true of the kallikrein in rat colon. The relative potencies of CGK and acrosin differ considerably: CGK is very potent and acrosin, being similar to trypsin, is a weak kininogenase (Schachter, 1980). The use of hyperimmune antisera raised against related enzymes and their failure to demonstrate a similar staining pattern to that of αCSK, αRIK and αRPK provides evidence that the reaction product observed in goblet cells is not due to a non-

Figure 7. Immunocytochemical localization of kallikrein in goblet cells of the rat colon. Semi thin resin section incubated with αRIK antiserum (1:1000) for 60 hours PAP technique. Tissue fixed as in Figure 4. Arrows = goblet cells, L = lumen. Magnification X570.

Figure 8. As Figure 7, substituting αRIK antiserum with αRPK antiserum (1:1000). Arrows = goblet cells, L = lumen. Magnification X570.

Figure 9. As Figure [illegible] substituting αRIK antiserum with αC GK antiserum (1:1000). Arrows = goblet cells, L = lumen. Magnification X5[illegible]

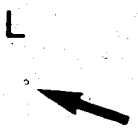


Figure 10. As Figure [illegible], substituting αRIK antiserum with αacrosin antiserum (1:1000). Arrows = goblet cells. L = lumen. Magnification X5[illegible]

specific interaction between mucus glycoproteins and hyperimmune antisera.

Absorption of specific antibody by pure antigen or crude extracts has previously been shown to abolish the specific immune reaction in colonic goblet cells (Schachter *et al.*, 1983). Therefore, in this study, it was not deemed necessary to perform such controls.

Employing a double staining technique, it was possible to stain both immunocytochemically and histologically goblet cells, such that the brown diaminobenzidine immunoproduct was superimposed on the pink/purple product of the periodic acid Schiff's reaction for carbohydrates.

3.2 Immunocytochemical Localization of Kallikrein in Goblet Cells of Rat Small Intestine

Kallikrein has been localized immunocytochemically in goblet cells of the rat small intestine employing two immunocytochemical techniques (Figures 11-13). A similar staining pattern was seen in all three segments of the small intestine, i.e. the duodenum, the jejunum and the ileum. The population of goblet cells in the small intestine is greatly reduced compared to the colon. Goblet cells in both the villi and crypts of Lieberkühn stained equally intensely. The brush border showed variable staining. No specific staining was associated with either columnar epithelial intestinal absorptive cells or endocrine cells which are present between the columnar epithelial cells at the base of the epithelium. No staining was observed in the lamina propria, muscularis mucosa, submucosa, muscle layers and their associated blood vessels and lymphoid tissue. As with the rat colon, mast cells associated with the lamina propria showed no specific staining.

The PAP and ABC methods yielded similar results, however the ABC method shortened primary antisera incubation time and affording greater sensitivity (Figures 12a and 13a). Using antisera raised against rat intestinal kallikrein and rat pancreatic kallikrein yielded a similar staining pattern to that observed with cat salivary kallikrein antisera (Figures 14 and 15). No specific staining was observed using antisera raised against CGK and acrosin (Figures 16 and 17).

Figure 11 Immunocytochemical localization of kallikrein in goblet cells of the rat ileum. Semithin resin section incubated with αCSK antiserum (1:1000) for 90 hours. PAP technique. Tissue was freeze-dried by copper block method (Edwards-Pearse freeze drier). Arrows = goblet cells; L = intestinal lumen. Magnification X300.

Figure 12a. Immunocytochemical localization of kallikrein in goblet cells of the rat ileum. As Figure 11. Arrows = goblet cells. L = intestinal lumen. Magnification X570.

Figure 12b. Control section for Figure 12a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification X570.

Figure 13a Immunocytochemical localization of kallikrein in goblet cells of the rat jejunum. Semi-thin resin sections incubated with αCSK antiserum (1:1000) for 40 hours. Avidin-biotin peroxidase technique. Tissue was freeze-dried by copper block method (Edwards-Pearse freeze-drier). Arrows = goblet cells, L = intestinal lumen. Magnification X570.

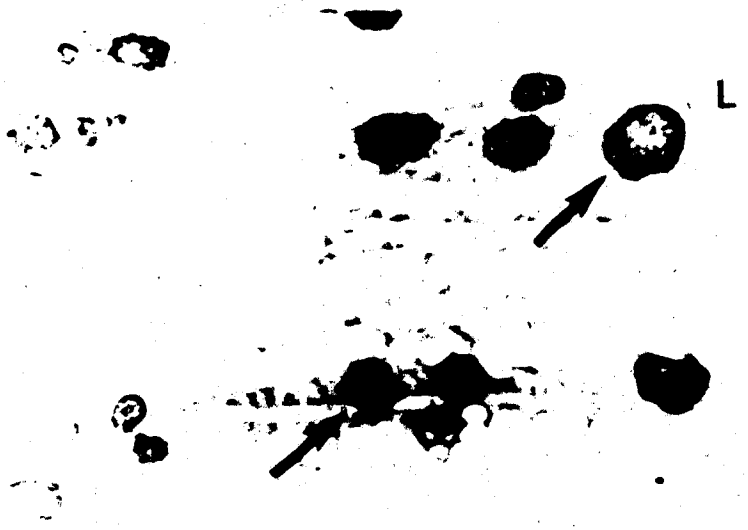


Figure 13b. Control section for Figure 13a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification X570.

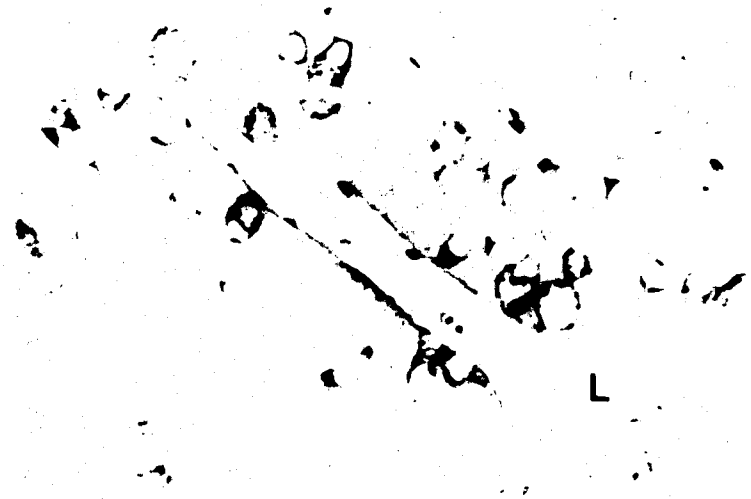
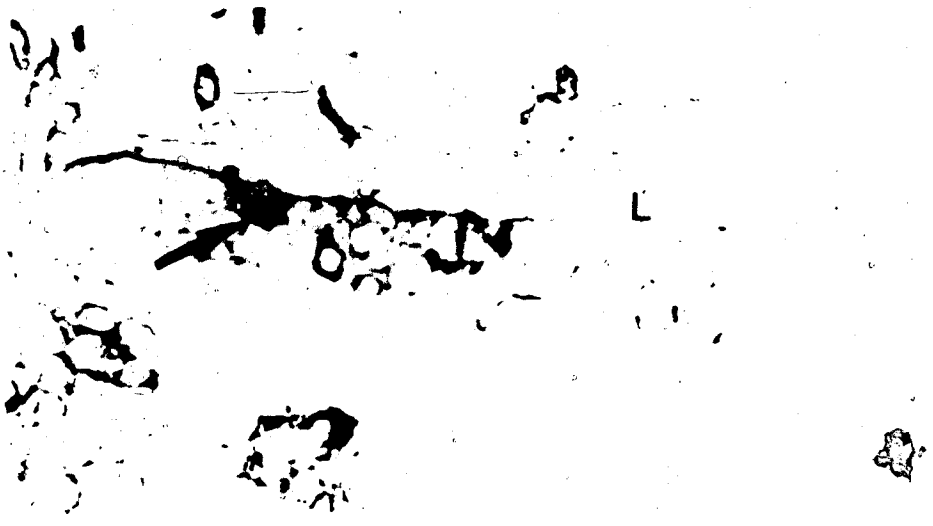


Figure 14. As Figure 12a, substituting αCSK antiserum with αRIK antiserum (1:1000). Arrows = goblet cells. L = intestinal lumen. Magnification X570.

Figure 15. As Figure 12a, substituting αCSK antiserum with αRPK antiserum (1:1000). Arrows = goblet cells. L = intestinal lumen. Magnification X570.

Figure 16. As Figure 12a, substituting αCSK antiserum with αCGK antiserum (1:1000). Arrows = goblet cells, L = lumen. Magnification X570.

Figure 17. As Figure 12a, substituting αCSK antiserum with αacrosin antiserum (1:1000). Arrows = goblet cells, L = lumen. Magnification X570.



The enzyme localized in the rat small intestine is immunogenically related to the enzyme localized in the rat colon based on their cross-reactivity to the same antisera and their lack of cross-reactivity to both CGK and acrosin antisera.

3.2.1 Stimulation of the Release of Kallikrein from Goblet Cells of the Rat Small Intestine

Following a bolus intraperitoneal injection of pilocarpine (a parasympathomimetic), an accelerated release of mucus by compound exocytosis from crypt (but not surface) goblet cells was observed throughout the small intestine. In all cases, the associated immunoreactive material was seen to move out of the crypt goblet cells within the small intestine into the crypt lumen, which then becomes expanded (Figure 18a). This finding suggests that the secretion of kallikrein from crypt goblet cells of the small intestine, as well as the associated mucus, is under parasympathetic control (Specian & Neutra, 1982). Goblet cells associated with the villi and surface were unaffected by pilocarpine, suggesting they are not under parasympathetic control.

3.3 Immunocytochemical Localization of kallikrein in Goblet Cells of Cat Small Intestine

Kallikrein has been localized in goblet cells of cat small intestine employing the PAP and ABC techniques (Figures 19-21). As with the rat small intestine, a similar staining pattern was seen in all three segments of the small intestine. Figures 19 through 21 show sections of cat duodenum. The brush border of the cat small intestine showed variable staining and was sometimes observed in control sections. No specific staining was observed in Brunner's glands, columnar epithelial absorptive cells, associated endocrine cells and mast cells within the lamina propria. Specific staining was absent from the lamina propria, muscularis mucosa, submucosa, muscle layers and their associated blood vessels and lymphoid tissue.

Figures 19 through 21 demonstrate the staining pattern observed using αCSK antisera. It is not known if a similar staining pattern is possible employing αRIK and αRPK antisera. However, in an earlier study, Schachter *et al.* (1983) reported that only weak specific staining

Figure 18a Immunocytochemical localization of kallikrein in goblet cells of rat jejunum following parasympathetic stimulation (pilocarpine). Semi-thin resin section incubated with αCSK antiserum (1:1000) for 60 hours. PAP technique. Tissue was fixed in 0.5% glutaraldehyde and 1.0% paraformaldehyde. Arrows = goblet cells, L = intestinal lumen (distended) Magnification X570.

Figure 18b Control section for Figure 18a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification X570.

Figure 19. Immunocytochemical localization of kallikrein in goblet cells of cat duodenum. Semi-thin resin section incubated with αCSK antiserum (1:1000) for 60 hours/PAP technique. Tissue was fixed by copper block method (Coulter-Terracio freeze-drier) and post-fixed with osmium vapour. Arrows = goblet cells, L = intestinal lumen. Magnification X300.

Figure 20a. As Figure 19. Arrows = goblet cells. L = lumen. Magnification X570.

Figure 20b. Control section for Figure 20a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification X570.

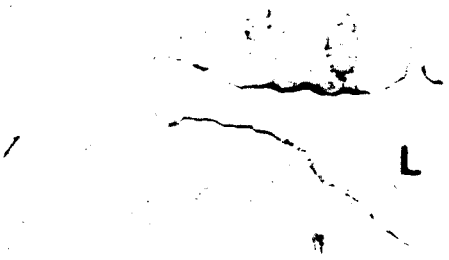
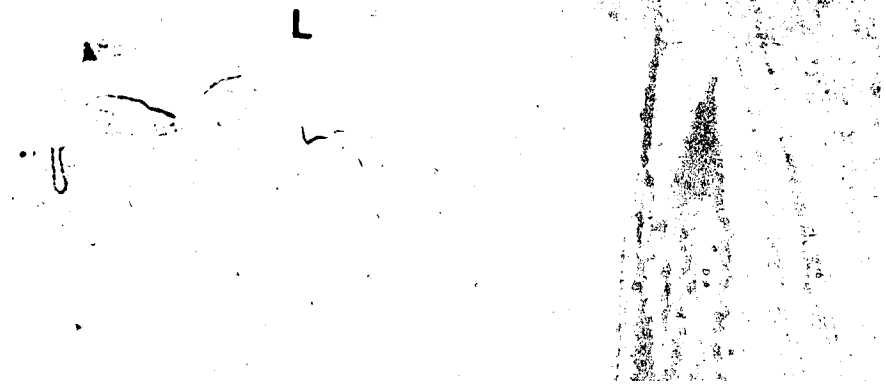


Figure 21a. As Figure 19, except primary antiserum incubation time 40 hours employing the ABC technique. Arrows = goblet cells, L = intestinal lumen. Magnification X570.

Figure 21b. As Figure 21a, substituting αCSK antiserum with normal rabbit serum (1:1000). L = lumen. Magnification X570.



was observed using αRIK and αRPK antisera in cat colon. Since similar techniques have been employed with regard to tissue fixation and immunocytochemistry, it is possible that a similar weak staining pattern may result in the cat small intestine goblet cells as that described for the cat colon.

4. DISCUSSION

The localization of kallikrein in goblet cells and on the luminal surface of epithelial cells (brush border) requires careful analysis, since false-positive staining is commonplace in immunocytochemical studies. The specificity of antisera to their specific antigen has been proven by the single arc precipitin line obtained for all antisera via Ouchterlony immunodiffusion. A previous study with these antisera employing preabsorbed or antigen-neutralized antisera and the subsequent negative staining pattern provides further proof of the specific nature of the antisera. (Schachter *et al.*, 1983). The use of hyperimmune specific antisera raised to similar but immunologically distinct enzymes and their failure to produce immune staining in goblet cells is further proof of a "true-positive" result. One could argue that the latter antisera used in this study, which were raised against acrosin and coagulating gland kallikrein (CGK), were not of high titre. This is not the case, since both antisera have been previously shown to be very effective in the localization of their specific antigens in sperm and coagulating gland respectively (Schachter *et al.*, 1978; Schachter, 1980b). The antigens employed in this study were all supplied by Dr. C. Moriwaki.

The significance of the localization of kallikrein in the mucous cells of the gastrointestinal tract and its possible physiological role(s) remains the most important question to be answered.

Recently, a serine protease with tissue kallikrein-like substrate specificity has been localized in our laboratory by an enzyme histochemical technique in mast cells of the rat colon and small intestine. These mast cells are associated with the lamina propria and align themselves near the base of the glandularis mucosa (Garrett *et al.*, 1982; Schachter, unpublished observations). Employing a different method of fixation and substrate incubation conditions, a serine-protease has been localized in goblet cells of rat colon and small intestine. Under the latter conditions, the mast cells failed to stain. These observations suggest that two related serine proteases are to be found in the small intestine and colon, one in goblet cells and one in mast cells, which are immunogenically distinct but enzymatically (with regard to substrate

specificity) similar.

In an earlier study, Woodbury *et al.* (1978) reported the immunofluorescent localization in small intestinal mast cells and goblet cells of a serine protease which had previously been named "group-specific protease" (GSP)(Katunuma *et al.*, 1975). This protease was known to be chymotrypsin-like with regard to chemical, physical, enzymatic, immunologic and inhibitor spectrum properties. Mast cells were also found to contain a related chymotrypsin-like protease which was named "mast cell protease". Thus, it is apparent that mast cells in the gastrointestinal tract contain a number of proteases with possibly different substrate specificities and immunological properties.

The first biochemical purification of a kininogenase from the gastrointestinal tract was reported by Seki *et al.* (1972). They demonstrated that the colonic mucosa contains a pre-kallikrein with properties similar to those of the plasma pre-enzyme and apparently different from tissue kallikrein. Previously, Zeitlin (1971) had shown that rat intestinal mucosal extracts contained a kinin-forming enzyme which was distinct (enzymatically and in inhibitory profile) from trypsin, plasmin and plasma kallikrein. Subsequently, Moriwaki *et al.* (1980) reported the first intensive biochemical purification of rat intestinal kallikrein (this preparation was used in the production of our αRIK antisera). They concluded that kallikrein was present in all parts of the intestine, i.e. both mucosal and muscle layers, but the greatest amount was present in the mucosal preparations. Rat intestinal kallikrein was found to exist in two forms, RIK-A and RIK-B, which were presumed to differ in carbohydrate content. Enzymatically, RIK-A and RIK-B were quite different from rat pancreatic kallikrein (RPK), suggesting that intestinal kallikrein is not derived from RPK but is produced by cells in the small intestine. Immunologically, RIK and RPK appear to be identical.

The initial observation of Seki *et al.* that colon kallikrein was similar to plasma kallikrein has since been refuted by Zimmerman *et al.* (1979) and Fujimori *et al.* (1985a) for man and cat respectively. Zimmerman *et al.* characterized human colon kallikrein as being very similar to human urinary kallikrein, both enzymatically and immunogenically. Fujimori *et al.*

report the purification of cat colon kallikrein and state that cat salivary and cat colon kallikrein are immunologically identical based on Ouchterlony immunodiffusion and enzyme-linked immunosorbent assay (ELISA) data. Schachter *et al.* (1983) also suggested that cat salivary kallikrein and cat colon kallikrein were immunologically similar.

It appears that there are intra- and inter-species similarities between tissue kallikreins, especially between rat and cat salivary kallikreins and gastrointestinal kallikreins. The fact that cat salivary kallikrein antiserum cross-reacts with rat intestinal and rat colonic kallikrein suggests that the three enzymes share antigenic determinants, while enzymatically they are quite distinct, especially with regard to inhibitor profile (Fujimori *et al.*, 1985a, b).

In search of a physiological role for gastrointestinal kallikrein, one must first answer some pertinent questions as to whether the kallikrein localized in goblet cells functions via the release of kinins from its physiological substrate, kininogen, or independently of the release of kinin. Kininogen was found in normal colonic tissue by Zeitlin and Smith (1973). However, in normal tissue, kininogen appeared only in the muscle layer, and none was detected in the mucosa. Subsequently, Geiger *et al.* (1977) demonstrated the presence of a "precursor of kinins" (kininogen) in gastric mucus. They found kinin activity after incubation of mucus with bovine trypsin and porcine pancreatic kallikrein. The suggestion that kininogen is associated with gastric mucus offers interesting possibilities, since kallikrein has been shown to be associated with mucus in the small intestine and colon.

Although stimulation of intestinal smooth muscle *in vitro* was one of the first recognized actions of kinins, the contribution of kinins to normal gastrointestinal function is still not established. Kinins are rapidly destroyed by kininases occurring in the blood and membrane-bound within the gastrointestinal tract. However, marked local effects within the gastrointestinal tract could be caused by free kinin within tissues in quantities too minute to produce detectable systemic effects. The first definitive proof of the presence of kininase II (angiotensin-converting enzyme) in the brush border of the intestine came from Ward and co-workers (1980). They demonstrated the presence of kininase II biochemically along the entire

length of the small intestine of the pig and speculated upon its role in protein metabolism in general, as well as on its possible action in regulating the metabolism of vasoactive peptides. Later, Defendini *et al.* (1983) localized kininase II immunocytochemically in the mucosa of the duodenum, jejunum and ileum, particularly on the free surface of the epithelial cell membrane. Thus, it is apparent that three components (kallikrein, kininogen and kininase) of the kallikrein-kinin system are present in the mucosa of the gastrointestinal tract and that kinin formation may be physiologically significant.

In search of a physiological role for pancreatic kallikrein, several groups of workers explored the possibility that kallikrein or kinins might affect intestinal absorption or secretion of various nutrients. Dennhardt and Haberich (1973) studied the influence of kallikrein on the absorption of water, electrolytes and hexoses from the rat intestine *in vivo*. Kallikrein, introduced into the intestinal lumen, enhanced water and electrolyte absorption from the jejunum and colon. Meng and Haberland (1973) independently demonstrated the same effects, but at higher concentrations of kallikrein the absorption of glucose was reversed. Moriwaki and associates (1973, 1977a) demonstrated that pancreatic kallikrein increased valine absorption from the everted intestine. Bradykinin was also effective. Kallikrein also enhanced transport of methionine and d-glucose from rat jejunum, as determined by recovery of labelled amino acids from the mesenteric vein, whereas other proteolytic enzymes such as trypsin and chymotrypsin had no effect. This action of kallikrein was blocked by aprotinin, implying that kinin formation is involved in the enhanced transport (Moriwaki and Fujimori, 1975). Other investigators failed to find an effect of kallikrein on transport of glucose and valine in the rat intestine (Caspary and Creutzfeldt, 1973). However, Caspary and Creutzfeldt used higher concentrations of kallikrein than did Moriwaki *et al.* (1973, 1977a, b) and Meng and Haberland (1973).

The site at which kinins are formed may determine its effect on ion, glucose and amino acid transport. Since kallikrein is localized in the "mucus" extruded from goblet cells, then kinins should be formed on the apical side of the intestinal mucosa. However, kallikrein isolated from the rat small intestine mucosa (RIK) has a more potent effect on valine transport in

the jejunum when it is added to the serosal side than when it is added to the mucosal side (Moriwaki *et al.*, 1977a). Similarly, bradykinin added to isolated intestinal segments on the serosal side stimulated sodium transport, whereas a mucosal application was ineffective.

More recently, Cuthbert and Margolius (1982) have demonstrated that kallidin (Lys-bradykinin) stimulates net chloride secretion in the isolated descending colon of the rat. Moreover, they have shown that kinins commonly exert their effects on chloride secretion when applied to the serosal side of their isolated epithelia, suggesting that the receptors for kinins are located on the basolateral membrane of the columnar epithelial cells, rather than being located apically. Independently, Manning *et al.* (1982), employing autoradiographical techniques, were able to determine the location of bradykinin receptors in the small intestine. Although their procedure does not allow the precise determination of the cellular location of receptors, bradykinin receptors in the lamina propria are probably located on the basolateral membranes of the epithelial cells (goblet and columnar absorptive). However, receptors may also occur on smooth muscle cells, blood vessels and/or sensory fibres in the villi.

The possibility that the tissue kallikrein-kinin system is involved intimately in membrane ion events seems strengthened by the evidence that kinins stimulate net chloride secretion and substantial amino acid and glucose movements. The problem still exists as to how a kallikrein associated with mucus in goblet cells and secreted into the lumen could influence electrolyte transport. One cannot discount the possible existence of a related enzyme in columnar epithelial cells, and our techniques have failed to detect this enzyme. The dual localization of kallikrein has already been reported with regard to pancreatic kallikrein. Ørstavik *et al.* (1980a) have reported the localization of kallikrein exclusively in acinar cells of the pancreas. However, employing different methods of tissue fixation and trypsin pretreatment, Pinkus *et al.* (1983) reported both acinar and islet beta cell localization. Thus, it is entirely possible that under different methods of fixation and enzymatic pretreatment, another related protease may be present within columnar epithelial cells of the gastrointestinal tract.

Many enzymes and peptide hormones are synthesized as inactive precursors that are proteolytically converted to their active forms. *In vivo* conversion of these precursor forms is irreversible and serves as a more rapid control mechanism for physiologic function than control at the level of gene transcription.

The serine proteases are known to activate proenzymes such as trypsinogen, chymotrypsinogen, etc., and plasma kallikrein itself activates pro-Hageman factor (see Schachter, 1980). The involvement of renal kallikrein in renin activation *in vitro* has been demonstrated (Sealey *et al.*, 1978a, b; Hseuh *et al.*, 1981). Hseuh *et al.* suggest that acid treatment activates the renin molecule via a conformational change and that kallikrein may then cleave a small peptide that permanently maintains acid-activated renin in an active state. Thus, renin zymogen must be in an active conformation, such as that induced by acid, to be "acted upon" by kallikrein. Therefore, kallikrein may be involved in renin activation *in vivo* (Hiwada *et al.*, 1983). There is evidence that kallikrein-like proteases, with their remarkably strict specificity, regulate the conversion of prohormones, like proinsulin and proglucagon, in the pancreas (Dorn *et al.*, 1978; ole-Moi Yoi *et al.*, 1979).

Recently, Powers and Nasjletti (1982) described a "novel" kininogenase in the porcine anterior pituitary and speculated upon its potential involvement in the processing of prohormones. The ability of this anterior pituitary kininogenase to act on protein substrates other than kininogens is unknown. Nevertheless, in the anterior pituitary, pro-opiomelanocortin (POMC) contains several Lys-Arg bonds, an Arg-Ser bond whose cleavage results in the formation of β-lipotropin, β-endorphin, β-melanotropin, α-melanocortin and corticotropin. Whether the kininogenase within the anterior pituitary has the conformational requirement essential for such bond cleavages remains unknown.

Several groups have described proteases in the anterior pituitary with trypsin-like activity which also convert β-lipotropin to β-endorphin or other opiates by cleavage at Arg-X residues (Graf *et al.*, 1977; Kenessey *et al.*, 1979; Orlowski and Wilk, 1981; McPartland *et al.*, 1981).

Serine proteases within the gastrointestinal tract may be involved in the processing of pre-secreted mucus, therefore modifying its composition prior to secretion. Naked mucin peptide segments are much more likely to be cleaved by proteases than their carbohydrate-decorated counterparts. Such is the case for porcine gastric glycoprotein mucins, which are cleaved by trypsin, pronase and pepsin, causing a decrease in the viscosity and gel-forming properties of the gastric mucus (Scawen and Allen, 1977). Presumably, trypsin cleaves a peptide sequence which is highly elaborated with saccharide chains, and in particular sialic acid, that conveys the viscosity to mucus, and their removal would lead to a decrease in mucus viscosity.

Thick, viscid mucus is the hallmark of cystic fibrosis, an inherited disease which is characterized by blockage of epithelium-lined ducts throughout the body. Cystic fibrosis mucins are more highly glycosylated than the mucins from controls, possibly via altered rates of synthesis and storage within goblet cells (Dische *et al.*, 1959; Clamp and Gough, 1979; Wesley *et al.*, 1981). At present, there is no evidence to suggest a connection between the manifestation of cystic fibrosis and discrepancies or abnormalities in the kallikrein-kinin system within the gastrointestinal tract.

The epithelial cells, both goblet and columnar absorptive, of the gastrointestinal tract have a high rate of turnover as a result of continuous shedding and replacement. Some investigators have suggested that pancreatic kallikrein is a physiological stimulus for mitotic activity in the gut and that either kallikrein or kinins are required for continual replacement of epithelial cells. Intraluminal application of pancreatic kallikrein in rats stimulated mitosis in the cells of the duodenum and jejunum, but not in the ileum or colon (Rohen and Peterhoff, 1973). The majority of mitotic cells are located in the lower third of the intestinal and colonic crypts (LeBlond and Walker, 1956; Messier, 1968; Sawicki *et al.*, 1971; Altmann, 1983). From this mitotic zone, the maturing cells must migrate upward to provide for the renewal of the surface epithelium. Since kallikrein has been localized in goblet cells at different stages of maturation, it is interesting to speculate at what stage kallikrein is synthesized, either prior to

goblet cell formation, i.e. within stem cells, or after goblet cell formation.

Other workers have shown that kallikrein or kinins have mitogenic effects on rat thymocytes and bone marrow cells and that these effects may depend on changes in cyclic adenosine monophosphate and calcium during the cell cycle (Rixon *et al.*, 1971; Perris and Whitfield, 1959, 1973; Boucek and Noble, 1973). However, it is not yet possible to link kinin formation, cAMP formation and cell division to a functional role in regeneration of intestinal epithelium.

Whatever the mechanism of its mitogenic effect, kallikrein has been reported to stimulate the proliferation of a wide variety of cells, both *in vitro* and *in vivo* (Schutte and Lindner, 1977). Some kallikreins, therefore, may be involved in differentiation, development and maturation of specific cells.

Some studies suggest that inappropriate formation or metabolism of kinins contribute to the pathogenesis of diseases such as carcinoid (Oates *et al.*, 1966; Oates and Butler, 1967), postgastrectomy dumping syndrome (Smith and Zeitlin, 1966; Zeitlin and Smith, 1966; Wong *et al.*, 1974; Chaimoff *et al.*, 1977), alcoholic liver disease (Wong *et al.*, 1972), and possibly ulcerative colitis (Zeitlin and Smith, 1973).

In conclusion, it is extremely difficult at present to assign a specific physiological role to kallikrein in the gastrointestinal tract. One must take into account the localization of kallikrein within goblet cells when assigning a specific role, but it is not possible to discount the existence of a related enzyme in mast cells and the possible existence within columnar epithelial absorptive cells. Earlier views assumed that kallikrein must function via the release of the peptide kinin, but it is now apparent that kallikrein may exert significant physiological actions independent of kinin release. As far as disease states within the gastrointestinal tract are concerned, it is impossible at present to definitively implicate deficiencies or abnormalities in the kallikrein-kinin system as being causal to or consequential of such diseases.

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SECTION B:

MEDIATORS OF VASODILATATION IN THE SUBMANDIBULAR GLAND OF THE CAT

1. INTRODUCTION

1.1 Historical Data

The dual neurovascular regulation of the submandibular gland was first demonstrated in 1858 by Claude Bernard, who showed that electrical stimulation of the sympathetic nerve to the submandibular gland of the dog reduced the venous outflow from the gland, whereas stimulation of the parasympathetic nerve greatly increased it. He concluded that there were vasodilator as well as vasoconstrictor nerves, and that the existence of both nerve types allowed a mechanism by which blood flow through the gland could be regulated locally.

In 1872, Heidenhain showed that the vasodilatation induced by parasympathetic nerve stimulation was not abolished by atropine in doses which blocked the concomitant secretion of saliva. As a result of these findings, it was assumed that the hyperemia in the submandibular gland was caused by vasodilator nerve fibres distinct from the secretory fibres.

In 1912, Barcroft and Piper challenged the view that the chorda tympani nerve (parasympathetic) did supply the submandibular gland with vasodilator nerve fibres. They showed that the chorda-induced vasodilatation was accompanied by an increase in oxygen consumption even in atropinized, non-secreting salivary glands. Barcroft thought, therefore, that the chorda-mediated vasodilatation was secondary to the increased metabolic activity of the gland. Later however, Barcroft (1914) did not discard the possible role of vasodilator nerves, stating "that under normal circumstances, dilatation may be instituted by dilator fibres and maintained by metabolic products". Later workers also thought that vasodilator fibres to the submandibular gland existed (Bayliss, 1923; Dale and Gaddum, 1930; Babkin, 1950). Terroux *et al.* (1959) were able to inhibit largely the metabolic activity induced by parasympathetic nerve stimulation with atropine while the vasodilatation persisted, suggesting that "true" vasodilator fibres exist. Subsequently, Garrett (1966a, b, c, d) concluded that there was an extensive innervation of the arteries and arterioles of the cat submandibular gland with cholinergic nerves using a histochemical technique for cholinesterase. Denervation studies also supported the

existence of a parasympathetic vasodilator innervation.

1.2 Acetylcholine as the Mediator

The concept of chemical neurotransmission emerged when it was first demonstrated that parasympathetic nerve stimulation caused effects like that of muscarine (Dixon, 1906; Dale, 1914). Dale, investigating the effects of the substance acetylcholine, originally isolated from ergot, found that it lowered blood pressure in the cat, inhibited the heart beat in the frog and caused contractions of frog intestinal muscles. He suggested that acetylcholine occurred naturally in the body, possibly acting as an antagonist to the effects of adrenaline and, as an inspired guess at the time, suggested that it was normally rapidly broken down by a hydrolytic enzyme; this would account for the fact that it had so far been impossible to isolate acetylcholine from the body. Dale's original ideas were extended by Loewi (1921), who demonstrated that the vagal stimuli to the heart were transferred by a chemical messenger which he named "Vagusstoff", and which is now known as acetylcholine (ACh). ACh was finally isolated from the spleen of cows and horses by Dale and Dudley (1929).

The postganglionic parasympathetic mediation of submandibular salivary secretion was shown to be via the release of ACh (Babkin, 1950). Subsequently, Emmelin and Muren (1950) showed that the preganglionic fibres also contain and release ACh upon stimulation. Thus, the local parasympathetic nervous control of the submandibular gland was found to be mediated by ACh, both at the preganglionic level, where the action of ACh is nicotinic, and at the post-ganglionic level, where the action of ACh is muscarinic. It is difficult, however, to account for the failure of atropine to block the vasodilatation which always accompanies the salivary secretion upon parasympathetic nerve stimulation. Dale and Gaddum (1930) postulated that ACh was released from vasodilator nerve endings in such great "intimacy with the receptor mechanism that atropine cannot prevent its access thereto".

However, following close arterial injection of acetylcholine, the vasodilator response induced could be abolished completely by atropine (Bhoola *et al.*, 1965). They suggested that

the relative resistance of chorda tympani-induced vasodilatation to atropine may be an extreme case of the variation in the sensitivity of cholinergic receptors to atropine (Ambache, 1955). Subsequently, it was demonstrated that at low frequencies of parasympathetic nerve stimulation, atropine reduces the vasodilator response, suggesting that a cholinergic vasodilator mechanism may be involved (Darke and Smaje, 1972).

1.3 The Kallikrein-Kinin System as a Mediator

Kallikrein was first described in salivary glands by Werle and Roden (1936) and shortly afterwards was shown to exert its hypotensive and other pharmacological effects by releasing an active fragment, bradykinin, from a globulin substrate in plasma, apparently by a proteolytic action (Werle *et al.*, 1937). In the same year, Ungar and Parrot (1936) suggested that kallikrein was released during salivary secretion and that it was the mediator of atropine-resistant vasodilatation produced by parasympathetic nerve stimulation in the submandibular gland. Ungar and Parrot's suggestion extended Barcroft and Piper's (1912) idea that vasodilation was caused by the secretory metabolic activity of gland cells, by suggesting kallikrein as the metabolic mediator.

Subsequently, experiments by Hilton and Lewis (1955a, b; 1956) indicated that kallikrein played a part in this vasodilatation. They perfused the cat submandibular gland with oxygenated Locke's solution and were able to demonstrate the presence in the venous effluent perfusate of a stable vasodilator material which formed bradykinin when incubated with a globulin preparation.

Later the view that release of a kinin-forming enzyme with a subsequent kinin formation is the cause of functional vasodilatation in the submandibular gland was strongly opposed by several authors (Bhoola *et al.*, 1965; Schachter and Beilenson, 1967; Beilenson *et al.*, 1968; Skinner and Webster, 1968a, b; Karpinski *et al.*, 1971; Schachter *et al.*, 1973; Darke and Smaje, 1973; Ferreira and Smaje, 1976). Bhoola *et al.* (1965) demonstrated that desensitization of the gland to bradykinin failed to influence the vasodilator response during chorda

stimulation. A marked vasodilatation could also still be obtained even if the cat submandibular gland was perfused with horse serum from which cat salivary kallikrein is unable to release bradykinin.

Skinner and Webster (1968a, b) demonstrated that following injection of carboxypeptidase B (a potent inactivator of kallidin and bradykinin), chorda-induced vasodilatation was unaffected, whereas the vasodilator response induced following bradykinin infusion was completely abolished.

Schachter *et al.* (1973) also demonstrated that the bradykinin potentiating peptide, BPP_{1a}, given intra-arterially, failed to enhance the vasodilator response to chorda stimulation, although it did increase the vasodilator response induced by bradykinin. Similar observations were reported by Ferreira and Smaje (1976) regarding atropine-resistant vasodilatation in the dog. Earlier, Schachter's group (Schachter and Beilenson, 1967; Beilenson *et al.*, 1968) demonstrated that following depletion of kallikrein in the cat submandibular gland, either by duct ligation, or by duct ligation and sympathetic nerve stimulation, the atropine-resistant vasodilatation induced by chorda nerve stimulation was unaffected.

Lundberg (1958), inserting intracellular electrodes into the secretory cells of the gland, noted a latency of 200-400 ms in secretory response induced by chorda nerve stimulation. Furthermore, Creed and Wilson (1969), performing similar experiments, noted a mean latency of 300 ms, which was similar to that reported earlier by Lundberg.

Gautvik (1970), noting delays between stimulation of the chorda nerve and onset of vasodilatation of 1-6 seconds, concluded that dilator nerve fibres initiate the vasodilatation via a cholinergic mechanism and that it is "maintained" by the "metabolite" kallikrein.

In a later study using more sensitive recording methods, Karpinski *et al.* (1971) noted a mean latency of 450 ms between nerve stimulation and vasodilatation, and suggested that the kallikrein-kinin system is not involved in the initiation of vasodilatation caused by stimulation of the chorda tympani nerve. Karpinski *et al.* (1971) stated that "it is most unlikely that there would be time for the activation of the secretory cell by acetylcholine, the release of kallikrein

into the interstitial fluid, the enzymatic release of kallidin and the final vasodilator action of kallidin".

Hilton and Lewis (1956) noted that kallikrein output from the gland was reduced after atropine; however, Ferreira and Smaje (1976) demonstrated a complete block of the release of kallikrein following atropine pretreatment and suggested that kallikrein cannot be involved in the vasodilator response after atropine administration.

1.4 Vasoactive Intestinal Polypeptide as a Mediator

1.4.1 Discovery and Chemistry

In 1969, Said and Mutt reported the extraction from normal lung tissue of a peptide which was capable of causing a gradual but prolonged peripheral vasodilatation. This finding led them to search for similar vasoactive substances in extracts of other metabolically active organs and, in 1970, they described the isolation of a potent peripheral and splanchnic vasodilatory peptide from hog small intestine which they named vasoactive intestinal peptide (VIP) (Said and Mutt, 1970a, b). Subsequent purification of VIP allowed determination of its amino acid sequence, which showed it to be a straight chain of twenty-eight amino acid residues with basic properties because of a predominance of arginine and lysine residues (Said and Mutt, 1972). A comparison of the amino acid sequence of VIP with those of the classical hormones secretin, pancreatic glucagon and the more recently characterized gastric inhibitory polypeptide, growth hormone-releasing factor, peptide histidine isoleucine (PHI) and peptide histidine methionine (PHM) reveals a marked degree of homology (Bryant, 1980; Tatemoto and Mutt, 1981; Tatemoto, 1984)

1.4.2 VIP as a Neurotransmitter/Modulator

It is now known that VIP-like immunoreactivity occurs not only in the intestine, but in the peripheral and central nervous systems (Said and Rosenberg, 1976; Bryant *et al.*, 1976;

Larsson *et al.*, 1976), and in this respect, VIP resembles a number of other peptides (somatostatin, substance P, cholecystokinin and enkephalin).

In many tissues, VIP-containing nerve fibres make contact with blood vessels (Larsson *et al.*, 1976; Edvinsson *et al.*, 1980; Edvinsson and Ekman, 1984; Järhult *et al.*, 1980, 1982; Uddman *et al.*, 1981; Gibbons *et al.*, 1984; Dey *et al.*, 1981). VIP-like immunoreactivity has been extensively mapped in the cholinergic neurons innervating exocrine glands of the cat, both at the light and electron microscopic levels (Wharton *et al.*, 1979; Uddman *et al.*, 1980; Lundberg *et al.*, 1979, 1980a, 1981c; Johansson *et al.*, 1981).

Lundberg *et al.* (1979) demonstrated an overlapping accumulation of VIP immunoreactivity and acetylcholinesterase staining in some postganglionic sympathetic and parasympathetic neurons, suggesting the presence of a VIP-like peptide in a population of sympathetic and parasympathetic cholinergic neurons. In a later study, Johansson *et al.* (1981) extended these findings and localized VIP-like immunoreactivity in large dense-cored vesicles (990 A) in association with acetylcholine in the parasympathetic nerve supply to the submandibular gland of the cat. The greatest accumulation of VIP-immunoreactive fibres and varicosities could be seen close to the secretory acini and more distant from the blood vessels, demilune cells and ductal system of the gland. Based on their immunocytochemical observations, Lundberg *et al.* (1979, 1980a) suggested "VIP released together with acetylcholine, may be responsible for the atropine-resistant vasodilatation in sweat glands and other exocrine glands".

1.4.3 VIP and Atropine-Resistant Vasodilatation

Considerable evidence has been provided to suggest that VIP may be a mediator (alone or in conjunction with acetylcholine) of atropine-resistant vasodilatation in the submandibular gland. Bloom and Edwards (1980) demonstrated that VIP was released from the cat submandibular gland following parasympathetic nerve stimulation in both the presence and absence of atropine. These authors also noted that close arterial infusions of VIP, which mimic the rise in the concentration of the peptide in the submandibular venous plasma during chorda

stimulation, also produce an increase in submandibular blood flow of the same order of magnitude as that observed during chorda stimulation at the same frequency. These observations were confirmed later by Lundberg (1981) and Lundberg *et al.* (1981a, b, 1982a) and extended by Shimizu and Taira (1979) to the dog submandibular gland, where they showed VIP was a potent vasodilator.

In a series of experiments, Lundberg (1981) and Lundberg *et al.* (1980a, 1981a, b, 1982a) extensively investigated the effect of VIP on atropine-resistant vasodilatation. These workers maintain that both acetylcholine and VIP are mediators of vasodilatation, with acetylcholine being the major mediator at low frequencies of chorda nerve stimulation and VIP being the major mediator at higher frequencies of chorda nerve stimulation. Furthermore, Lundberg and coworkers demonstrated that following VIP antiserum infusion, both chorda-induced and VIP-induced vasodilator responses were abolished. In spite of a dense innervation of VIP-immunoreactive neurons around secretory elements in the cat submandibular gland, exogenous VIP has been shown to have no secretory effect *per se* (Lundberg *et al.*, 1980a, 1982a). However, VIP injected intravenously was found to induce a flow of saliva from both the parotid and the submandibular gland of the rat (Ekstrom *et al.*, 1983).

VIP and acetylcholine have also been shown to be co-released from the submandibular gland of the cat during parasympathetic nerve stimulation (Lundberg *et al.*, 1982b). A more recent study demonstrated the co-release of two related peptides from the cat submandibular gland, namely VIP and PHI. These workers concluded that parasympathetic control of salivary gland function may involve a multimessenger system, acetylcholine and the peptides VIP and PHI (Lundberg *et al.*, 1984). In earlier attempts to block atropine-resistant vasodilatation, Lundberg and coworkers (1980b) focussed their interest on avian pancreatic polypeptide (APP). APP is a thirty-six amino acid polypeptide (Kimmel *et al.*, 1975) which greatly differs in amino acid sequence from pancreatic polypeptides isolated from other species (Lin and Chance, 1974; Kimmel *et al.*, 1975). Lundberg *et al.* demonstrated that avian pancreatic polypeptide reversibly inhibits chorda-induced and VIP-induced vasodilator responses in the cat

submandibular gland. This work provided more evidence for the possible involvement of VIP. Avian pancreatic polypeptide also inhibited the chorda-induced secretory response, possibly via an effect on blood flow. This finding creates an anomaly, since it has long been presumed that the secretory response of the cat submandibular gland is totally cholinergic. Throughout their studies on atropine-resistant vasodilatation, Lundberg and coworkers stated "interactions with cholinergic mechanisms cannot be excluded".

1.5 Adrenergic Vasodilator Mediators

Skinner and Webster (1968a, b) suggested that beta-adrenergic mechanisms may, in association with a cholinergic mechanism, mediate atropine-resistant vasodilatation in the cat submandibular gland. These workers showed that a marked vasodilatation was produced by a close arterial or intravenous injection of isoprenaline, and these vasodilator responses could be blocked by propranolol (a beta adrenoceptor blocking drug). They also showed that propranolol reduced but did not abolish the vasodilatation induced by chorda stimulation and in this respect was similar to atropine. While neither atropine nor propranolol abolished chorda-induced vasodilatation, the combination of these two drugs was more effective than either one alone.

However, in an earlier study, Davey *et al.* (1965), by pretreating cats with reserpine (1.0 mg kg^{-1}) 24 hours prior to experimentation, were unable to reduce or abolish atropine-resistant vasodilatation induced by chorda stimulation, even though the vascular response to sympathetic nerve stimulation was abolished. Davey *et al.* suggested that it "would therefore seem that an adrenergic mechanism is not involved in the atropine-resistant vasodilatation in the submaxillary gland of the cat".

Schachter and Beilenson (1968) confirmed the results of Davey *et al.* with reserpine. However, they only occasionally observed some suppression of chorda-induced vasodilatation by propranolol, contrary to other workers (Skinner and Webster, 1968a, b). Schachter and Beilenson (1968) demonstrated that this reduction was always paralleled by a corresponding

reduction in the vasodilatation produced by an injection of acetylcholine close arterially and suggested that these effects of propranolol may be due to an anticholinergic action of this drug.

1.6 Purinergic Mediation of Atropine-Resistant Vasodilatation

The idea of purinergic neurotransmission was first developed by Burnstock (1972, 1975). He suggested that the principal active substance released by non-cholinergic, non-adrenergic inhibitory neurons is a purine nucleotide, probably ATP.

Subsequently, Jones and Mann (1977) suggested that cyclic AMP may act as a secondary messenger in both sympathetic and parasympathetic vasodilator responses, since cAMP mimics the nerve-induced responses in the cat submandibular gland and these responses were potentiated by phosphodiesterase inhibitors. In a later study, Jones *et al.* (1980) demonstrated that intra-arterial administration of a number of purine compounds to the cat submandibular gland led to an increased blood flow, with ATP and ADP being the most potent, while dibutyryl cAMP and cGTP were essentially ineffective.

1.7 Other Vasodilator Mechanisms

As previously mentioned, during salivary secretion the metabolic activity of the salivary gland is greatly increased (Terroux *et al.*, 1959). The possibility therefore exists that besides the aforementioned "specific" dilator mechanisms, metabolic factors could be linked to the hyperemic response following nerve stimulation. In exercising skeletal muscle, the markedly increased metabolism leads to a pronounced regional hyperosmolality, which has been shown to be an important causal factor in the exercise hyperemia response (Mellander *et al.*, 1967; Lundvall, 1972). Subsequently, Lundvall and Holmberg (1974) showed that a pronounced tissue hyperosmolality develops in the cat's submandibular gland during parasympathetic activation and suggested that this factor contributes to the functional vasodilator response as well.

1.8 Aim of the Present Study

The purpose of the present study was to re-examine the old idea of acetylcholine (ACh) as a major transmitter for vasodilatation in the cat submandibular gland using selective muscarinic antagonists, depleters and potentiators of cholinergic responses. Also, the possible contribution of vasoactive intestinal polypeptide (VIP) and a number of other putative transmitters were investigated.

2. MATERIALS AND METHODS

2.1 Animals and Anesthesia

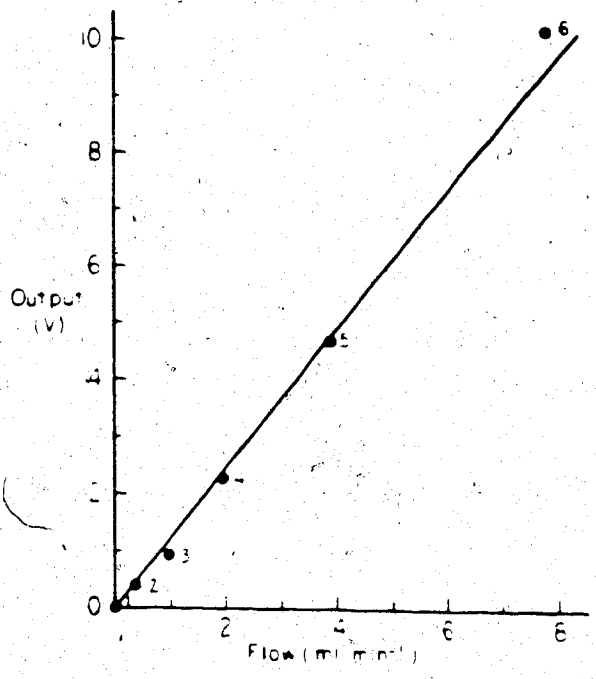
Cats of either sex were starved overnight and allowed water *ad libitum*. Anesthesia was induced in adult cats (2.7-5.3 kg) of either sex by chloroform (J.T. Baker Chemical Co.) in a closed box (aerated with 100% oxygen). Ether (Fisher Scientific Co.) on a face mask was used for temporary maintenance of anesthesia until a venous cannula was inserted. Anesthesia was maintained throughout the experiment by chloralose (Sigma Chemical Co.) (80 mg kg^{-1}). Cats were intubated via the trachea to aid artificial respiration when required.

In chronic experiments, cats were anesthetized with sodium pentobarbitone (Nembutal; 35 mg kg^{-1} i.p.) (MTC Pharmaceuticals).

2.2 Blood Flow Measurements

Blood flow through the submandibular gland was measured using a forced convection flowmeter with a probe in the external jugular vein (Karpinski, 1971; Karpinski and Vaneldik, 1971). All veins draining into the external jugular vein were ligated, except that from the submandibular gland. The flowmeter is of a thermal type, designed to record rapidly a wide range of volume flows. The sensor device is a thermistor kept at a constant temperature utilizing negative feedback. As blood flows past the sensor, it cools by forced convection. The voltage which controls the amount of power required to maintain the thermistor at a constant temperature is a function of the velocity of the blood and shows a linear relationship (Figure 1). A flow-insensitive reference thermistor compensates for any change in blood temperature. Both thermistors are contained in a nylon probe which is a tube 2 cm in length and 1.5-2.0 mm internal diameter. This tube size is chosen so as not to increase venous resistance, and since the probe has a constant area, the device can be calibrated in terms of volume flow. Before insertion into the external jugular vein, the probe was initially filled with 1% heparin in 0.9% saline to aid in the prevention of clot formation.

Figure 1. Calibration curve for forced convection flowmeter and probe. The calibrating fluid is saline. Each point (1—6) is the mean of five measurements. The SEM's corresponding to the different points (1—6) are ±0.02, ±0.01, ±0.02, ±0.04, ±0.14 and ±0.02.



Calibration curves for each probe were obtained using a calibrated syringe driver and 0.9% saline or blood as the calibrating fluid (Figure 1). The probe and tubing were maintained at 37°C in a constant temperature bath during the calibration procedure. The output voltage was measured using a digital volt meter. The sensitivity of the flowmeter system is high and can measure flows as low as 0.01 ml min^{-1} up to 40 ml min^{-1} with appropriate probes. Changes in flow rate were measured on a Sanborn 7700 series recorder (see Figure 2 for experimental set-up).

2.3 Nerve Stimulation

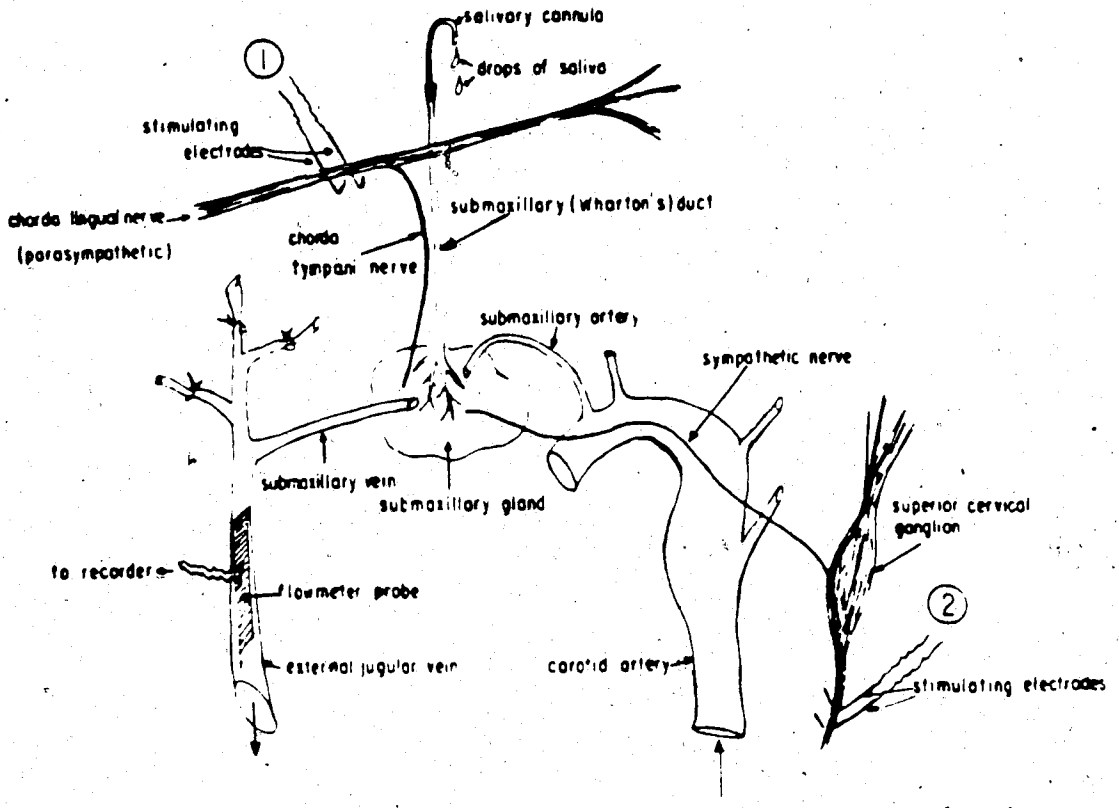
2.3.1 Parasympathetic

The parasympathetic nerve supply to the cat submandibular gland runs in the chorda tympani nerve, a branch of the chorda lingual nerve. The chorda tympani nerve was exposed in the region where it crosses the submandibular and sublingual ducts and, following further dissection, was cut as near as possible to its point of exit from the skull. Care was taken during dissection not to damage the submandibular duct. The distal end of the cut nerve was mounted on bipolar platinum electrodes and immersed in a pool of liquid paraffin. In all cases, parasympathetic nerve stimulation was assumed to be preganglionic. The nerve was stimulated supramaximally (7-10 V) with square wave pulses of 0.5 msec duration at a frequency of either 10 or 20 Hz using a WP Instruments stimulating isolator, for periods ranging from 1 second to 60 seconds. Stimulation was either continuous or intermittent, with the intermittent stimulation being interrupted either manually or by using a WP Instruments interval generator.

2.3.2 Sympathetic

In the cat, the cervical sympathetic nerve could be exposed running near the carotid artery. The sympathetic nerve could be readily separated from the vago-sympathetic trunk and was stimulated as described for the parasympathetic nerve; however, in all cases, a frequency of

Figure 2. Schematic representation of the experimental set-up for studying blood flow. 1. Stimulation of parasympathetic innervation, 7—10 V, 0.5 msec, 10 or 20 Hz. 2. Stimulation of sympathetic innervation, 7—10 V, 0.5 msec, 20 Hz.



20 Hz was employed for periods of 2 to 5 seconds.

2.4 Salivary Duct Cannulation

The cat submandibular duct was separated from the sublingual duct, and identified by stimulation of the chorda tympani, which caused the duct to expand due to nerve-induced salivary secretion. The duct was then cannulated with a fine glass cannula approximately 10 mm rostral to the point at which the duct was crossed by the chorda lingual nerve.

2.5 Blood Pressure and Drug Administration

Blood pressure was monitored from the femoral artery using a Statham P23 (0-75 cm Hg) transducer and recorded on a Sanborn 7700 series recorder. All cannulae were flushed with 1% heparin in 0.9% saline. Intravenous injections were made through a cannula in a femoral vein. Heparin (10 mg kg^{-1}) was administered intravenously. Close arterial injections were given retrogradely via the ipsilateral lingual artery. Intraductal injections were made via the ipsilateral submandibular duct cannula which, in all cases, had been previously clamped to ensure access of the drug to the gland. Table 1 lists the drugs used and the companies or individuals from whom they were obtained or donated.

Table 1. Table of Drugs Used and Sources From Which They Were Obtained

Drugs (and Abbreviations)	Source(s)
Acetylcholine chloride (ACh)	Sigma Chemical Corp.
Adenosine triphosphate (ATP)	Sigma Chemical Corp.
Atropine sulphate	Nutritional Biochemicals Corp.
Avian pancreatic polypeptide (APP)	Dr. J.R. Kimmel University of Kansas School of Health Sciences Peninsula Laboratories
Bradykinin (BK)	Sigma Chemical Corp. Boehringer Mannheim
Choline chloride	MC/B Reagents
4-diphenylacetoxy-N-methylpiperidine (4-DAMP)	Dr. R. Barlow University of Bristol
Pentamethylene-bis-4-diphenyl-N-methyl-piperidine (bis, 4-DAMP)	Dr. R. Barlow University of Bristol
Eserine sulphate	Nutritional Biochemicals Corp.
Hemicholinium-3	Sigma Chemical Corp.
Heparin	Sigma Chemical Corp.
Hexamethonium (HX)	Sigma Chemical Corp.
Secoverine HCl	Duphar Pharmaceuticals Ltd., The Netherlands

Substance P (SP)	Sigma Chemical Corp.
Vasoactive intestinal polypeptide (VIP)	Dr. V. Mutt Karolinska Institute Boehringer Mannheim Sigma Chemical Corp.
VIP antiserum	Calbiochem Ltd.

3. RESULTS

3.1 Blood Flow Through the Cat Submandibular Gland — Resistance to Inhibition by Atropine

Experiments were performed on a total of thirty nine cats. Resting blood flow through the submandibular gland of the cat showed a range of 0.6—2.5 ml min^{-1}. Representative traces of a number of experiments will be shown.

Stimulation of the parasympathetic nerve supply to the submandibular gland (chorda lingual nerve, 10 or 20 Hz, 8 V, 0.5 msec pulse width for 1 to 20 seconds) caused salivation and a marked increase in blood flow (3 to 35-fold) which was usually rapid in onset (latency of less than 1 second) and subsided upon cessation of stimulation (Figure 3). Stimulation of the sympathetic innervation to the submandibular gland (20 Hz, 8 V, 0.5 msec pulse width for 5 seconds) caused salivation and an intense vasoconstriction, followed by a variable vasodilatation upon cessation of stimulation (Figure 3).

Following the administration of atropine (0.5 mg kg^{-1} i.v.), the increase in blood flow and salivation induced by a close arterial infusion of acetylcholine are abolished. The vasodilatation induced by parasympathetic (chorda) nerve stimulation is usually reduced slightly. The vascular effects induced by sympathetic nerve stimulation are unaffected by atropine given intravenously, intra-arterially or intra-ductally. The vasodilatation induced by vasoactive intestinal polypeptide given intra-arterially is unaffected by atropine. The secretory response associated with parasympathetic nerve stimulation is totally abolished by atropine, suggesting a totally cholinergic mechanism for parasympathetic induced salivary secretion. This is in accord with electrophysiological studies in which it was demonstrated that the main activation of acinar and ductal cells is via ACh since atropine blocks the secretory potentials in submandibular cells which normally occur during parasympathetic nerve stimulation (Lundberg, 1958).

Figures 4 and 5 show the effects of intravenous (0.5 mg kg^{-1}) administration of atropine on chorda-induced vasodilatation at varying durations of stimulation. It is clear that

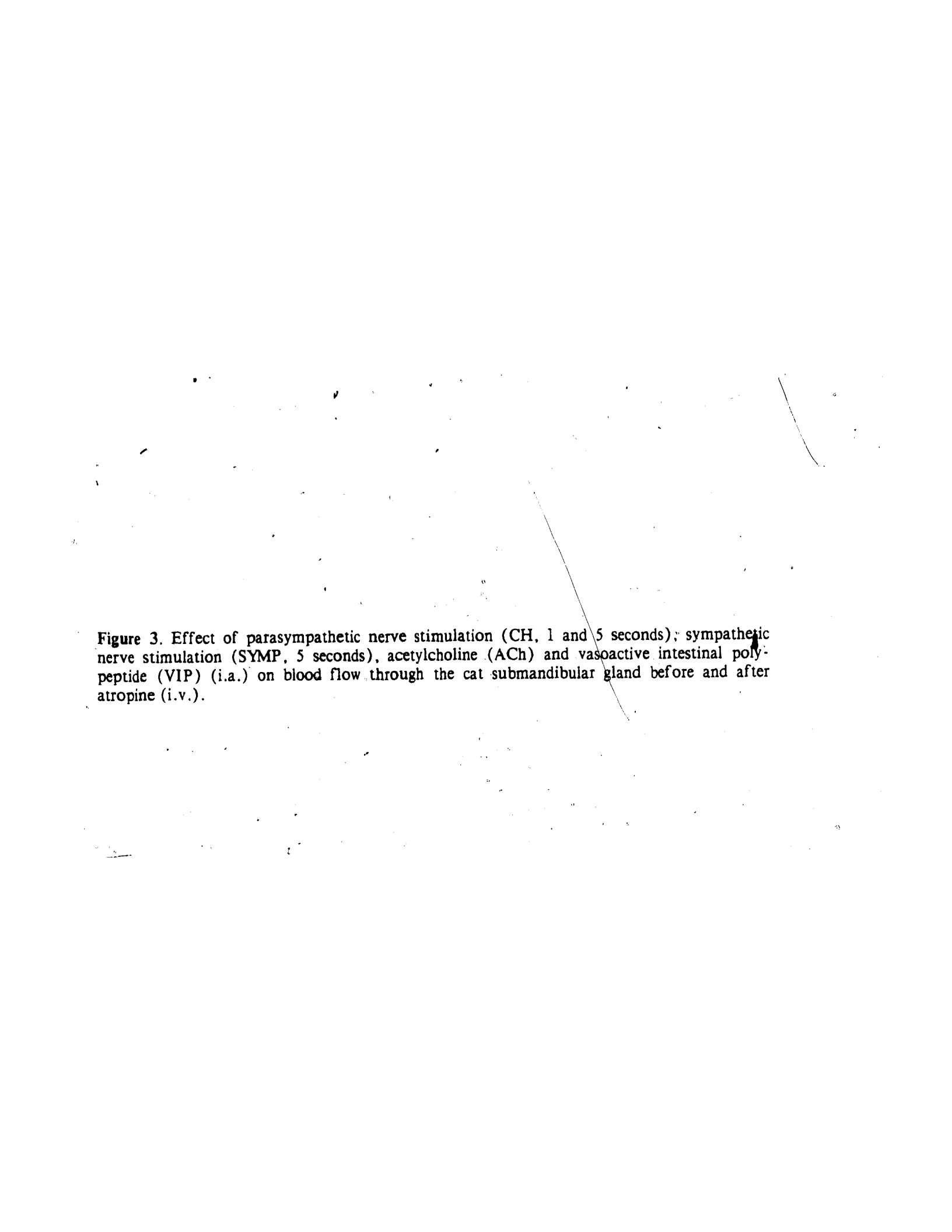


Figure 3. Effect of parasympathetic nerve stimulation (CH, 1 and 5 seconds), sympathetic nerve stimulation (SYMP, 5 seconds), acetylcholine (ACh) and vasoactive intestinal polypeptide (VIP) (i.a.) on blood flow through the cat submandibular gland before and after atropine (i.v.).

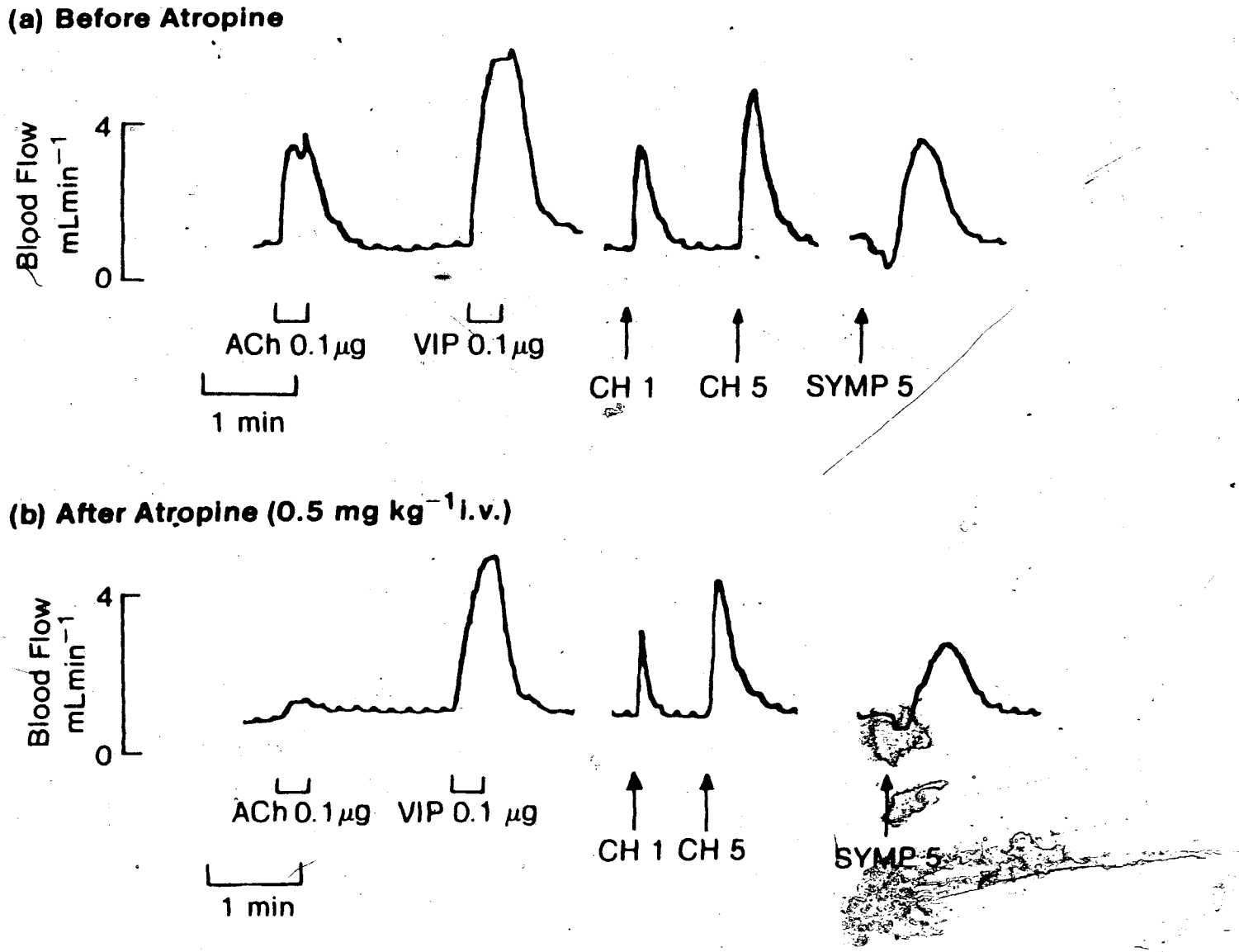
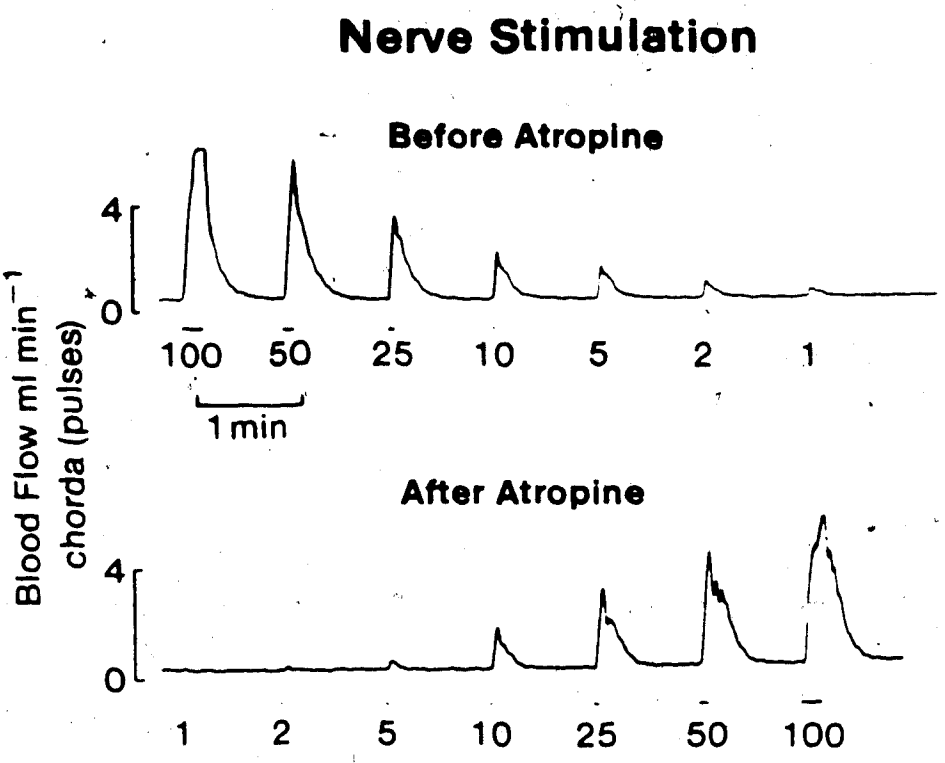

(a) Before Atropine
Blood Flow
mLmin⁻¹
4
0
ACh 0.1μg
VIP 0.1μg
CH 1
CH 5
SYMP 5
1 min
(b) After Atropine (0.5 mg kg⁻¹ I.v.)
Blood Flow
mLmin⁻¹
4
0
ACh 0.1μg
VIP 0.1 μg
CH 1 CH 5
SYMP 5
1 min

Figure 4. Representative trace from one experiment of the effect of atropine (i.v. 0.5 mg kg^{-1}) on chorda-induced vasodilatation at different durations of stimulations but constant frequency (1—100 pulses).



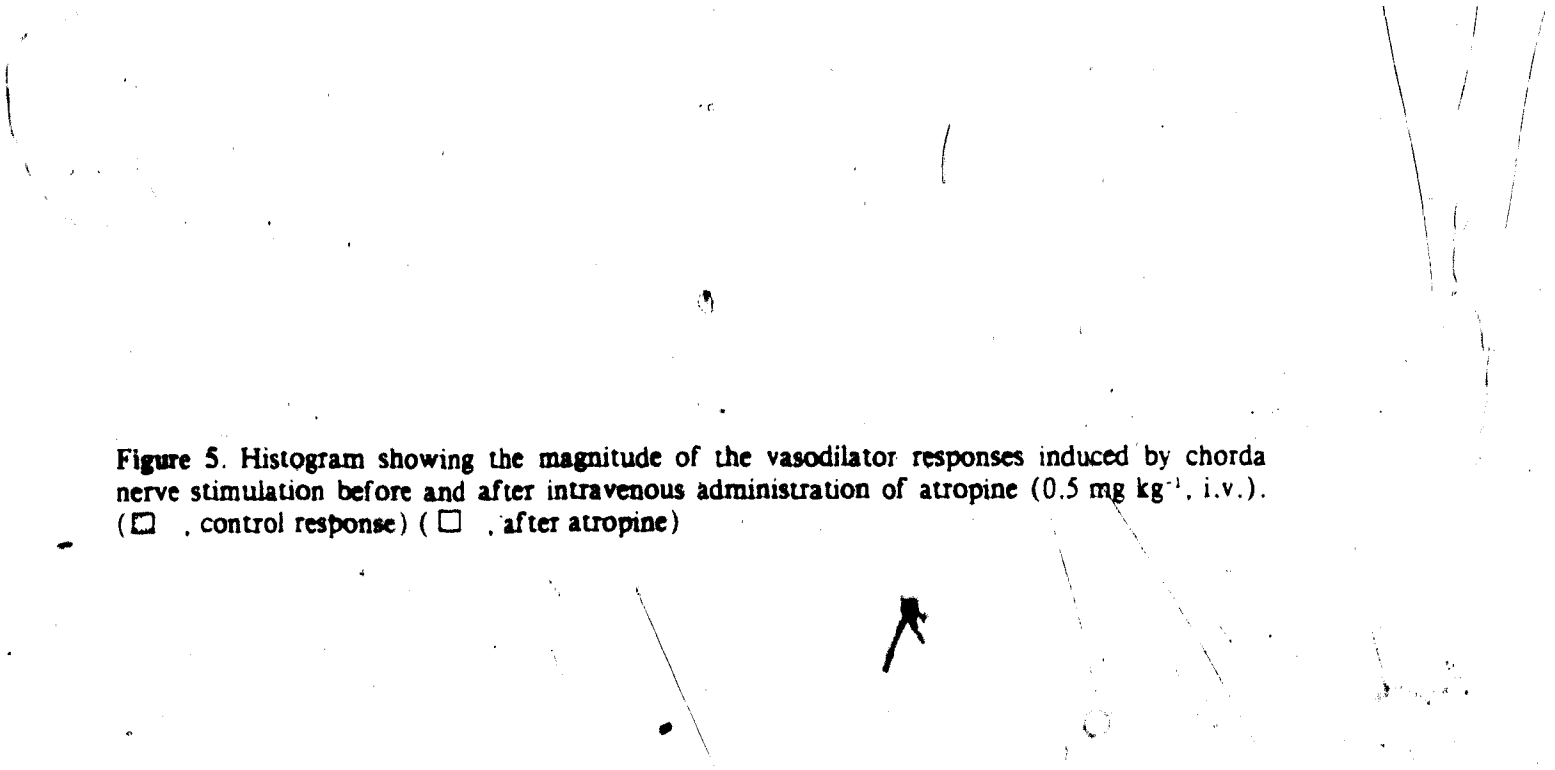
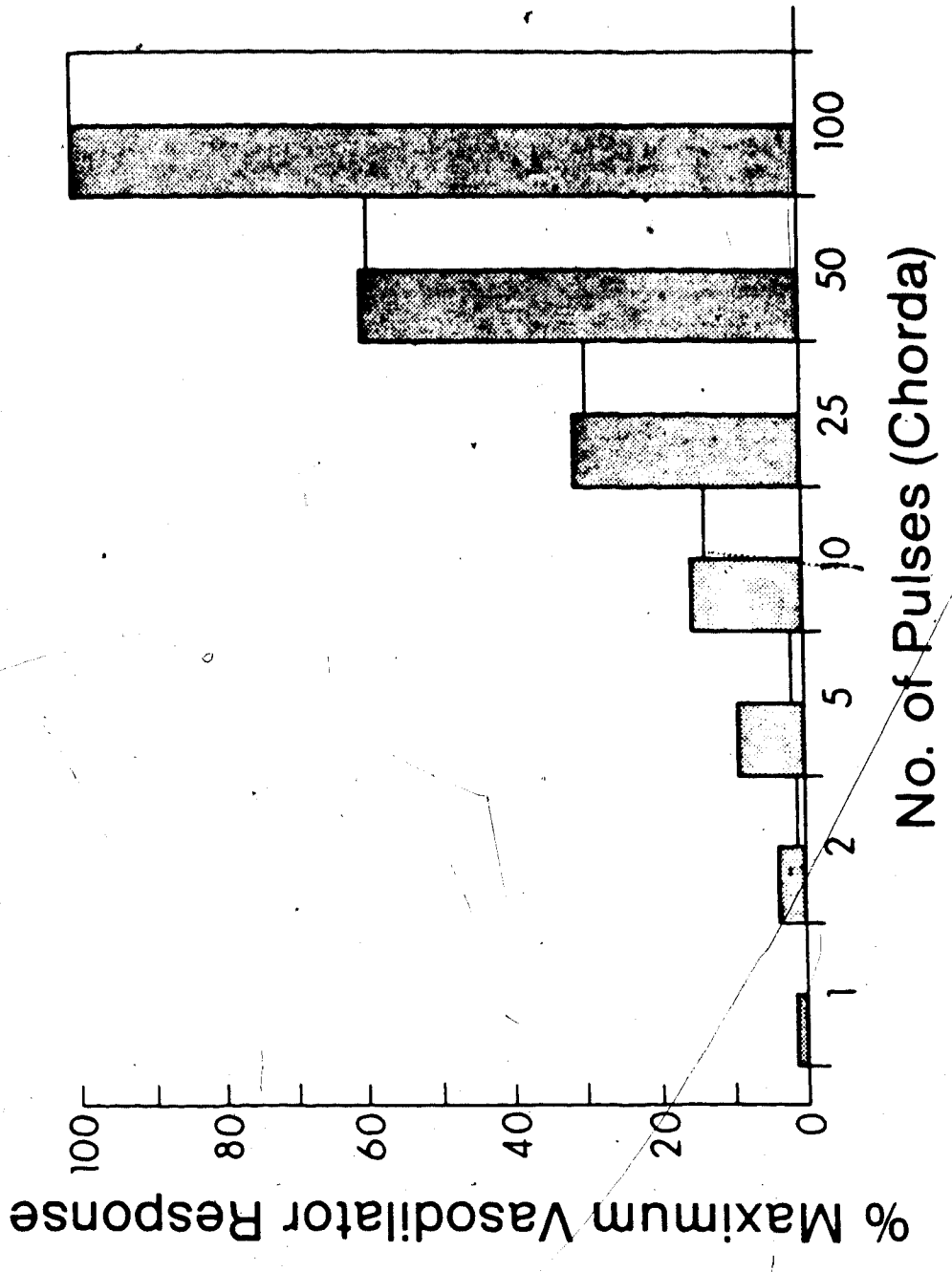


Figure 5. Histogram showing the magnitude of the vasodilator responses induced by chorda nerve stimulation before and after intravenous administration of atropine (0.5 mg kg^{-1}, i.v.). (■ , control response) (□ , after atropine)


% Maximum Vasodilator Response
0
20
40
60
80
100
No. of Pulses (Chorda)
1
2
5
10
25
50
100

atropine given intravenously significantly reduces these vascular responses, particularly at low stimulation duration (i.e. 1—5 pulses). These results are in accord with a previous report where an appreciable reduction in the vasodilator response induced by chorda nerve stimulation was observed, especially when low frequencies of stimulation were used (Darke & Smaje, 1972).

Intraductal atropine effectively abolishes chorda-induced vasodilatation at 2 pulses (Figures 6 and 7). Even at higher stimulation durations of 10, 25, 50 and 100 pulses, the vasodilatation is reduced by at least 50%. It appears that intra-ductal atropine is affecting only postganglionic muscarinic receptors, since it is now accepted that most of the parasympathetic ganglia are located outside the gland (see Section 3.3.1).

It should be noted that the effects of atropine on vasodilator responses induced by parasympathetic nerve stimulation vary markedly between animals. However, it is apparent that atropine, especially at low frequencies or duration of stimulation, effectively reduces chorda-induced vasodilator responses.

3.2 Effects of Putative Transmitters on Blood Flow

Acetylcholine, bradykinin and VIP, when injected intra-arterially, caused dose-dependent increases in blood flow through the submandibular gland (Figures 8 and 9). Whereas all three substances caused an increase in blood flow, only acetylcholine caused salivation. The rapidity of onset of the vasodilatation caused by a close-arterial infusion of acetylcholine closely resembles the response following chorda stimulation. However, the vasodilatation caused by VIP was usually slower in onset, more protracted and more variable than that caused by acetylcholine.

On a molar basis, VIP was the most potent of these three vasodilator substances, being four to five times more potent than bradykinin and twenty to thirty times more potent than acetylcholine, whilst bradykinin is five times more potent than acetylcholine. The vasodilatation caused by both VIP and bradykinin was atropine resistant and was not subject to tachyphylaxis upon repeated infusions of small doses.

Figure 6. Representative trace from one experiment of the effect of atropine (i.d. 0.5 mg) on chorda-induced vasodilatation at different durations of stimulation but constant frequency (2—100 pulses).

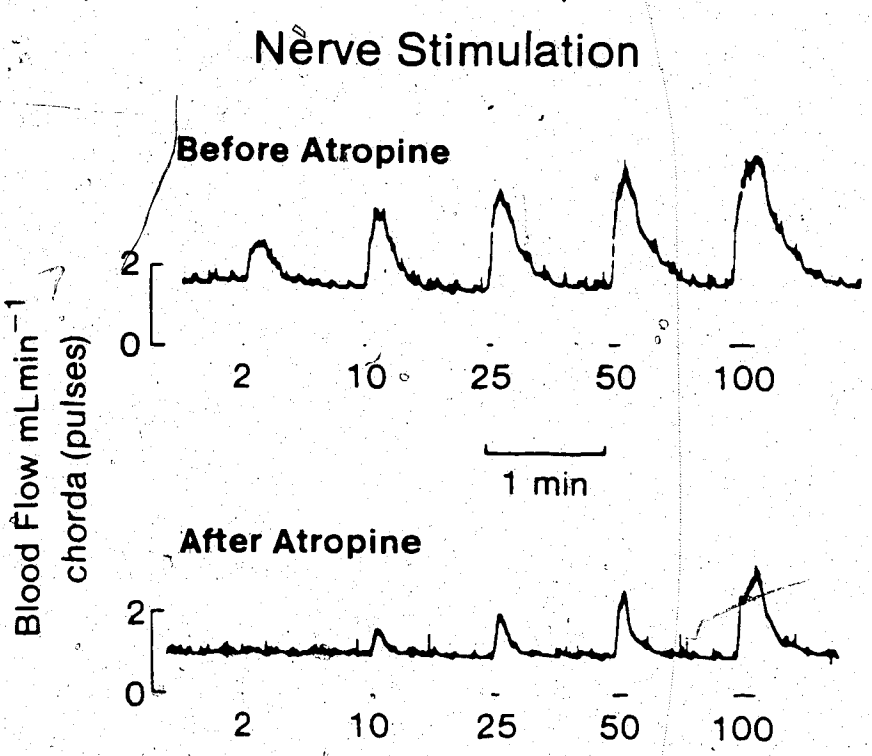


Figure 7. Histogram showing the magnitude of the vasodilator responses induced by chorda nerve stimulation before and after intraductal administration of atropine (0.5 mg) (□ , control response) (□ , after atropine).

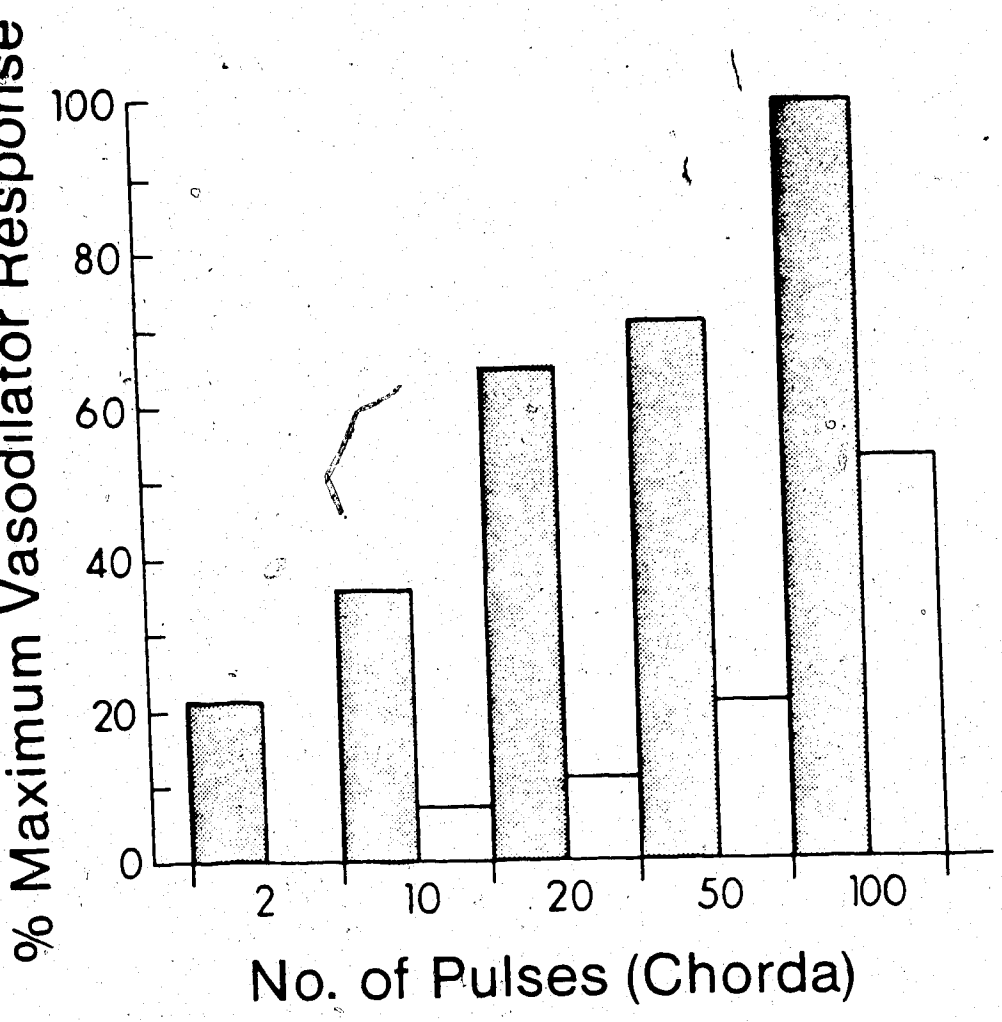
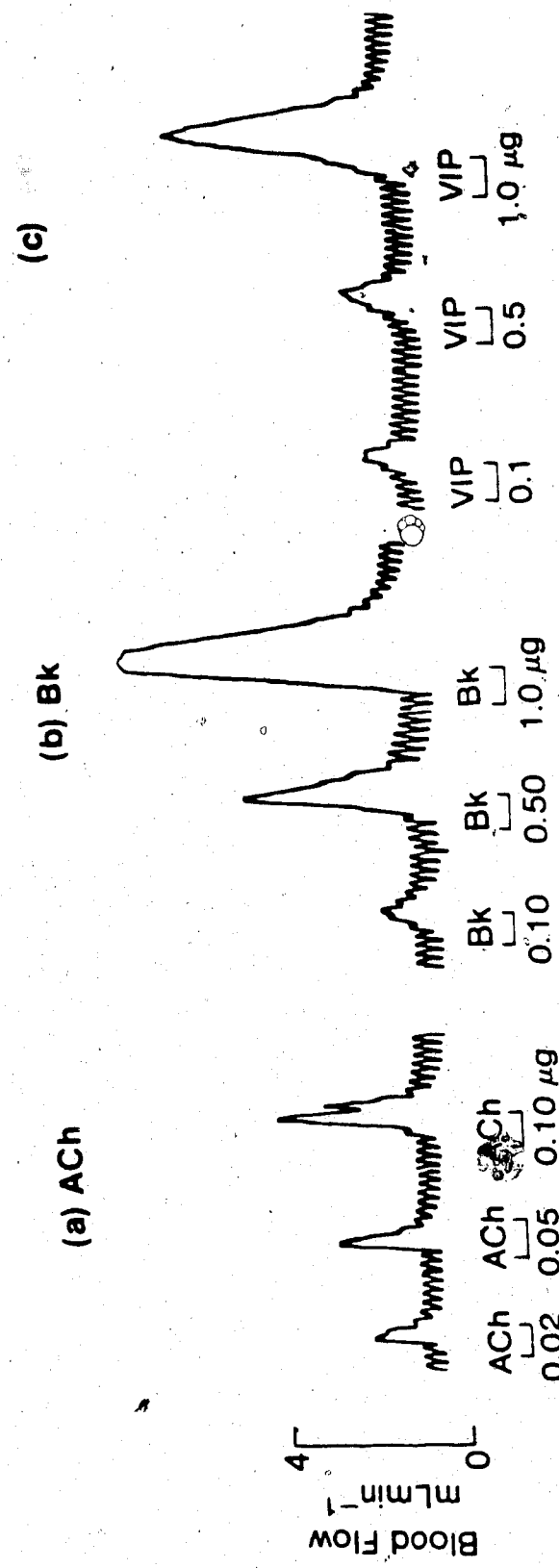


Figure 8: Representative trace of the dose-dependent increases in blood flow through the cat submandibular gland induced by close-arterial injections of (a) acetylcholine (ACh), (b) bradykinin (Bk), and (c) vasoactive intestinal polypeptide (VIP) (in the same cat).


(a) ACh
(b) Bk
(c)
Blood Flow
mLmin⁻¹
4
0
1 min
ACh 0.02
ACh 0.05
ACh 0.10 μg
Bk 0.10
Bk 0.50
Bk 1.0 μg
VIP 0.1
VIP 0.5
VIP 1.0 μg

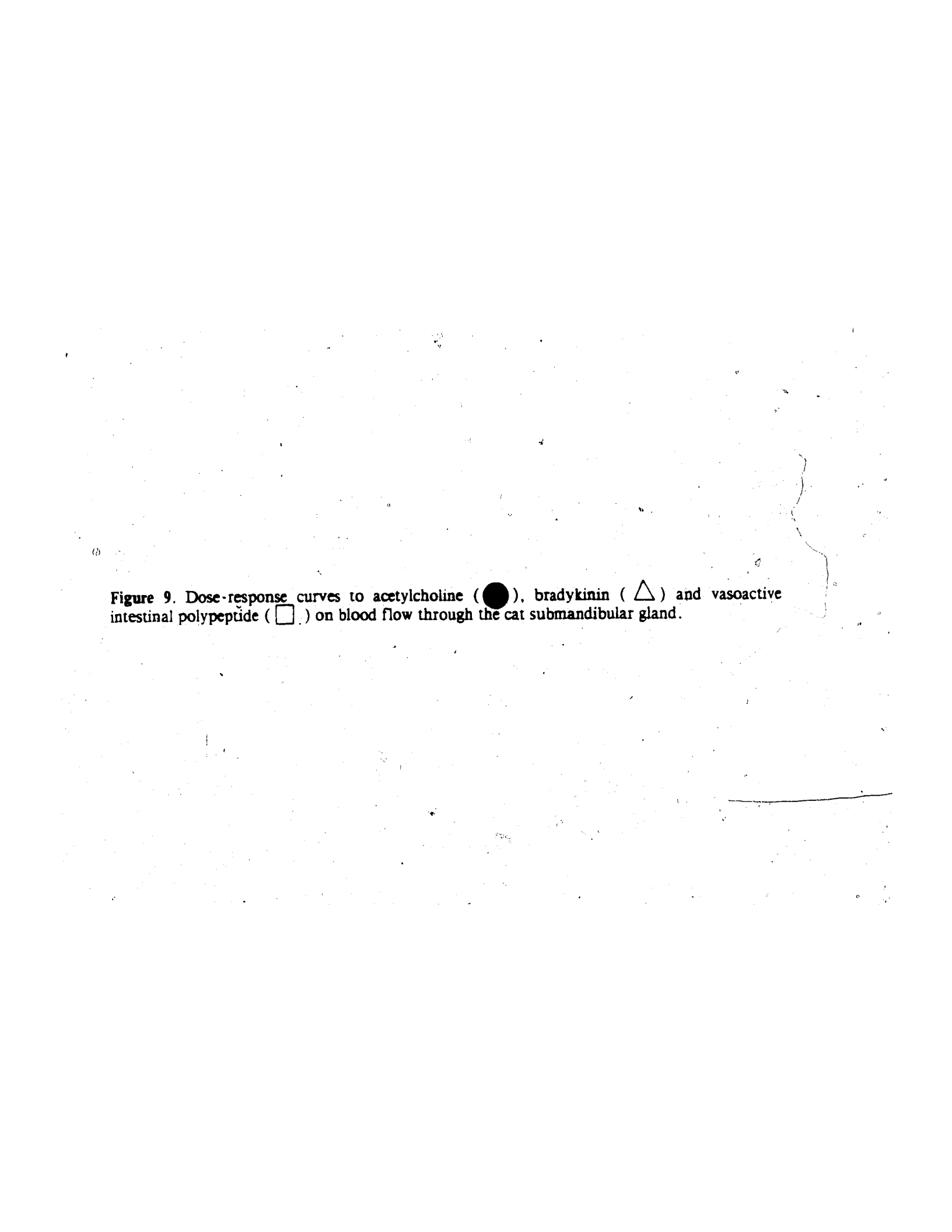
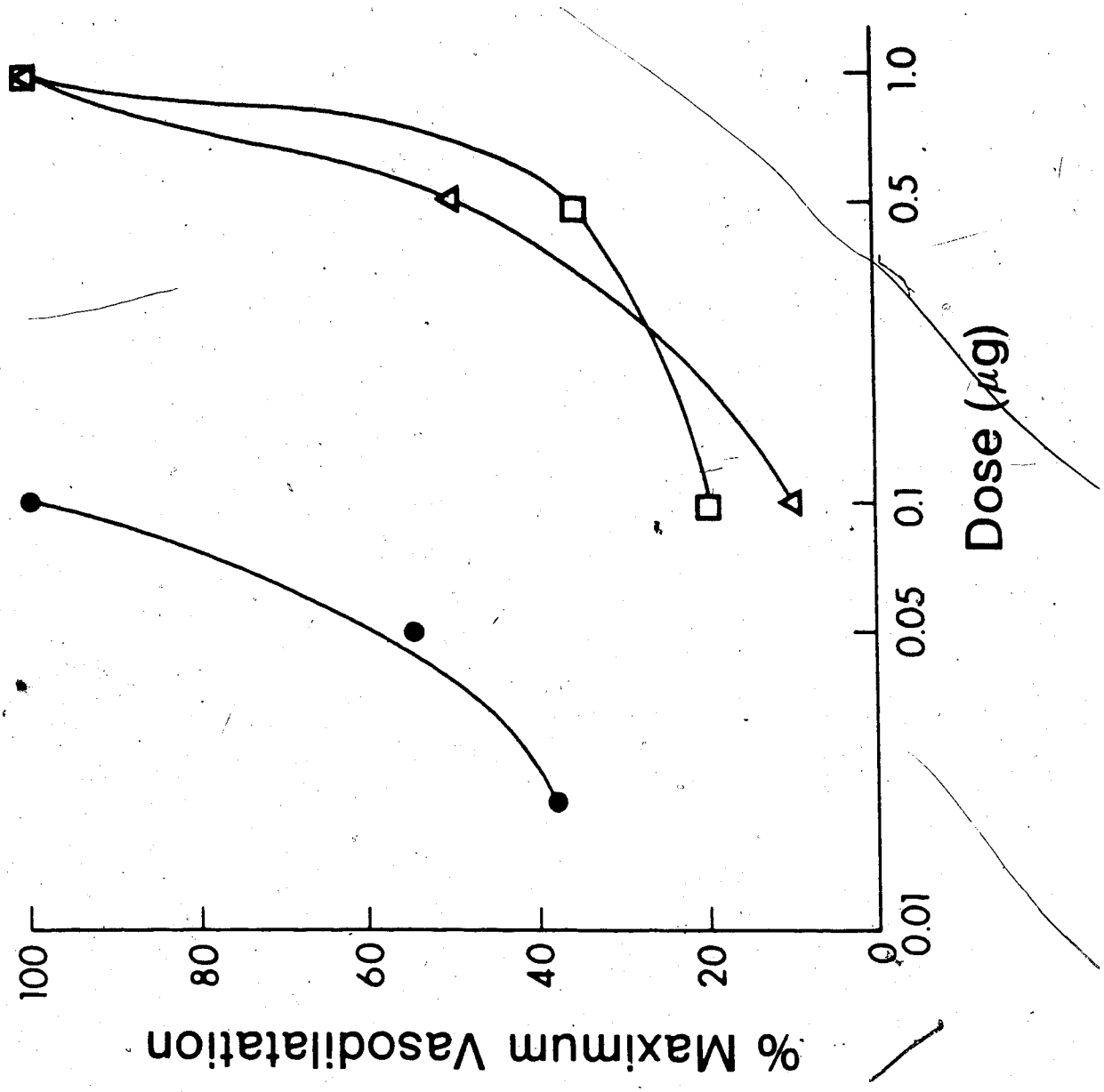


Figure 9. Dose-response curves to acetylcholine (●), bradykinin (△) and vasoactive intestinal polypeptide (□) on blood flow through the cat submandibular gland.


% Maximum Vasodilatation
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1.0
Dose (μg)

Close-arterial injections of ATP (0.2—50μg; Figure 10) caused small, slow and variable increases in blood flow which were often accompanied by falls in systemic blood pressure (not shown). the responsiveness between animals varied markedly, such that in some cases no vasodilatation could be observed following ATP. ATP did not induce salivation. In an attempt to desensitize the submandibular gland vasculature to ATP, it was noted that even during a continuous infusion of ATP (2.5 mg; 0.4 mg min^{-1}; Figure 11), it was still possible to elicit "normal" chorda vasodilatation compared to control chorda responses in the same animal. In an earlier study, Jones *et al* (1980) reported that ATP given intra-arterially was the most potent of the purine compounds and that phosphodiesterase inhibitors (theophylline and 3-isobutyl-1-methylxanthine) potentiated the effects of chorda vasodilatation.

Close-arterial injections of substance P (0.5—2.0 μg; Figure 12) also caused variable changes in blood flow which were always accompanied by falls in systemic arterial blood pressure. Even when substance P did not cause a change in blood flow (0.5 μg i.a.), a fall in blood pressure was observed. The fact that substance P infusions were always accompanied by drops in blood pressure suggests that substance P is an unlikely candidate as the mediator of atropine-resistant vasodilatation in the submandibular gland of the cat.

3.3 Evidence For and Against Acetylcholine as a Transmitter

There is some evidence for ACh being the transmitter of atropine-resistant vasodilatation, but there are also some compelling arguments against it. In favour of ACh is the fact that its close-arterial injection mimics the time course of nerve-induced vasodilatation, both in onset and duration (Bhoola *et al.*, 1965; Schachter & Beilenson, 1968). However, an important objection is the fact that whereas this nerve-induced vasodilatation is largely resistant to atropine, the vasodilator effects of ACh injected close-arterially are readily blocked by small doses of this drug (Bhoola *et al*, 1965; Figure 3).

Despite the above reservations, the following results provide evidence in support of ACh as a mediator.

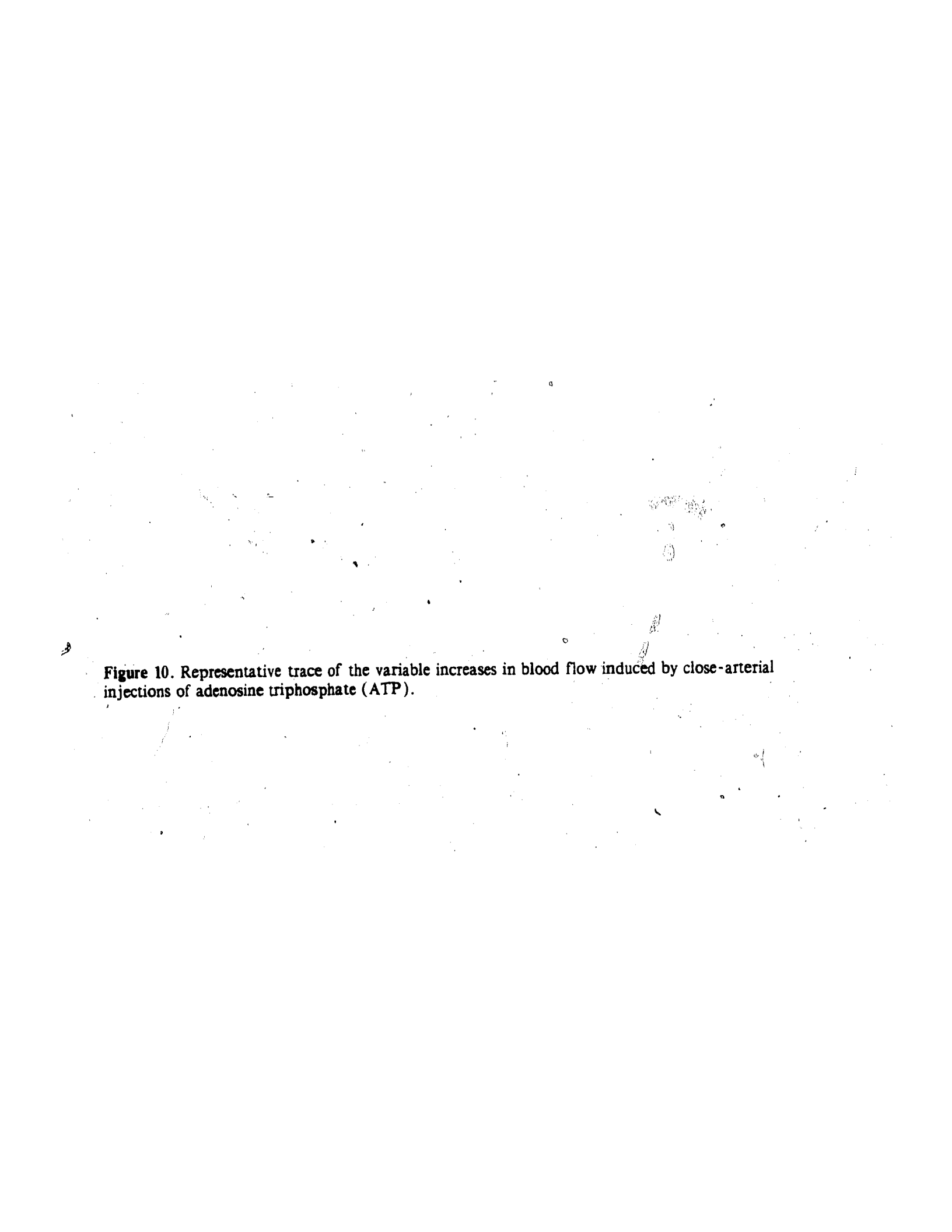


Figure 10. Representative trace of the variable increases in blood flow induced by close-arterial injections of adenosine triphosphate (ATP).


Blood Flow
mL min⁻¹
4
0
1 min
ATP 0.2 μg
ATP 1.0 μg
ATP 10 μg
ATP 50 μg

Figure 11. Superimposed chorda-induced (CH, 5 and 10 seconds) vasodilator responses during a continuous close-arterial infusion of ATP (2.5 mg).

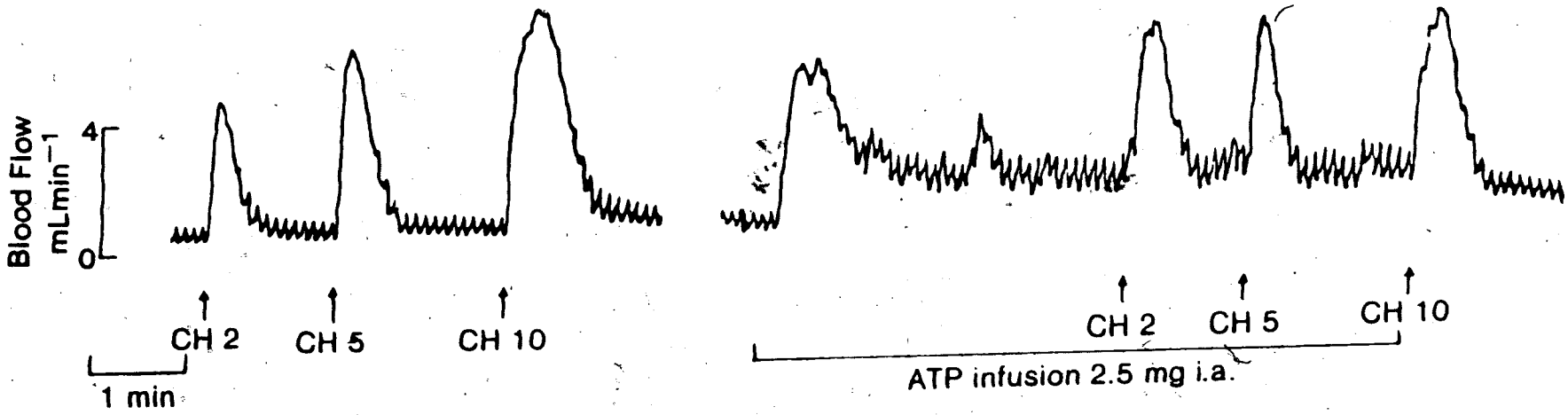
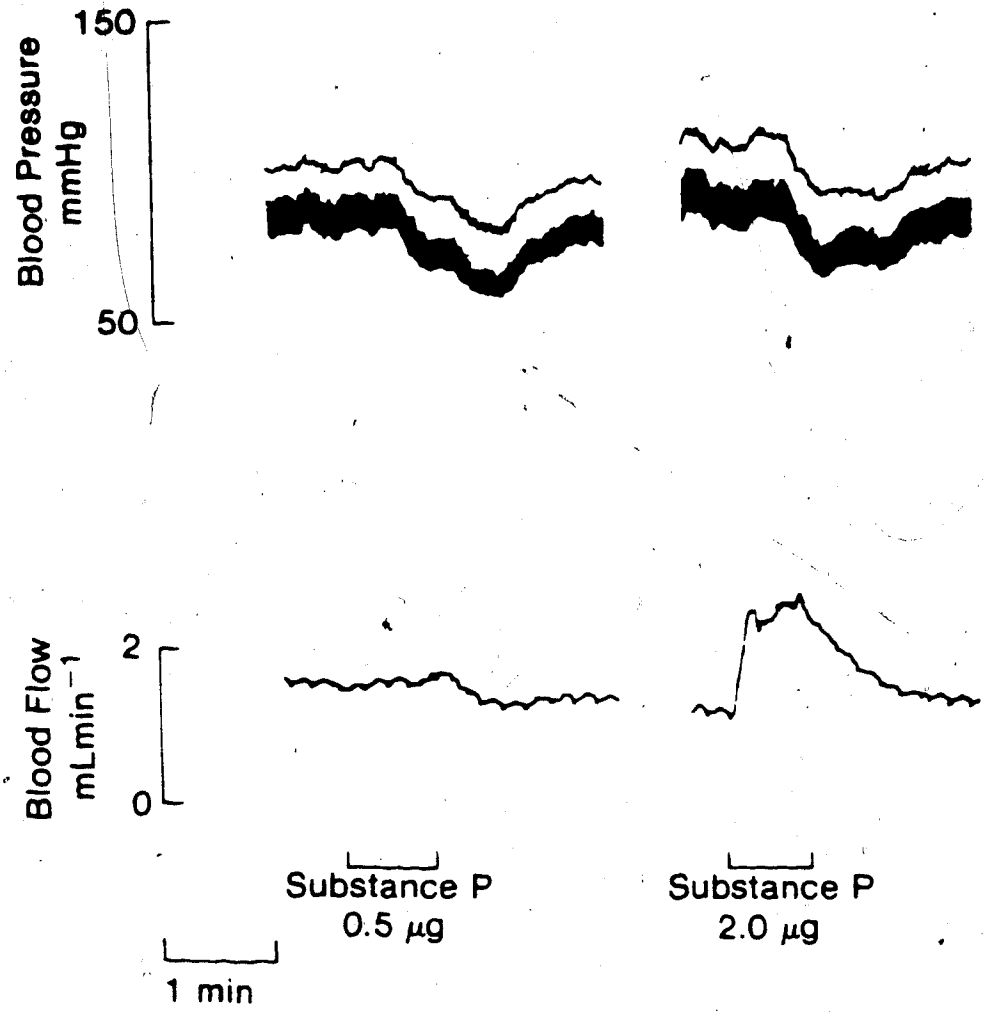

Blood Flow
mLmin^{-1}
4
0
CH 2
CH 5
CH 10
1 min
CH 2
CH 5
CH 10
ATP infusion 2.5 mg i.a.

Figure 12. Representative trace of the variable increases in blood flow caused by close-arterial injections of substance P (0.5—2.0 μg i.a.). Note: Drop in systemic blood pressure.



3.3.1 Effects of Hexamethonium

Both the secretory and vasodilator effects of chorda stimulation can be blocked readily by an intravenous injection of hexamethonium (a bisonium compound which has a highly specific action on ganglionic nicotinic receptors; Figure 13). This is in agreement with earlier findings of other workers (Hilton & Lewis, 1956; Lundberg *et al.*, 1980a). However, the intra-ductal administration of hexamethonium causes only a small decrease in chorda vasodilatation (Figure 13) and a small decrease in salivation (Emmelin *et al.*, 1954). This is consistent with the generally accepted view that the majority of parasympathetic ganglia are outside the gland, and would suggest that a drug administered intra-ductally would have a minimal effect on pre-ganglionic nicotinic or muscarinic receptors.

3.3.2 Effects of Hemicholinium

In contrast to the limited effect of intra-ductal hexamethonium, the intra-ductal administration of hemicholinium-3 (1 mg i.d., 50 μg/min for 20 minutes) accompanied by parasympathetic nerve stimulation to facilitate depletion of stores of acetylcholine completely abolished the vasodilator effect of chorda nerve stimulation (Figure 14). During the depletion experiments, atropine was given intravenously (500 $\mu g\ kg^{-1}$ i.v.) to prevent salivation during parasympathetic (chorda) nerve stimulation, thus preventing the loss of hemicholinium. Hemicholinium-3, which has some structural similarities to choline (the precursor for acetylcholine), appears to compete for the choline uptake carrier in the nerve membrane, thus preventing the synthesis and replacement of depleted acetylcholine stores. The vasodilatation could be restored by intra-ductal administration of choline chloride (1 mg). The conclusion one is led to from these observations is that at least part of the action of hemicholinium is due to the depletion of ACh at postganglionic sites where it normally participates in nerve-induced vasodilatation.

Figure 13. Representative trace of the effects of hexamethonium given intravenously and intraductally on chorda-induced vasodilatation (CH 1 and 5 seconds).

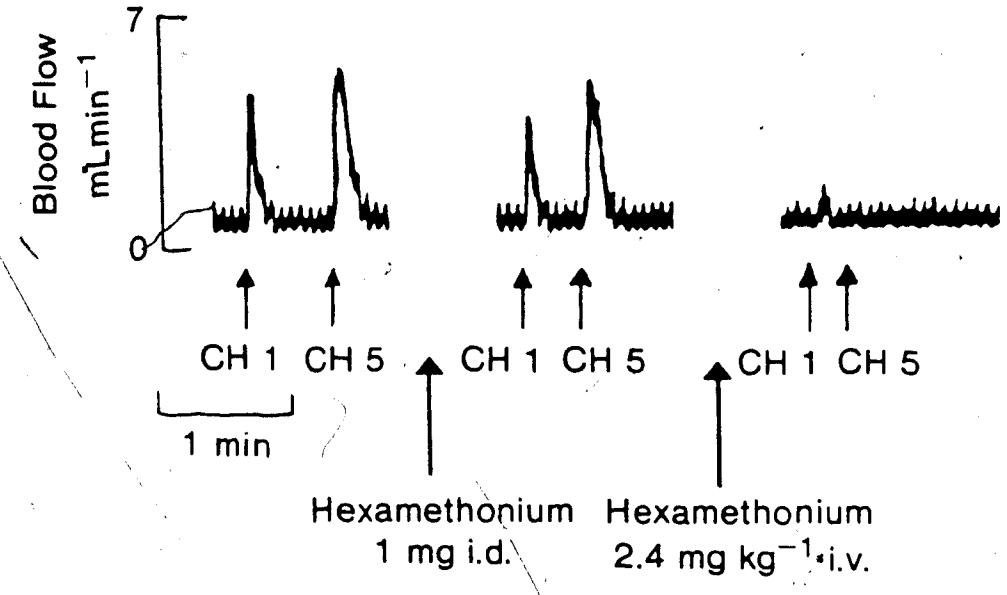
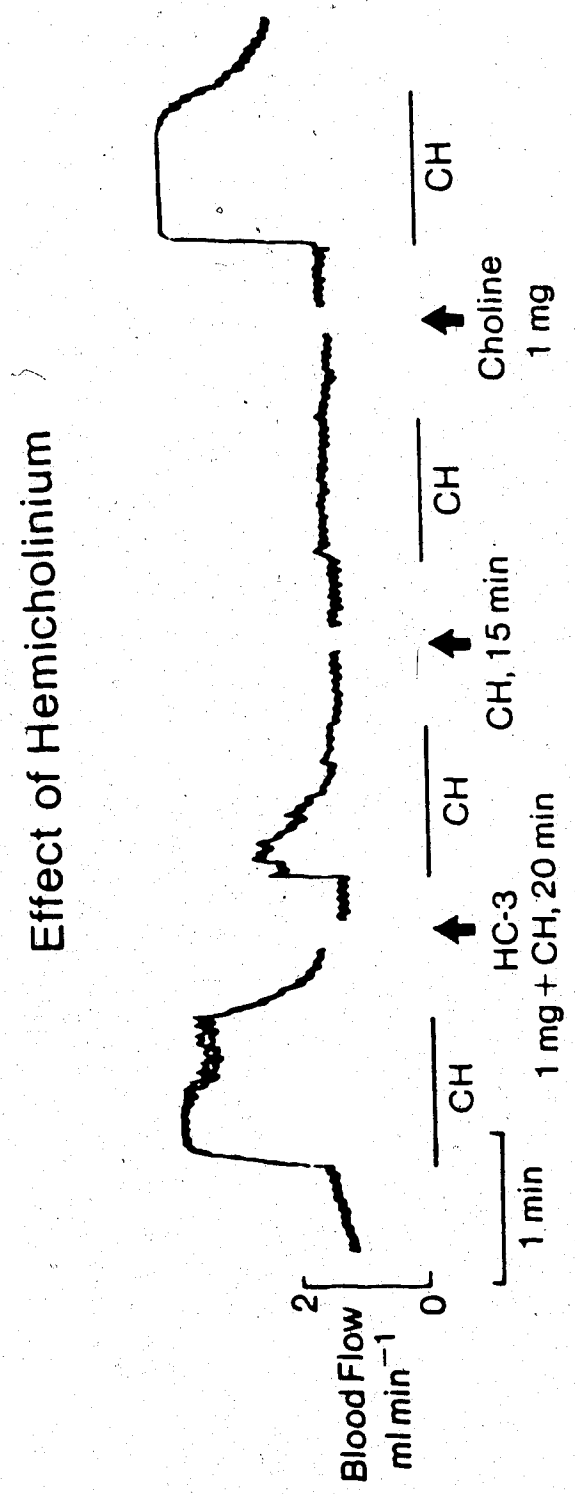


Figure 14. Representative trace of the effects of intraductal hemicholinium (HC-3, 1 mg i.d.) on chorda-induced vasodilatation. Chorda stimulation causes a vasodilatation which can be blocked by a combination of hemicholinium (1 mg) plus prolonged, intermittent chorda stimulation (20 and 15 minutes), and restored by choline (1 mg i.d.) (the vasodilator response after choline appears "flat" on top because the recording device reached maximal deflection at this flow rate).


Effect of Hemicholinium
Blood Flow
ml min⁻¹
2
0
1 min
CH
CH
CH
CH
HC-3
1 mg + CH, 20 min
CH, 15 min
Choline
1 mg

3.3.3 Effects of Eserine

Intravenous eserine (100 $\mu g\ kg^{-1}$) has been shown to potentiate the vasodilatation induced by chorda nerve stimulation at all stimulation durations (Figure 15). The administration of eserine intra-arterially (1 mg) potentiates chorda-induced vasodilatation only at low stimulation durations (Figure 15). Eserine given by both of these routes potentiates chorda vasodilatation at both nicotinic (ganglionic) and muscarinic (both ganglionic and post-ganglionic) sites. In an attempt to eliminate the effects of eserine at nicotinic sites, eserine was given intra-ductally. Since the majority of the ganglia are outside the gland, it can be assumed that intra-ductal eserine exerts its effects mostly at postganglionic muscarinic sites. Intraductal eserine has been shown to potentiate markedly (between 25 and 150%) the vasodilator effects of chorda nerve stimulation: this potentiation was abolished at low stimulation duration (2 pulses) and reduced at higher stimulation duration (25 pulses) by intravenous atropine (Figure 16). The observation that this potentiation by eserine is atropine-sensitive lends some support to the suggestion that at least part of the postganglionic effector mechanism mediating vasodilatation is cholinergic.

3.3.4 Effect of Other Anti-muscarinic Antagonists

4-Diphenyl acetoxy-N-methylpiperidine (4-DAMP) is a specific muscarinic receptor antagonist with no known anti-nicotinic actions. Based on studies performed on muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells and in ileum, Barlow and co-workers (1976) demonstrated that 4-DAMP showed a much higher affinity for receptors (20-fold) in the ileum than those in the atria. Based on Barlow's findings on the possible existence of more than one muscarinic receptor subclass, it was decided to examine the effects of 4-DAMP and an analogue, pentamethylene-bis-4-diphenyl acetoxy-N-methylpiperidine (bis 4-DAMP), on atropine-resistant vasodilatation in the submandibular gland of the cat. The structures of 4-DAMP and bis 4-DAMP are shown in Figure 17 and compared to other anti-muscarinics and acetylcholine.

Figure 15. Histogram depicting % potentiation of chorda-induced vasodilatation at different durations of stimulation (0.25—20 seconds) by eserine given intravenously (□ , 100 $\mu g\ kg^{-1}$) and intra-arterially (□ , 1 mg).

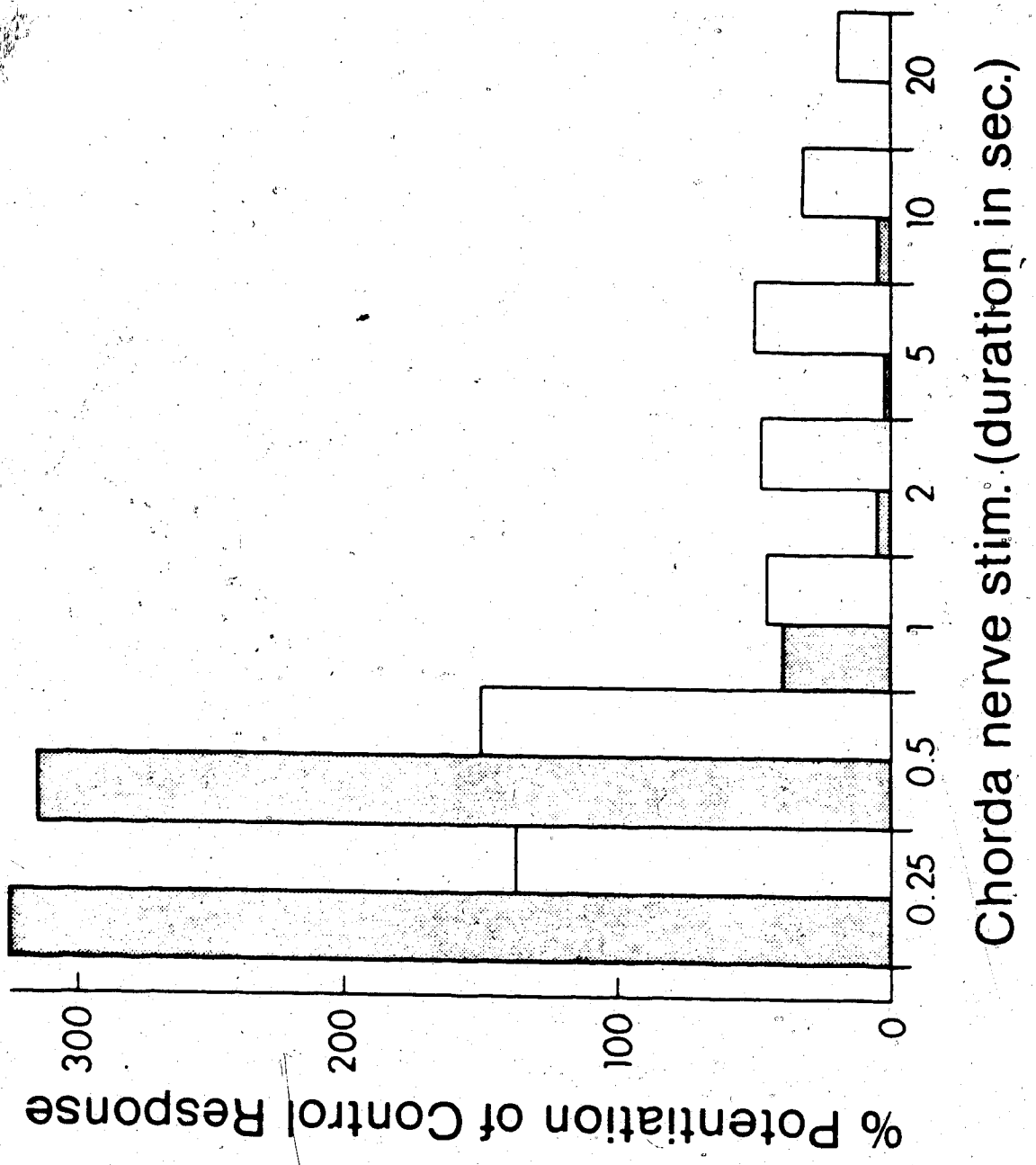
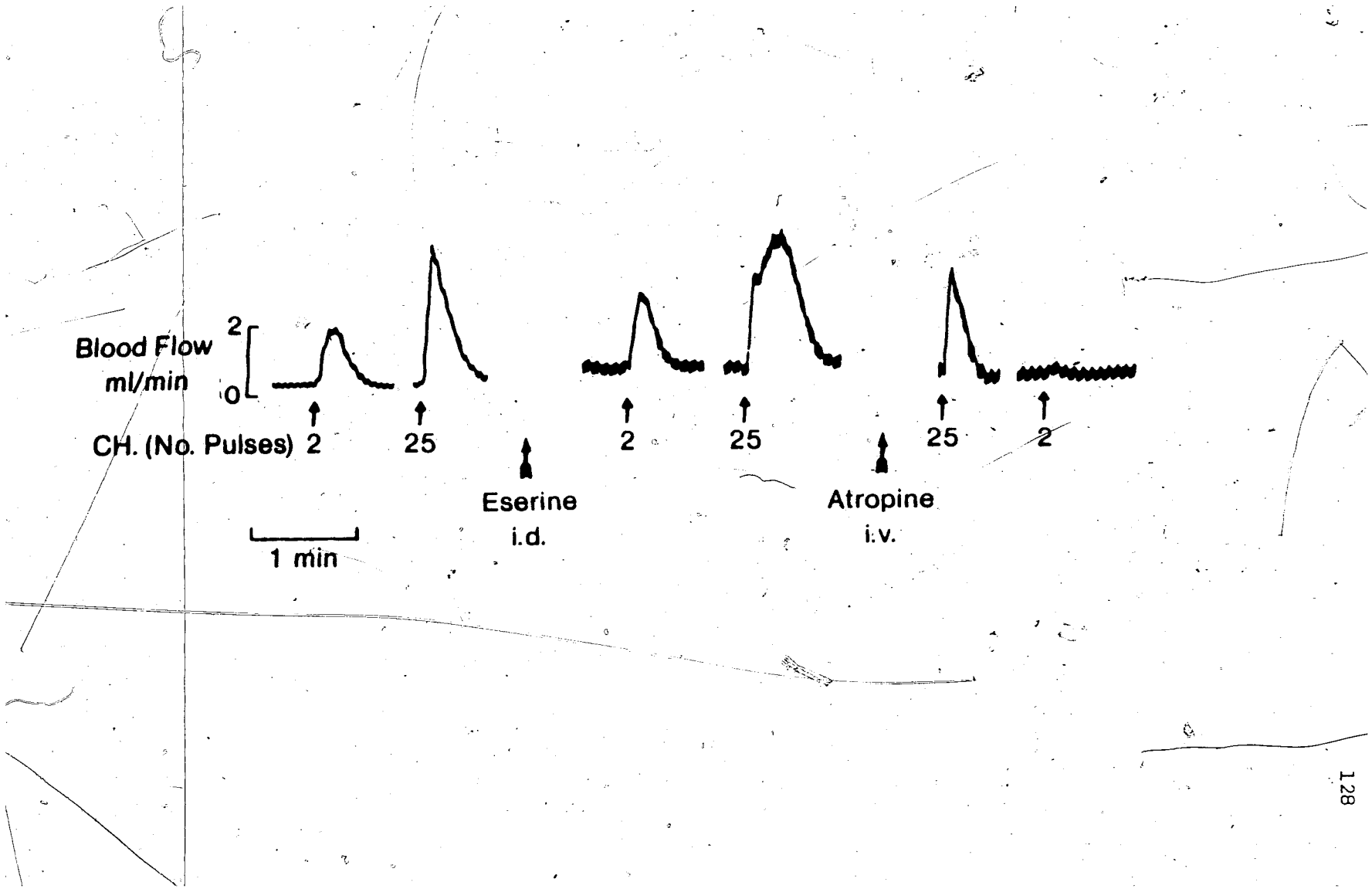

% Potentiation of Control Response
300
200
100
0
0.25
0.5
1
2
5
10
20
Chorda nerve stim. (duration in sec.)

Figure 16. Representative trace of the effect of intraductal eserine (1 μg) on chorda-induced vasodilatation. The chorda-induced vasodilatation (2 and 25 pulses) is potentiated by eserine and is reduced below pre-eserine values by atropine (0.5 mg kg^{-1}).


Blood Flow
ml/min
2
0
CH. (No. Pulses) 2
25
2
25
25
2
Eserine
i.d.
Atropine
i.v.
1 min

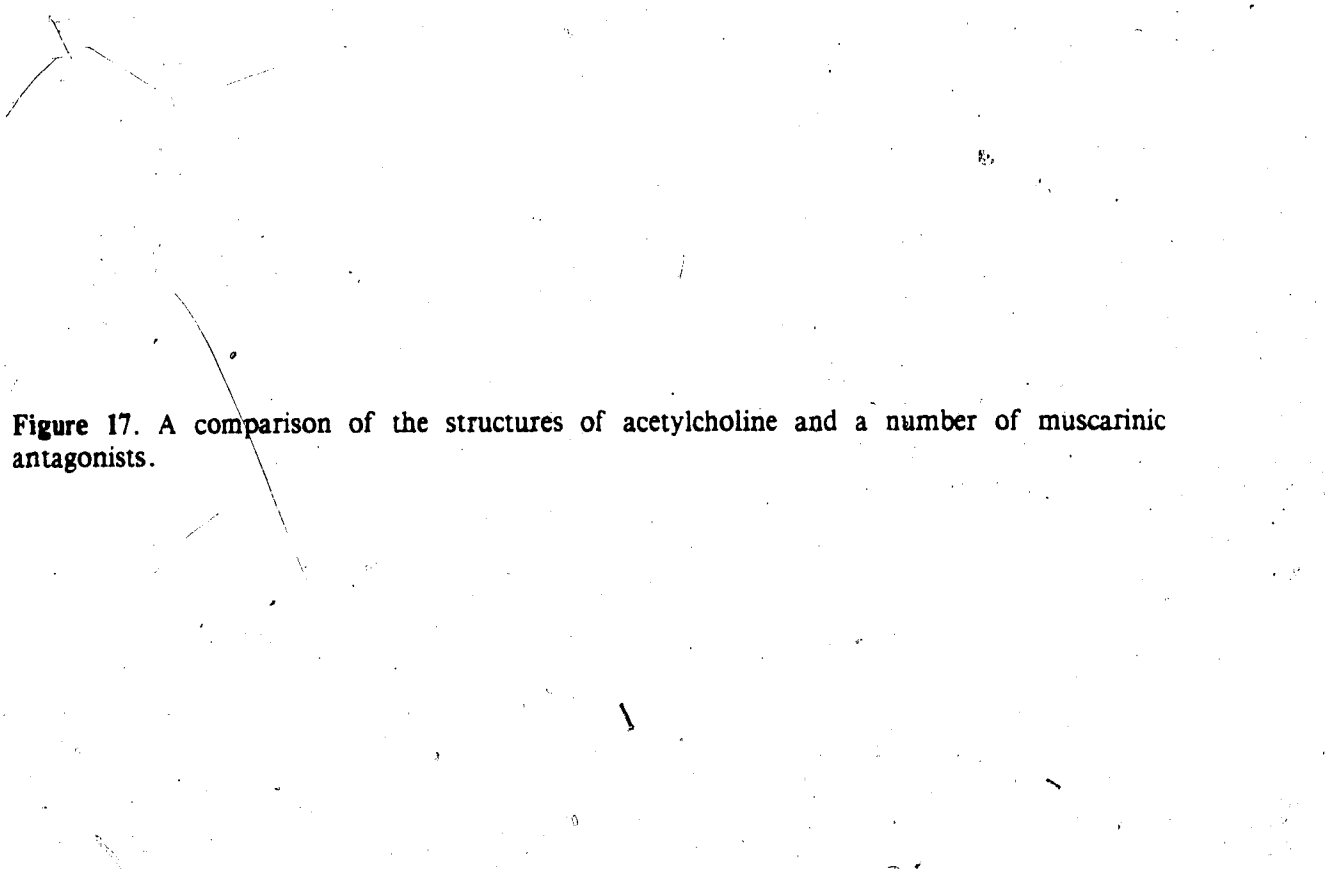
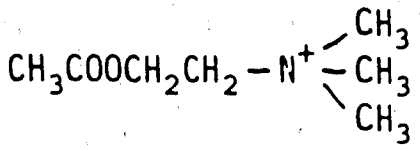
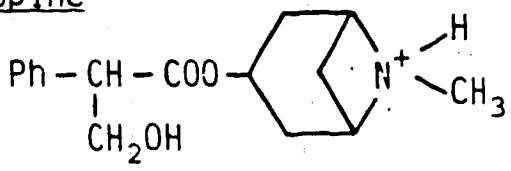


Figure 17. A comparison of the structures of acetylcholine and a number of muscarinic antagonists.

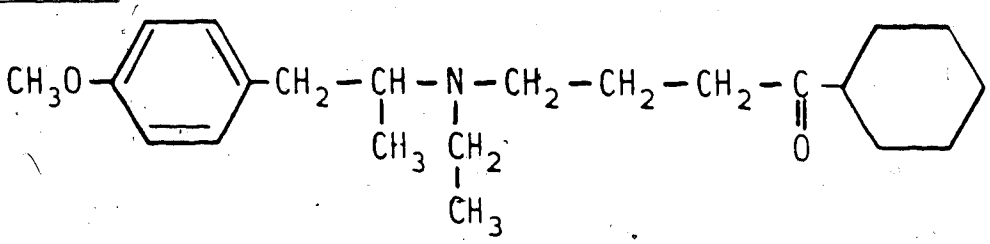
Acetylcholine



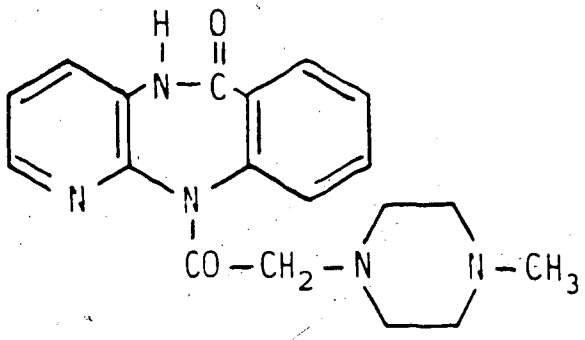
Atropine



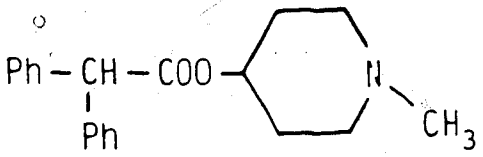
Secoverine



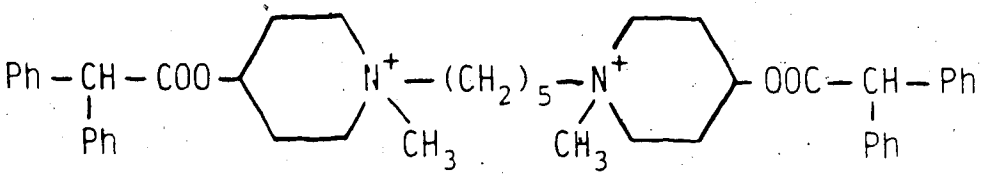
Pirenzepine



4-DAMP



bis, 4-DAMP



4-DAMP given intravenously (1 mg kg^{-1}) abolishes completely the vasodilatation induced by a close-arterial injection of acetylcholine while having no effect on the vasodilatation induced by both bradykinin and VIP. 4-DAMP significantly reduces the vasodilatation (up to 70%) induced by chorda nerve stimulation at 2, 5 and 10 seconds duration (Figure 18). 4-DAMP completely abolishes salivation induced by chorda nerve stimulation. Following a further close-arterial injection of 4-DAMP 0.5 mg i.a.), the vasodilatation induced by chorda nerve stimulation is completely abolished (Figure 18).

Figure 19 shows the effects of a cumulative close-arterial injection of 4-DAMP. Following the first injection (1 mg i.a.), the vasodilatation induced by chorda stimulation was reduced up to 70% (Figure 19b). The initial reduction in chorda-induced vasodilatation appears transient, since partial recovery is observed (Figure 19c). Following a second infusion (2 mg i.a.), the vasodilatation induced by chorda stimulation (5 and 20 seconds) is completely abolished, but returns partially over a period of time. The results presented here are representative of a number of experiments (eight). Although the extent to which 4-DAMP reduces chorda-induced vasodilatation varies between animals, it consistently reduces chorda-induced vasodilatation by at least 50%.

4-DAMP given intra-ductally (1 mg i.d.) abolishes completely the effects of a close-arterial injection of acetylcholine, whilst having no effect on the vasodilatation induced by VIP. 4-DAMP reduces up to 75% the vasodilatation induced by chorda nerve stimulation (Figure 20a and 20b). (Note — in this case the sympathetic after-dilatation is completely abolished, similar effects were seen in a number of experiments, but this block appeared transient).

Pentamethylene bis, 4-diphenylacetoxy-N-methylpiperidine (bis, 4-DAMP) given intravenously and intra-arterially completely abolishes the vasodilatation induced by a close-arterial injection of acetylcholine. Bis, 4-DAMP also significantly reduces (in this experiment) the vasodilatation induced by VIP. The reproducibility of this effect is very inconsistent. The significance and mechanism by which bis, 4-DAMP affects VIP-induced vasodilatation

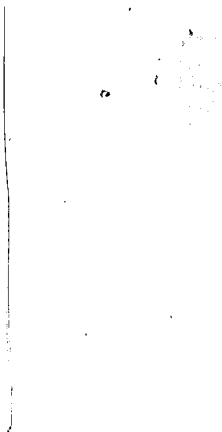
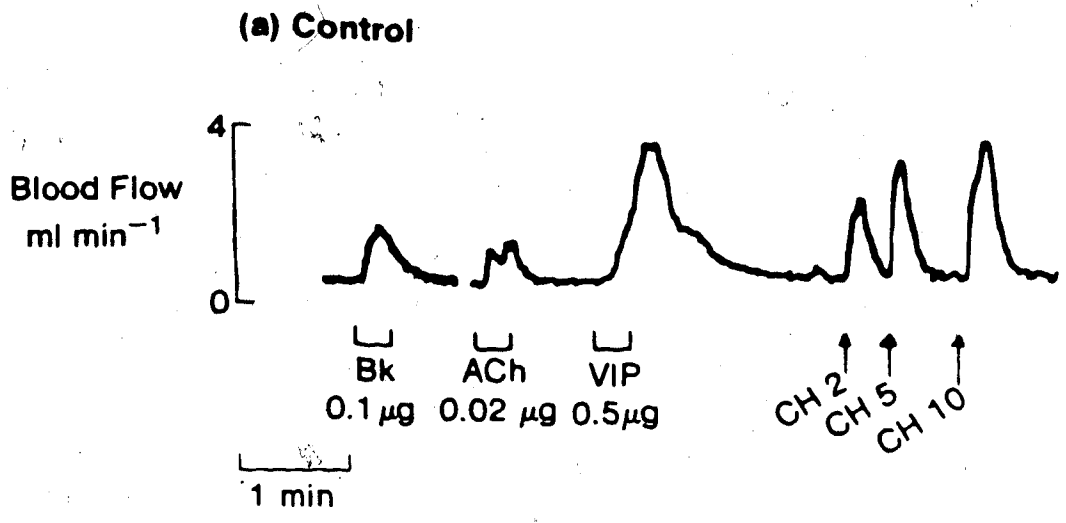
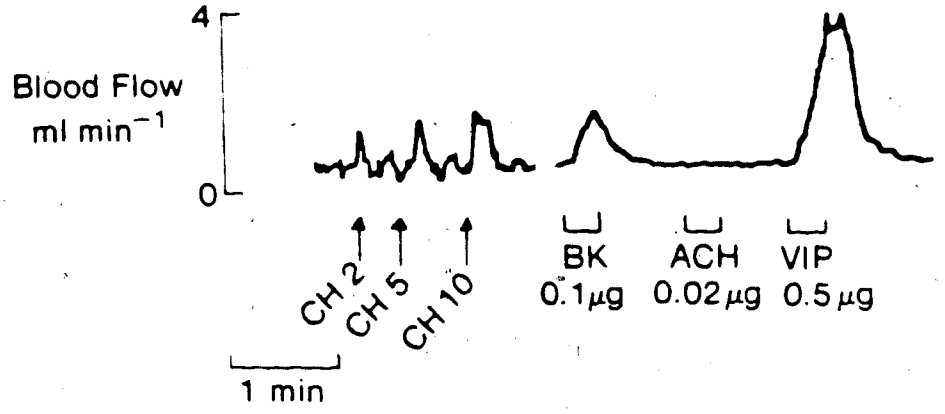


Figure 18. (a) Control responses to chorda nerve stimulation (2, 5 and 10 seconds). Bk, ACh and VIP given intra-arterially. (b) Responses after 4-DAMP (1 mg kg^{-1} i.v.). (c) Responses after 4-DAMP (0.5 mg i.a.).


(a) Control
Blood Flow
ml min⁻¹
4
0
Bk
0.1 µg
ACh
0.02 µg
VIP
0.5µg
CH 2
CH 5
CH 10
1 min


(b) After 4-DAMP 1 mg kg⁻¹ iv.
Blood Flow
ml min⁻¹
4
0
CH 2
CH 5
CH 10
BK
0.1µg
ACH
0.02µg
VIP
0.5µg
1 min

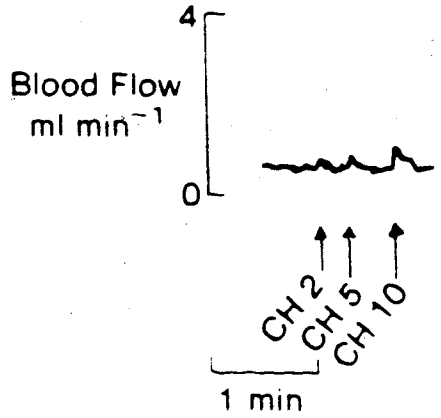

(c) After 4-DAMP 05 mg i.a.
Blood Flow
ml min⁻¹
4
0
CH 2
CH 5
CH 10
1 min

Figure 19. (a) Control response to chorda nerve stimulation (5 and 20 seconds). (b) After 4-DAMP (1 mg i.a.). (c) Almost complete recovery. (d) After 4-DAMP (2 mg i.a.).

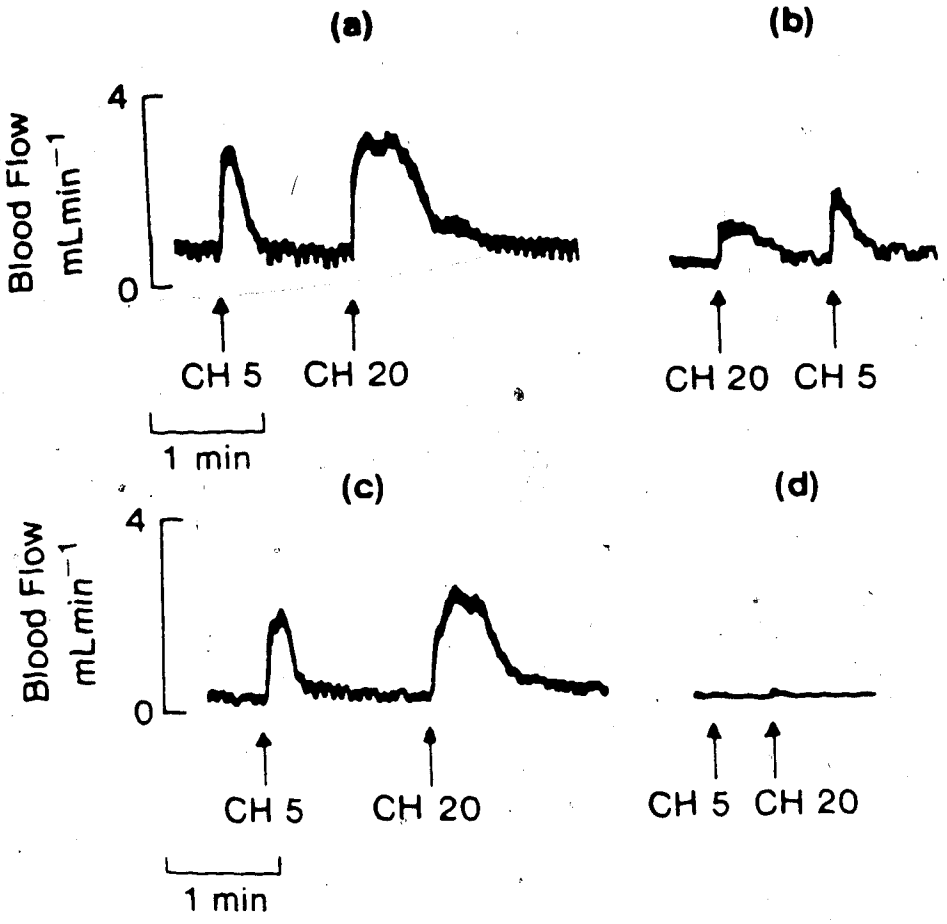
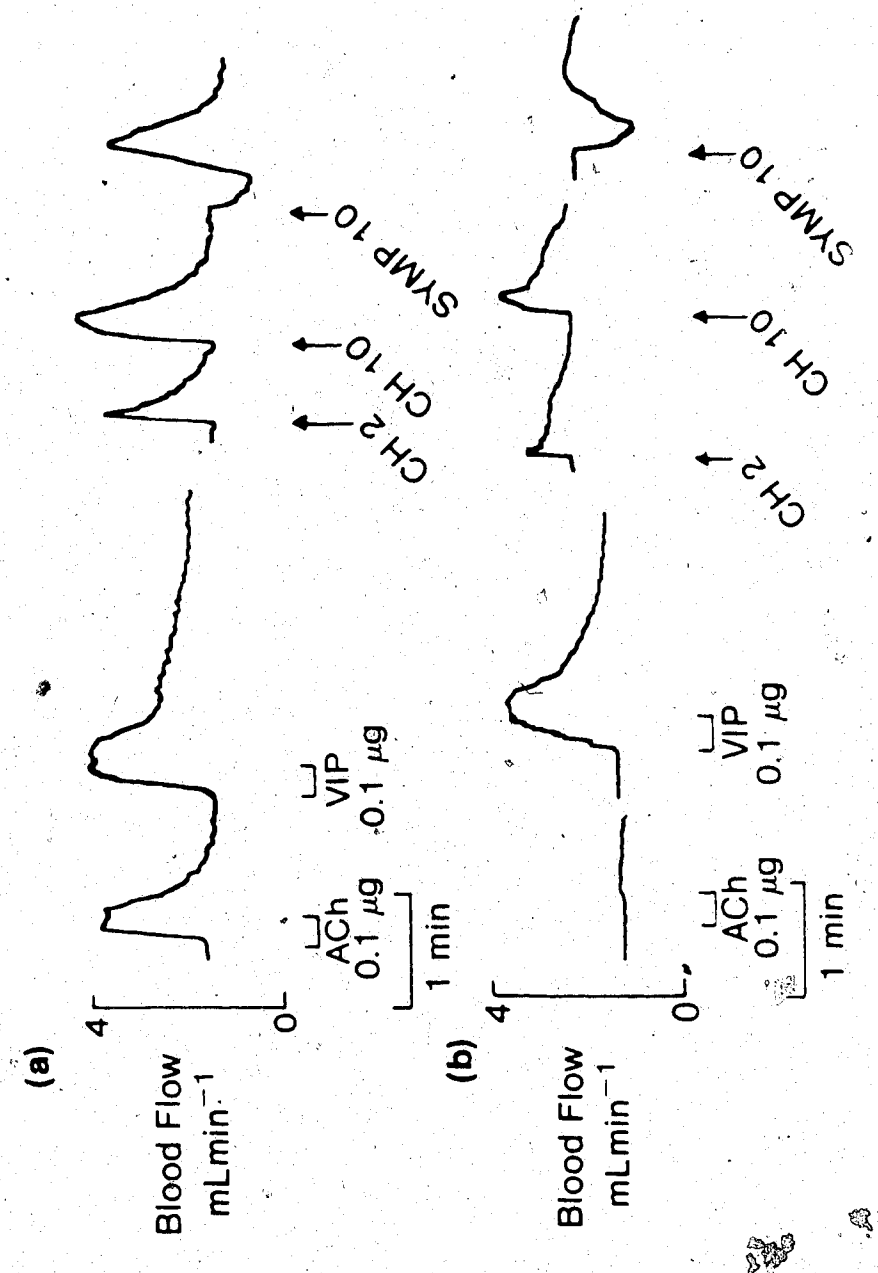


Figure 20. (a) Control responses to acetylcholine, VIP, chorda nerve stimulation (2 and 10 seconds) and sympathetic nerve stimulation (10 seconds). (b) After 4-DAMP (1 mg i.d.). Note: Sympathetic after-dilatation is completely abolished.


(a)
Blood Flow
mLmin⁻¹
4
0
SYMP 10
CH 10
CH 2
VIP
0.1 μg
ACh
0.1 μg
1 min
(b)
Blood Flow
mLmin⁻¹
4
0
SYMP 10
CH 10
CH 2
VIP
0.1 μg
ACh
0.1 μg
1 min

remains unknown (Figure 21). Chorda-induced vasodilatation at 1 second duration is completely abolished, while at 5 seconds duration the vasodilator response is reduced by over 70%. (Note — bis, 4-DAMP, like 4-DAMP, reduces the after-dilatation following sympathetic nerve stimulation). The time-course of block of chorda-induced vasodilatation by 4-DAMP and bis, 4-DAMP is of interest since the block in some cases is partial, transient and shows some recovery. Although recovery is partial, the vasodilatation induced by chorda nerve stimulation never returns to pre-4-DAMP or pre-bis, 4-DAMP levels.

Secoverine hydrochloride is thought to be a selective muscarinic antagonist which inhibits cholinergically-induced gastrointestinal motility (Sanger & Bennett, 1981) at doses which have no effect on salivary and gastric secretions (Zwagemakers & Claassen, 1980).

Secoverine given intravenously has no effect on chorda-induced vasodilatation while blocking the secretion induced by chorda nerve stimulation (Figure 22). The vasodilatation induced by acetylcholine is reduced, then abolished following a further intravenous dose of secoverine. The effects of secoverine on acetylcholine-induced vasodilatation have proven very inconsistent. Secoverine has no effect on sympathetic-induced after-dilatation.

In an earlier study, Schachter *et al.* (unpublished observations) demonstrated that pirenzepine, a selective M_1 muscarinic antagonist (Birdsall *et al.*, 1980), had no effect on chorda-induced vasodilatation but blocked the concomitant salivary secretion. Pirenzepine was also shown to be only moderately effective in blocking the vasodilatation induced by a close-arterial injection of acetylcholine (much the same as secoverine).

3.4 Evidence For and Against VIP as a Transmitter

3.4.1 Does Avian Pancreatic Polypeptide (APP) Inhibit the Effects of VIP and Chorda-induced Vasodilatation?

It has previously been reported that avian pancreatic polypeptide (APP) inhibits VIP-induced and chorda-induced vasodilatation in the cat submandibular gland (Lundberg *et al.*,

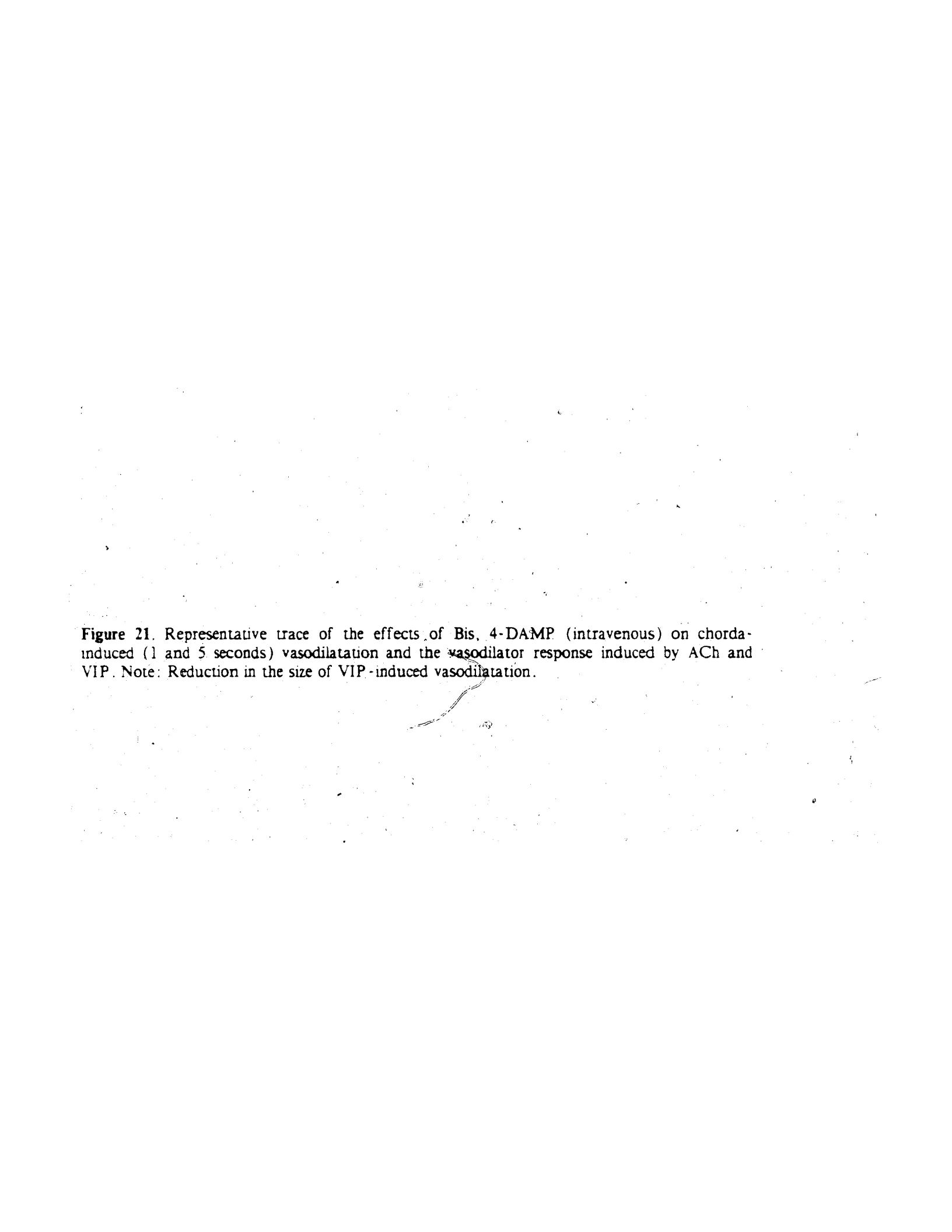
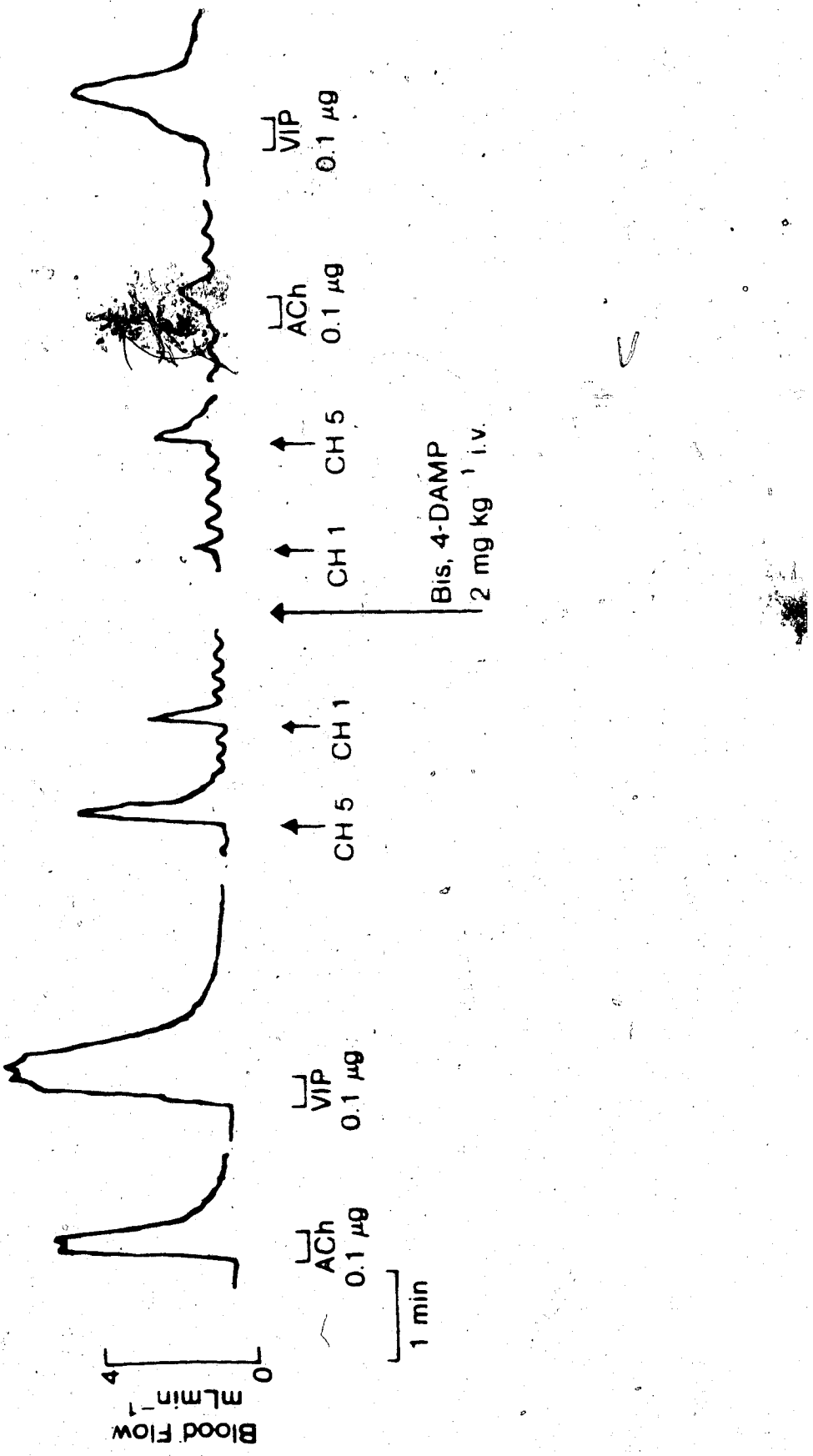


Figure 21. Representative trace of the effects of Bis, 4-DAMP (intravenous) on chorda-induced (1 and 5 seconds) vasodilatation and the vasodilator response induced by ACh and VIP. Note: Reduction in the size of VIP-induced vasodilatation.


Blood Flow
mLmin⁻¹
4
0
1 min
ACh
0.1 μg
VIP
0.1 μg
CH 5
CH 1
CH 1
CH 5
Bis, 4-DAMP
2 mg kg⁻¹ i.v.
ACh
0.1 μg
VIP
0.1 μg

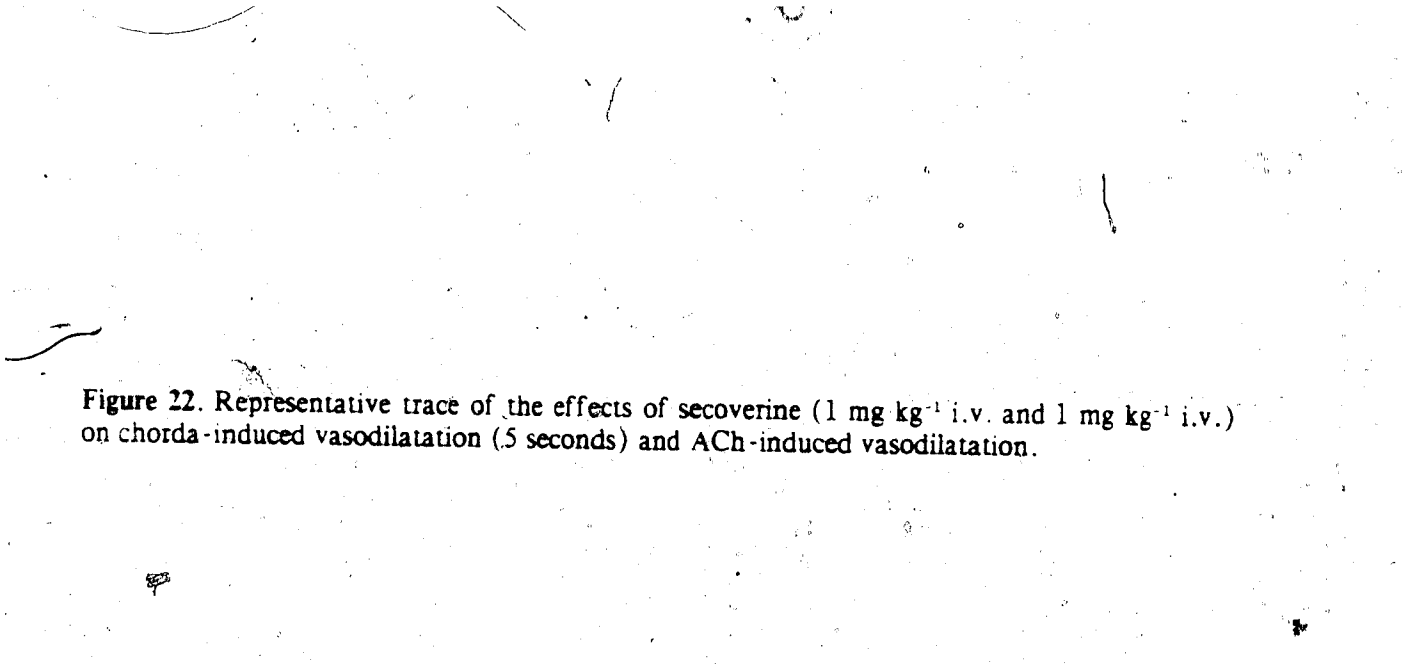
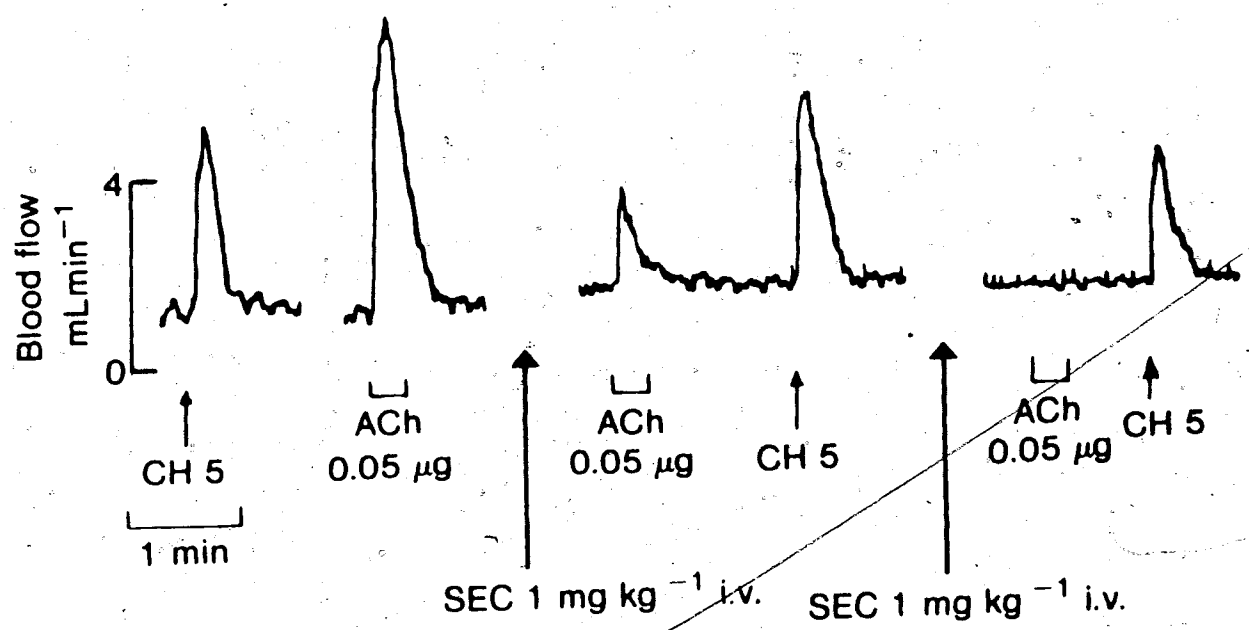


Figure 22. Representative trace of the effects of secoverine (1 mg kg^{-1} i.v. and 1 mg kg^{-1} i.v.) on chorda-induced vasodilatation (5 seconds) and ACh-induced vasodilatation.


Blood flow
mL min⁻¹
4
0
CH 5
1 min
ACh
0.05 μg
ACh
0.05 μg
CH 5
ACh
0.05 μg
CH 5
SEC 1 mg kg⁻¹ i.v.
SEC 1 mg kg⁻¹ i.v.

1980b; Lundberg, 1981). In the present experiments, four different batches of highly purified APP were used (2—100 μg i.a.), but chorda-induced vasodilatation was neither abolished nor significantly reduced by any of these preparations. APP is very inconsistent in its effects on VIP-induced vasodilatation, as can be seen in Figures 23 and 24. In Figure 23, APP (2 μg i.a.) completely abolished the effects of VIP, whereas APP (5 μg i.a.) in another experiment had no effect on the vasodilatation induced by VIP (Figure 24). A cumulative close-arterial infusion of APP gradually reduces the VIP-induced vasodilatation but has no effect on chorda-induced vasodilatation (Figure 25).

In a total of nine experiments, APP reduced or sometimes abolished the effect of VIP (four experiments), but in five other experiments the APP was ineffective. Even when the response to VIP was abolished by APP, the block was short-lived and could not always be repeated by a further dose of APP. During a close-arterial infusion of APP (10 μg i.a.) superimposed chorda nerve stimulation-induced vasodilator responses, were essentially unchanged (Figure 26). When the submandibular vasculature was unresponsive to injected VIP, chorda nerve stimulation always increased blood flow. The avian pancreatic polypeptide used in these experiments was purified and provided by Dr. Joe Kimmel, who also provided the APP used by Lundberg and coworkers (Lundberg *et al.*, 1980b; Lundberg, 1981).

3.4.2 Desensitization of the Submandibular Gland to VIP

Since a specific antagonist to VIP is not available at present, a different approach to the problem of inhibiting VIP was used, i.e. "desensitization" of the submandibular gland vasculature to VIP by close-arterial infusion of large doses of the peptide (25 μg—100 μg i.a., total; 2.5 μg/min i.a.) (Figure 27). If the blood pressure fell during this procedure, the infusion was stopped until the blood pressure returned to normal. The infusion caused a marked vasodilatation at first, but towards the end of the infusion period blood flow returned to control levels. The vasodilator response to chorda nerve stimulation was not abolished following VIP desensitization, even though the gland no longer responded to VIP injected

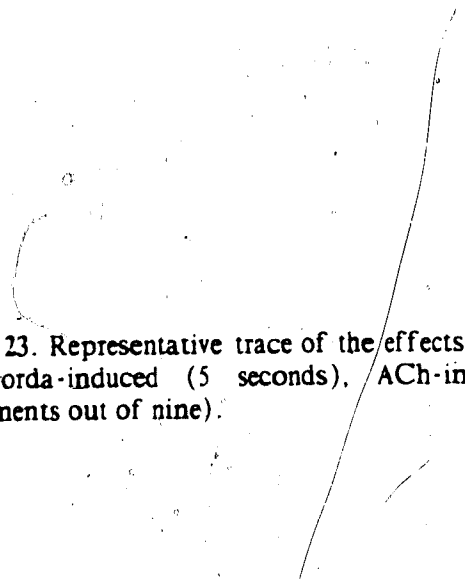


Figure 23. Representative trace of the effects of avian pancreatic polypeptide (APP, 2 μg i.a.) on chorda-induced (5 seconds), ACh-induced and VIP-induced vasodilatation (four experiments out of nine).

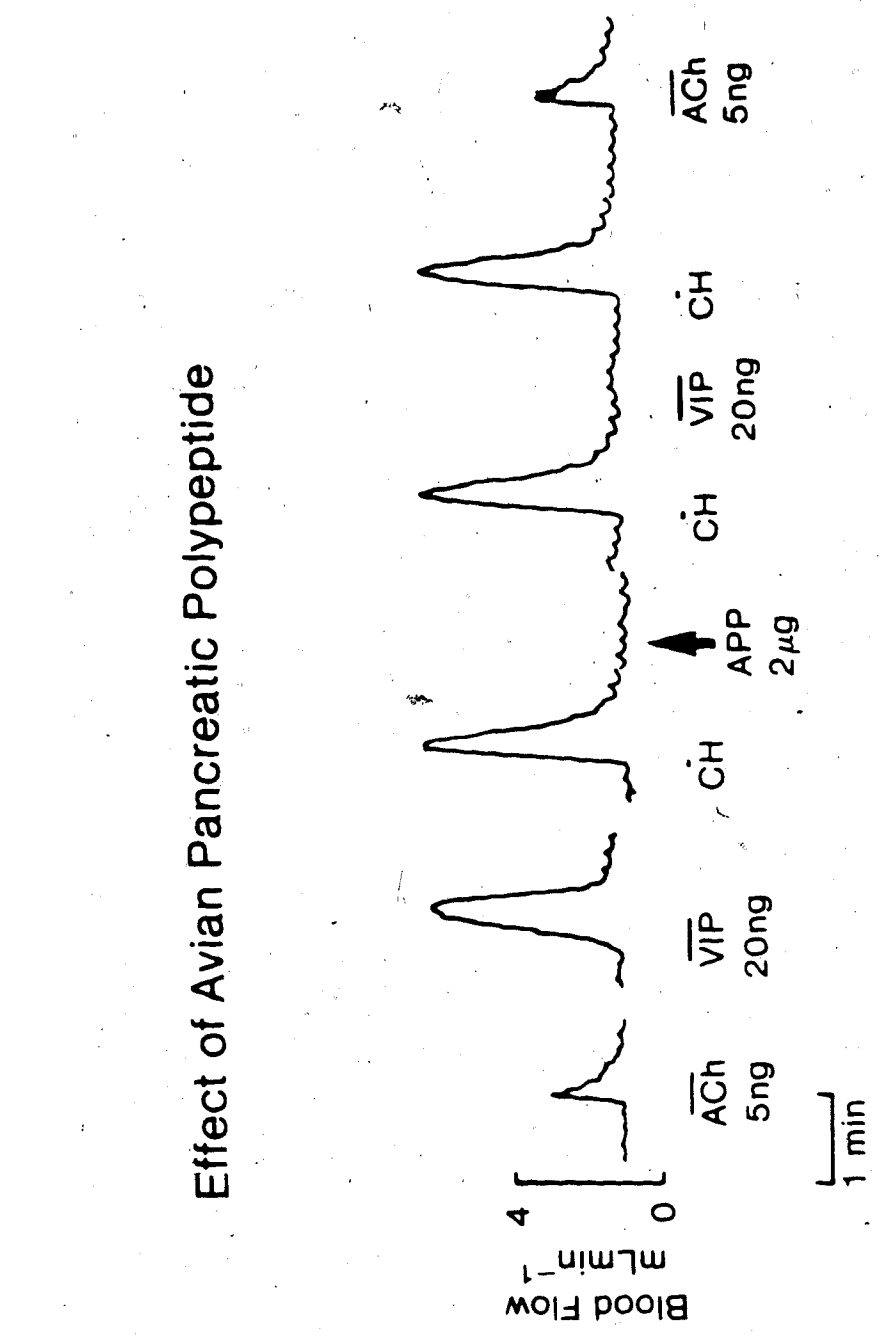

Effect of Avian Pancreatic Polypeptide
Blood Flow
mLmin⁻¹
4
0
1 min
ACh 5ng
VIP 20ng
CH
APP 2μg
CH
VIP 20ng
CH
ACh 5ng

Figure 24. Another experiment in which APP (5 μg i.a.) failed to significantly affect chorda-induced (20 seconds), ACh-induced and VIP-induced vasodilatation (five experiments out of nine).

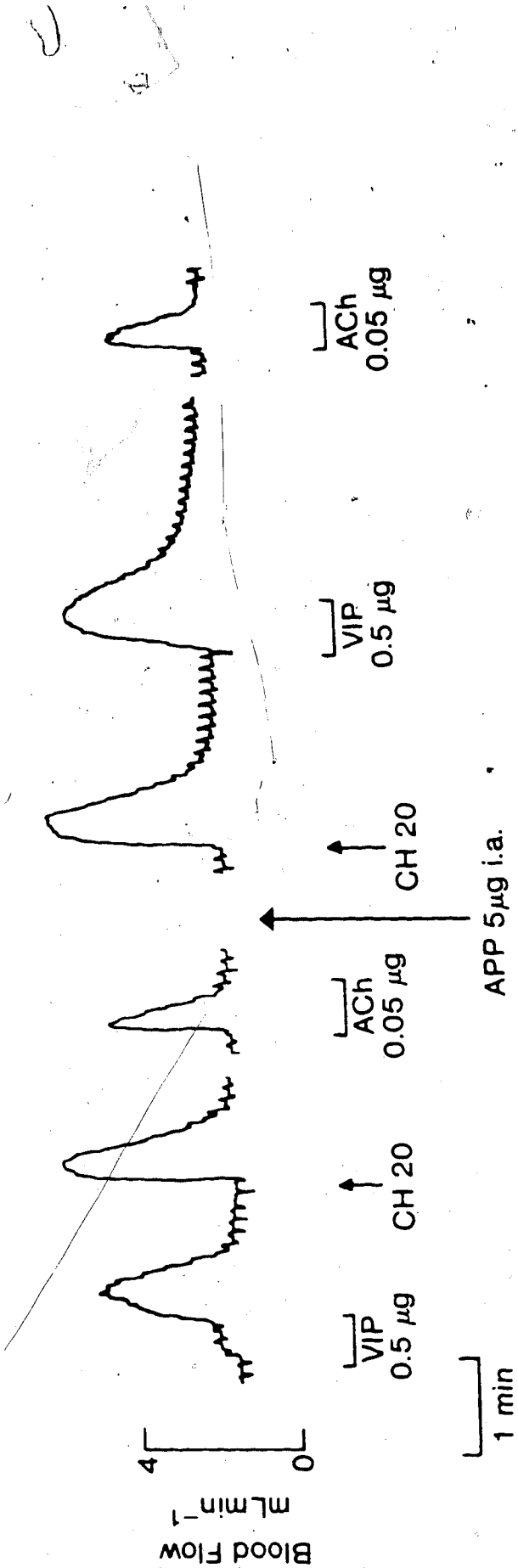

Blood Flow
mL min⁻¹
4
0
1 min
VIP
0.5 µg
CH 20
ACh
0.05 µg
APP 5µg i.a.
CH 20
VIP
0.5 µg
ACh
0.05 µg

Figure 25. Effects of a cumulative close-arterial infusion of APP (2.5, 5.0 and 20.0 μg) on chorda-induced (5 seconds) and VIP-induced vasodilatation.

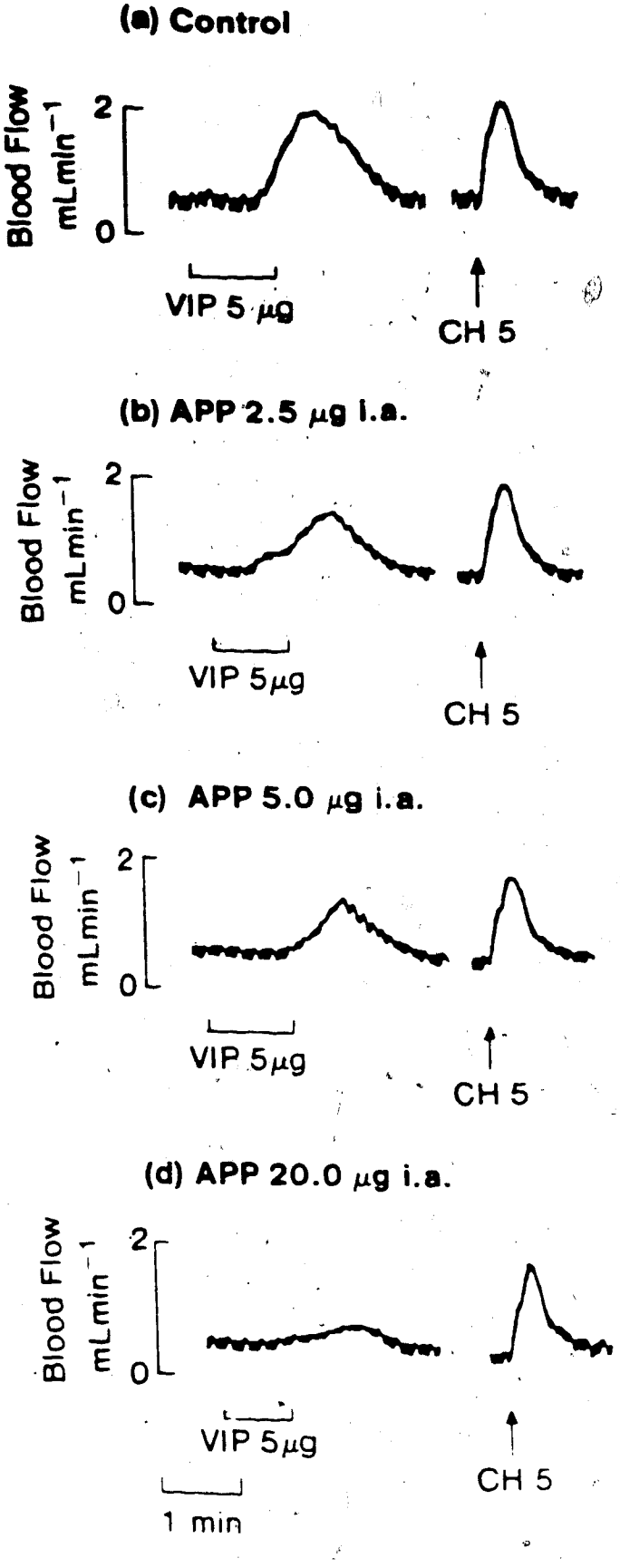

(a) Control
Blood Flow
mLmin⁻¹
2
0
VIP 5 µg
CH 5
(b) APP 2.5 µg i.a.
Blood Flow
mLmin⁻¹
2
0
VIP 5µg
CH 5
(c) APP 5.0 µg i.a.
Blood Flow
mLmin⁻¹
2
0
VIP 5µg
CH 5
(d) APP 20.0 µg i.a.
Blood Flow
mLmin⁻¹
2
0
VIP 5µg
CH 5
1 min

Figure 26. Superimposed chorda-induced vasodilatation (5 seconds) during a continuous close-arterial infusion of APP (10 μg).

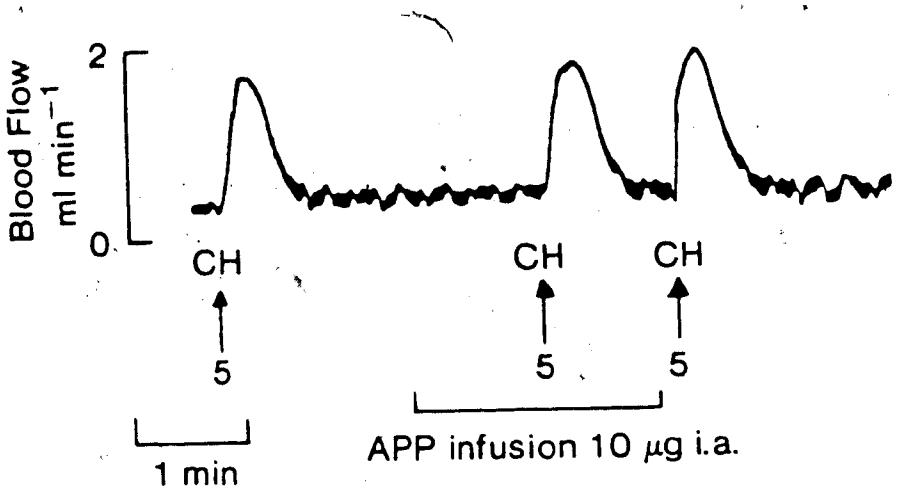
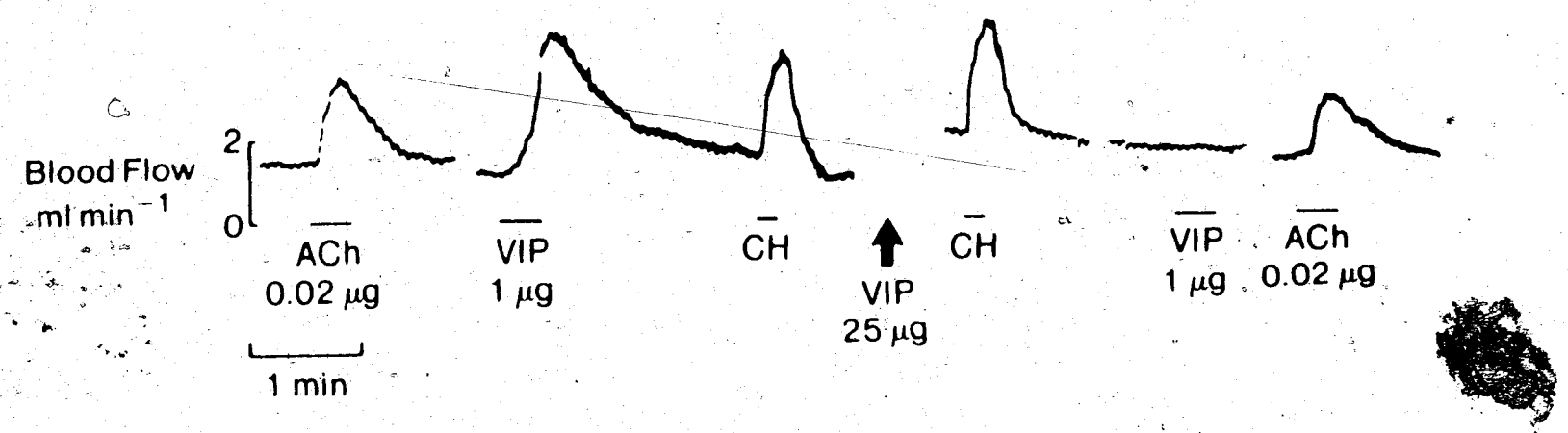


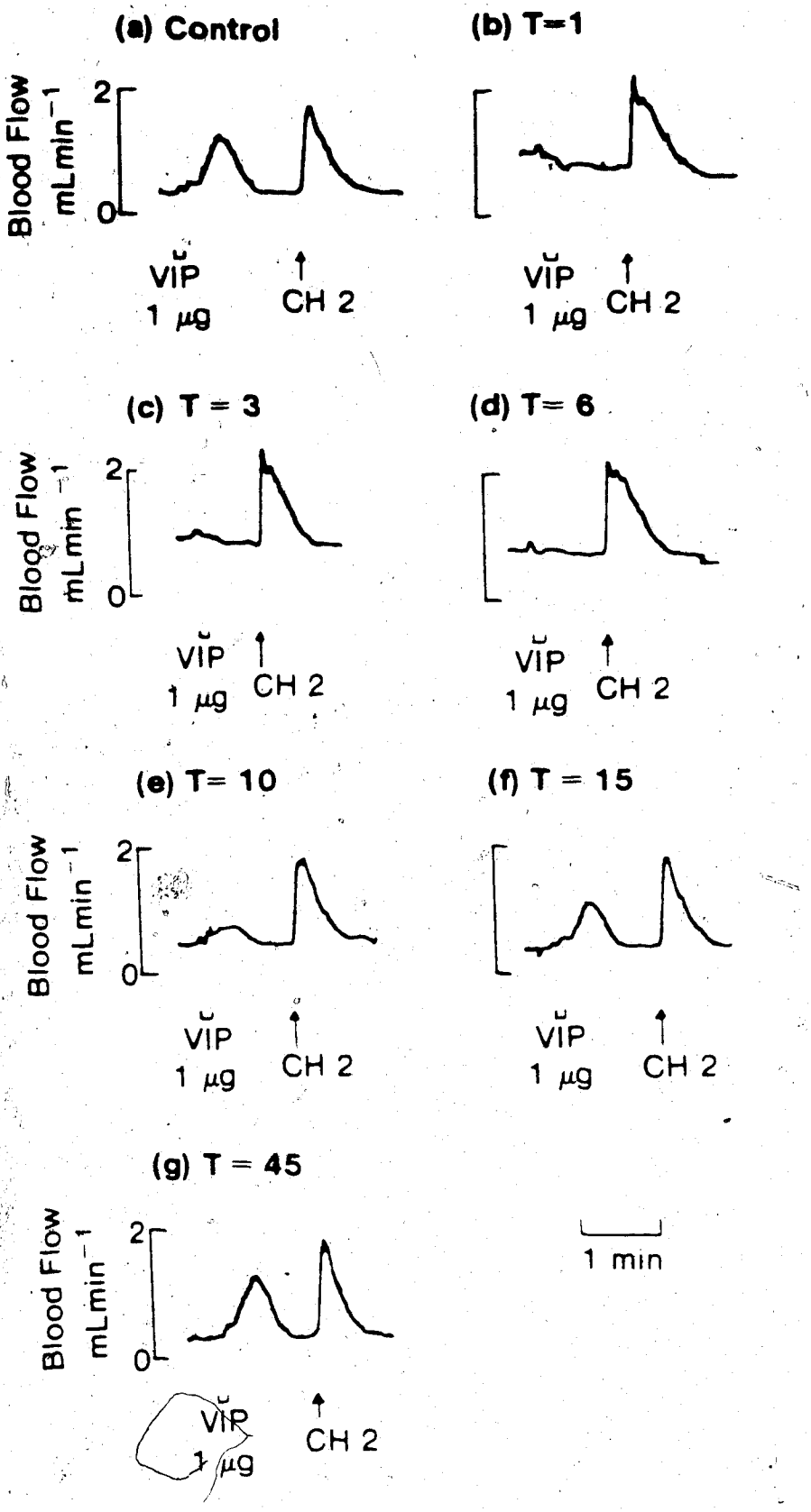
Figure 27. Desensitization of the submandibular gland vasculature to VIP (25 μg i.a.; 25 μg min^{-1}) and its effects on chorda-induced (10 seconds), ACh-induced and VIP-induced vasodilatation.

Desensitization to VIP



close arterially (Figure 27). The submandibular gland vasculature recovered from desensitization (Figure 28). The time-course of desensitization recovery appeared to be dependent upon the desensitizing dose of VIP given, such that following a desensitizing dose of 25 μg VIP i.a., recovery occurred after only 3—5 minutes, whereas after a desensitizing dose of 100 μg i.a., recovery was not complete until after 45 minutes (Figure 28).

Figure 28. Time course of recovery of the submandibular gland vasculature to VIP following a desensitizing dose of 100 μg i.a. (10 μg min^{-1}). T = time in minutes after desensitization.


(a) Control
(b) T=1
(c) T = 3
(d) T= 6
(e) T= 10
(f) T = 15
(g) T = 45
Blood Flow
mLmin⁻¹
2
0
VIP
1 μg
CH 2
1 min

4. DISCUSSION

The results presented here suggest that despite its atropine resistance, the major mediator of parasympathetic vasodilatation in the cat submandibular gland is acetylcholine. At the present time, however, one cannot discount the possible contribution of VIP.

Contrary to the vasodilatation, it is quite clear that atropine abolishes submandibular secretion at all frequencies and durations of stimulation. However, atropine reduces, or even abolishes, the vasodilator responses at lower stimulation frequency, suggesting an atropine-sensitive cholinergic mechanism at low stimulation frequency (Darke and Smaje, 1972). In favor of acetylcholine being a major mediator is the fact that intra-ductal eserine potentiated chorda-induced vasodilatation, particularly at lower stimulation duration, and this potentiation was reduced by atropine. However, it can be debated whether the potentiation of vasodilatation by eserine occurs at pre- or postganglionic storage sites of acetylcholine. In favor of eserine exerting its effects at postganglionic sites, however, is the observation that intra-ductal administration of hexamethonium (a ganglion-blocking drug) has very little effect on chorda-induced vasodilatation, suggesting that intra-ductal eserine acts at postganglionic effector sites (Emmelin *et al.*, 1954; Hilton and Lewis, 1956; Lundberg *et al.*, 1980a).

In contrast to this limited effect of intra-ductal hexamethonium, the intra-ductal administration of hemicholinium accompanied by some parasympathetic nerve stimulation to facilitate depletion of stores of acetylcholine, completely abolished the vasodilator effects of nerve stimulation. The vasodilatation could be restored by intra-ductal administration of choline chloride. The conclusion one is led to from these observations is that the action of hemicholinium is due to the depletion of acetylcholine at postganglionic sites, where it normally participates in chorda-induced vasodilatation.

The effects of other muscarinic antagonists on chorda-induced vasodilatation requires careful analysis. The concept of heterogeneity of muscarinic receptors was first proposed by Burgen and coworkers (Birdsall *et al.*, 1978). These findings were substantiated significantly by the discovery of certain antagonists, such as pirenzepine, that discriminate between high and

low affinity subtypes (Hammer *et al.*, 1980; Birdsall *et al.*, 1980). On the contrary, the classical antimuscarinic agent, atropine, shows virtually the same affinity for muscarinic receptor subtypes, a finding that probably has delayed for years the recognition of receptor heterogeneity. The distribution of these receptor populations varies in different organs. Thus, muscarinic receptors showing high affinity for pirenzepine prevail in discrete areas of the mammalian brain and in peripheral autonomic ganglia, while low affinity sites are present in the heart, in the smooth muscle of the upper gastrointestinal tract and bladder (Hammer *et al.*, 1980; Birdsall *et al.*, 1980).

Similar observations were noted by Rattan and Goyal (1984) working with the lower esophageal preparation. These workers termed the muscarinic ganglionic receptors M_1 and those of smooth muscle M_2. They also demonstrated that pirenzepine selectively antagonizes the M_1 receptor subclass and 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) the M_2 receptor subclass. This extended the earlier findings of Barlow *et al.* (1976) and Brown *et al.* (1980), who established that 4-DAMP acts selectively on "so-called" M_2 receptors. Our results with 4-DAMP and its analogue, bis.4-DAMP, demonstrate a reversible inhibition of chorda-induced vasodilatation, as well as that induced by a local injection of acetylcholine. 4-DAMP irreversibly inhibited chorda-induced salivary secretion. Thus, it is possible that the muscarinic mechanism involved following chorda-induced vasodilatation is 4-DAMP-sensitive but atropine-resistant. However, an anomaly exists, since it has been shown for the lower esophageal sphincter preparation of the opposum that both atropine and 4-DAMP are effective in antagonizing the contraction induced by bethanechol (Goyal *et al.*, 1980; Rattan and Goyal, 1984). One could argue that this observation is similar to the effect of atropine and 4-DAMP on acetylcholine-induced vasodilatation but is not representative of the obviously complex events which are occurring at the postganglionic effector sites within the submandibular gland. The reversibility of the effects of 4-DAMP on chorda-induced vasodilatation suggests a transient binding to postganglionic muscarinic receptors (M_2 type) in the submandibular gland, and upon repeated chorda nerve stimulation, 4-DAMP is displaced from acetylcholine binding

sites by released acetylcholine. 4-DAMP possesses the necessary quaternary ammonium structure required for binding to muscarinic receptors, but the specific mechanisms of binding are unknown, and binding permanency is unclear at present. Another possibility is that 4-DAMP may be interacting with muscarinic (M_1 type) receptors at ganglia. This possibility can be excluded by the fact that sympathetic nerve-induced salivation and pupillary reflexes are unaffected by 4-DAMP.

Pirenzepine has no significant effect on chorda-induced vasodilatation, but is moderately effective in reducing the concomitant salivary secretion and vascular effects induced by acetylcholine. Thus, it is apparent that chorda-induced vasodilatation is not mediated by M_1 receptors at postganglionic effector sites.

Secoverine is totally ineffective in reducing chorda-induced vasodilatation, and only marginally effective on the vascular effects induced by acetylcholine.

Most recently, Eglen and Whiting (1985) showed that 4-DAMP has the highest affinity for muscarinic receptors mediating vasodilatation in the rabbit aorta and dog femoral artery, and concluded that the receptor profile for vascular smooth muscle mediating vasodilatation appears to differ from that described for the central nervous system, myocardium and gastrointestinal smooth muscle.

In some of our experiments, 4-DAMP was shown to exert limited effects on VIP-induced vasodilatation. In some cases, 4-DAMP would enhance the VIP-induced vasodilatation, and at other times it appeared to attenuate the VIP response. Interpretation of these findings may prove difficult. However, one can speculate on the possible interactions between VIP receptors and muscarinic receptors. Previously, Lundberg (1981) demonstrated that, in the presence of VIP, ACh-induced secretion showed more than 100% increase over control levels. However, we were unable to detect any significant potentiation of salivary secretion by VIP.

Combined histochemical and immunohistochemical data indicate the coexistence of acetylcholine and VIP in postganglionic nerves innervating the submandibular gland in the cat and rat (Lundberg *et al.*, 1980a; Lundberg, 1981). A possible molecular mechanism for the

synergistic actions of ACh and VIP is the 10,000-fold increase induced by VIP in the affinity of ACh for muscarinic receptors (Lundberg *et al.*, 1982c). Subsequently, Hedlund *et al.* (1983) have shown that long-term treatment with atropine induces not only an increase in muscarinic receptors, but also in the number of VIP receptors in the salivary gland of the rat. Recently, Eva *et al.* (1985) suggested that VIP inhibits the turnover of acetylcholine and therefore participates in the feedback regulation of ACh metabolism. As suggested by Lundberg and co-workers, VIP could shift muscarinic receptors from a low to a high affinity conformation. Hence, the VIP-induced inhibition of turnover of ACh might reflect either an interaction between ACh and VIP at the level of presynaptic autoreceptors or an interaction at the post-synaptic recognition sites. According to this novel view, compounds stored in neurons and released extracellularly by nerve impulses can be considered to act as mediators and modulators. Thus, it is possible that VIP may function in the submandibular gland as a modulator for the mediating action of ACh, which is atropine-resistant but relatively sensitive to 4-DAMP.

Of all the vasodilator agents used in this study, VIP was the most potent, causing a dose-related vasodilatation which subsided slowly upon cessation of infusion. VIP did not cause salivation. VIP is twenty to thirty times more potent than acetylcholine on a molar basis. In addition, parasympathetic nerve stimulation produces an abrupt rise in the output of VIP in the venous effluent from the gland in the presence or absence of atropine (Bloom and Edwards, 1980; Lundberg *et al.*, 1981a; Uddman *et al.*, 1980). Immunohistochemically, VIP-ergic nerves have been shown to be present around acini, ducts and blood vessels in the cat submandibular gland (Wharton *et al.*, 1979; Lundberg *et al.*, 1980a; Uddman *et al.*, 1980; Johansson and Lundberg, 1981). It must be emphasized, however, that the vast majority of these nerves occur around acini and ducts. Thus, the VIP in plasma probably originates from nerves innervating tissues other than blood vessels. This creates an anomaly: since VIP does not cause salivary secretion, why is there a rich VIPergic innervation of secretory elements? Is it possible that VIP released from glandular nerves, after diffusion delays reaches vascular receptors and contributes to the vasodilator response via a paracrine VIP action? However, the

onset of the vasodilatation associated with chorda nerve stimulation is almost immediate.

The observed VIP levels in the venous effluent depend on two factors. (1) VIP diffusion from the site of synaptic release to blood vessels may be limited, since VIP is a fairly large water-soluble peptide. (2) Local enzymatic degradation prior to arrival at receptor sites in the vasculature takes places within the tissue and in the blood (Edwards *et al.*, 1978). During prolonged stimulation, a gradual decline in VIP output was noticed, but the size of the vasodilator response remained unchanged, suggesting that the amount of VIP available for release is limited (Uddman *et al.*, 1980). The VIP output after 1 hour was only 20% of the maximal value. This means either not all glandular VIP is available for release, and or only a fraction of the released VIP was recovered in the venous outflow due to degradation.

In our experiments, during desensitization, when the submandibular vasculature was unresponsive to VIP, the vasodilator response to chorda nerve stimulation was not abolished. The submandibular vasculature showed variable time courses of recovery (5—45 minutes). This finding supports the idea that VIP cannot be the only, or major, mediator involved. The mechanism by which VIP exerts its vasodilator effects is unknown at present. It is unclear whether VIP interacts with its own receptors, causing direct changes in membrane channel conductances, or indirectly affects membrane channel conductances via alterations in cAMP or Ca^{2+} metabolism.

Avian pancreatic polypeptide is a thirty-six amino acid polypeptide (Kimmel *et al.*, 1975) with a markedly different amino acid composition compared to pancreatic polypeptides from other species (Lin and Chance, 1974; Kimmel *et al.*, 1975). Immunohistochemical evidence suggests that an APP-immunoreactive substance is present in both central and peripheral neurons (Loren *et al.*, 1979). Furthermore, APP-like immunoreactivity has been shown to coexist with catecholamines or other neuropeptides in certain neurons, particularly around arteries and arterioles of exocrine glands such as the nasal mucosa and submandibular gland of the cat (Lundberg *et al.*, 1980c; Vincent *et al.*, 1982).

In this study, APP was found to be very inconsistent in reducing VIP-induced vasodilatation. This finding is in sharp contrast to that reported by Lundberg and associates (Lundberg, 1981; Lundberg *et al.*, 1980a), who stated that APP reversibly inhibits both VIP-induced vasodilatation and salivation. According to Lundberg and coworkers, APP reduces VIP-induced salivation, but we have shown that injected VIP does not cause salivation in the cat submandibular gland. Lundberg also showed that APP reversibly inhibits chorda-induced vasodilatation, which we have been unable to demonstrate. The inconsistent effects of APP on VIP-induced vasodilatation are probably due to a non-specific steric interaction with VIP receptors or postsynaptic effector mechanisms within the submandibular vasculature. Furthermore, it is difficult to contemplate a specific receptor interaction, due primarily to the marked structural dissimilarities between VIP and APP and the ultimate lack of quaternary binding conformation. If, as has been suggested by Lundberg, APP is a specific reversible inhibitor of VIP, then vasodilatation induced by nerve stimulation would be reversibly inhibited by APP if VIP were a major mediator of atropine-resistant vasodilatation.

The localization of an APP-like peptide within catecholamine-rich neurons offers interesting possibilities as to its functional significance. Since APP has been localized within adrenergic neurons within the submandibular gland and nasal mucosa vasculature, one would expect an exogenous application of the peptide to enhance or augment the response induced by sympathetic nerve stimulation or a local application of catecholamines. However, it is apparent that APP has no effect on sympathetic nerve-induced vasoconstriction, after-dilatation and salivation.

The possibility of using antisera as pharmacological blocking agents at synapses was originally suggested by the findings that antiserum against nicotinic receptors could paralyze skeletal muscle *in vivo* (Patrick and Lindstrom, 1973; Heilbronn and Mattsson, 1974). Also, antibodies from myasthenia gravis patients could penetrate into the synaptic cleft of the neuromuscular junction, bind to postsynaptic membranes, and subsequently block skeletal muscle endplate potentials. Employing a VIP antiserum preparation, we have been unable to block or

significantly reduce VIP-induced vasodilatation or chorda-induced vasodilatation and salivation (not shown). However, Lundberg *et al.* (1981b) demonstrated that following a close-arterial infusion of antiserum against VIP, chorda-induced vasodilatation and salivation were significantly reduced or abolished. This finding was in agreement with the earlier work of Goyal *et al.* (1980), who demonstrated that immuno-antagonism of VIP with a high-titre antiserum antagonized a VIP-induced fall in lower esophageal sphincter pressure in the opposum. However, Goyal used extremely low dilutions (1:1) of VIP antiserum to observe any effect on lower esophageal relaxation induced by intramural nerve stimulation (Goyal, personal communication). This minor effect of VIP antiserum may be because the large size of the antibody makes it difficult for the antibody molecule to achieve significantly high concentrations in the extracellular space at the site of neuromuscular transmission. Moreover, the time course and the affinity of binding of exogenous VIP with the antiserum compared to that with VIP receptor sites may also influence the degree of antagonism. More work is required to see if similar arguments could apply to the inconsistent effects seen when VIP antisera are employed in the submandibular gland.

In 1907, Carlson supplied evidence for the possible existence of vasodilator fibres in the sympathetic nerves running to the submandibular gland of the cat. By stimulation of the cervical sympathetic trunk, Carlson noted a significant increase in the venous outflow in addition to the previously-noted vasoconstriction. Oborin (1952) later confirmed Carlson's findings and detected a sympathomimetic substance in venous effluent from the gland which he characterized as being adrenaline. Despite this observation, Oborin concluded that the sympathetic vasodilatation was mainly a reactive hyperemia, since short-term occlusion of the arterial supply was followed by a dilatation similar to that seen on sympathetic stimulation. A minor contribution to this reactive hyperemia was thought to be due to the presence of a weak, adrenergic vasodilator component.

Subsequently, Bhoola *et al.* (1965) examined the effects of α- and β-adrenoceptor antagonists and guanethidine on the sympathetic after-dilatation. They concluded that the

secretory, constrictor and vasodilator responses due to sympathetic nerve stimulation were all related to the release of noradrenaline and/or adrenaline. Furthermore, since secretion and vasoconstriction were separable by the actions of α-adrenoreceptor antagonists, they concluded that separate sympathetic secretory and vasoconstrictor fibres existed. The sympathetic after-dilatation was most probably due to the action of noradrenaline on vascular β-adrenoceptors, although this effect was less clear.

In this study, 4-DAMP (and bis.4-DAMP) has consistently been shown to reduce the after-dilatation which follows the vasoconstriction associated with sympathetic nerve stimulation. Interpretation of this finding is very difficult in view of the previously postulated β-adrenergic mechanism. It is unlikely that 4-DAMP has any direct effect on adrenergic mechanisms via receptor binding (Barlow, personal communication). Fray and Leaders (1967) and Ehinger *et al.* (1970) postulated a model for neurochemical interactions between cholinergic and adrenergic mechanisms. Their formulation circumvents the need for acetylcholine, choline acetyltransferase and acetylcholine esterase in sympathetic postganglionic fibres and suggests that the liberation of acetylcholine from peripheral parasympathetic neurons facilitates the release of noradrenaline in adjacent sympathetic fibres, possibly by parasympathetic post-ganglionic nerve cross-over. The possibility of the existence of such an interactive mechanism derives support primarily from the observation of extensive overlap in the terminal fields of postganglionic parasympathetic and sympathetic fibres in many tissues. Thus, it is possible that 4-DAMP may interact with the cholinergic mechanisms involved in facilitating the release of noradrenaline at sympathetic nerve terminals and thus reduce or abolish the concomitant sympathetic nerve-induced vasodilatation. This model cannot always account for cholinergic-adrenergic interactions in the peripheral nervous system, however, since such interactions are noted in peripheral tissues in which adrenergic axons are not accompanied by cholinergic neurons (Silver, 1974). It may seem superfluous to have neurotransmitter effects dependent on the action of yet another substance, the presynaptic or postsynaptic neuromodulator. The neuromodulator "interface" between the action potential and transmitter release and

postsynaptic actions, however, may permit "fine-tuning" of postsynaptic information.

At present, based on the inconsistent vasodilator effects of substance P and ATP, they appear to be unlikely candidates for the neurotransmitter involved. However, one cannot discount a secondary messenger role for ATP (cAMP) possibly being involved in VIP-induced vasodilatation.

In conclusion, it appears that acetylcholine is the major mediator, especially at lower frequencies (duration) of nerve stimulation, but one cannot discount the fact that VIP is a potent vasodilator, is present within selective postganglionic cholinergic nerve terminals, and is detectable in the venous effluent following chorda nerve stimulation. However, the present work with a "so-called" VIP antagonist APP, and the densensitization data suggest that VIP is unlikely to be the major mediator. It is evident that at higher frequencies (and duration) of nerve stimulation, chorda-induced vasodilatation becomes progressively atropine-resistant, but it appears that 4-DAMP almost completely abolishes this atropine-resistant component, suggesting a 4-DAMP-sensitive, atropine-resistant muscarinic receptor mechanism. At present, there is no substantial data on the muscarinic receptor subclasses at postganglionic cholinergic nerve terminals in the cat submandibular gland. It would be of interest to determine what muscarinic receptor subtypes predominate at these sites utilizing autoradiographic techniques in association with labelled (^{3}H) quinuclidinyl-benzilate (QNB) and specific muscarinic agonist/antagonist binding studies, as has been so elegantly performed for the rat brain by Potter *et al.* (1984). The use of isolated submandibular artery (*in vitro*) would provide further insight as to the muscarinic receptor population present within the artery and a possible correlation to the observations presented here. However, this may prove difficult, due to the inaccessibility and small size of the submandibular gland artery in the cat.

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