

Innervation of the Gallbladder: Structure, Neurochemical Coding, and Physiological Properties of Guinea Pig Gallbladder Ganglia

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ABSTRACT The muscle and epithelial tissues of the gallbladder are regulated by a ganglionated plexus that lies within the wall of the organ. Although these ganglia are derived from the same set of precursor neural crest cells that colonize the gut, they exhibit structural, neurochemical and physiological characteristics that are distinct from the myenteric and submucous plexuses of the enteric nervous system. Structurally, the ganglionated plexus of the guinea pig gallbladder is comprised of small clusters of neurons that are located in the outer wall of the organ, between the serosa and underlying smooth muscle. The ganglia are encapsulated by a shell of fibroblasts and a basal lamina, and are devoid of collagen. Gallbladder neurons are rather simple in structure, consisting of a soma, a few short dendritic processes and one or two long axons. Results reported here indicate that all gallbladder neurons are probably cholinergic since they all express immunoreactivity for choline acetyltransferase. The majority of these neurons also express substance P, neuropeptide Y, and somatostatin, and a small remaining population of neurons express vasoactive intestinal peptide (VIP) immunoreactivity and NADPH-diaphorase enzymatic activity. We report here that NADPH-diaphorase activity, nitric oxide synthase immunoreactivity, and VIP immunoreactivity are expressed by the same neurons in the gallbladder. Physiological studies indicate that the ganglia of the gallbladder are the site of action of the following neurohumoral inputs: 1) all neurons receive nicotinic input from vagal preganglionic fibers; 2) norepinephrine released from sympathetic postganglionic fibers acts presynaptically on vagal terminals within gallbladder ganglia to decrease the release of acetylcholine from vagal terminals; 3) substance P and calcitonin gene-related peptide, which are co-expressed in sensory fibers, cause prolonged depolarizations of gallbladder neurons that resemble slow EPSPs; and 4) cholecystokinin (CCK) acts presynaptically within gallbladder ganglia to increase the release of acetylcholine from vagal terminals. Results reported here indicate that hormonal CCK can readily access gallbladder ganglia, since there is no evidence for a blood-ganglionic barrier in the gallbladder. Taken together, these results indicate that gallbladder ganglia are not simple relay stations, but rather sites of complex modulatory interactions that ultimately influence the functions of muscle and epithelial cells in the organ.

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INTRODUCTION

Contractile and epithelial activities in the gallbladder are known to be influenced by circulating hormones such as cholecystokinin (CCK) (see Ryan, 1987); however, until recently, the contribution of the nervous system to the control of gallbladder function had not been extensively explored. Nevertheless, the gallbladder contains a well defined ganglionated plexus, which lies at the outer surface of the muscularis, as well as two extensive axonal plexuses which lie on the outer surface of the muscularis and in the submucosa (Cai and Gabella, 1983). Several lines of evidence indicate that these neural elements of the gallbladder play a crucial role in the regulation of smooth muscle and epithelial function in the gallbladder. For example, CCK appears to act within the ganglia to cause the gallbladder to contract (Bauer et al., 1991; Hanyu et al.,

1990; Mawe, 1991; Mawe et al., 1994; Takahashi et al., 1991). Furthermore, disruption of the neural input to the organ can lead to gallbladder malfunction (Pellegrini and Patti, 1981).

Over the past several years, efforts in this laboratory and others have concentrated on elucidating the properties of the ganglia that lie in the wall of the guinea pig gallbladder. This article includes a summary of the major structural, neurochemical, and electrophysiological findings that we and others have reported within the past few years, and it includes novel findings with

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regard to the chemical coding of gallbladder neurons and the absence of a blood-plexus barrier in this system.

MATERIALS AND METHODS

Immunohistochemistry and Histochemistry

The immunohistochemical and histochemical methods that have been used in this study have been described elsewhere in detail (Mawe and Gershon, 1989; Talmage and Mawe, 1993; Talmage et al., 1992).

Sources and dilutions of primary antisera used in this study are listed in Table 1. Species specific antisera that were labeled with either Cy3 or fluorescein isothiocyanate (FITC) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

The mounted preparations were examined with a Zeiss fluorescence photomicroscope equipped with an HBO 100 W UV light source. A rhodamine (565 nm primary filter/590 nm secondary) filter combination was used to visualize Cy3. A fluorescein (485 nm primary filter/520 nm secondary) filter combination was used to visualize FITC.

Blood-Plexus Barrier Studies

Electron Microscopy. Gallbladder preparations were pinned out in iced Krebs solution, and fixed overnight at 4°C in a solution containing 3% paraformaldehyde-2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. Following osmication with a 1% solution of OsO₄ in 0.1 M PO₄ buffer (pH 7.4) for 1 hour, tissues were dehydrated, treated with 2% uranyl acetate in 100% ethanol, and finally embedded in an EPON-Araldite resin between two glass slides. Areas of interest were cut from the whole mount with a razor, and remounted on a beam capsule. Thin (70 nm) serial sections were taken using a Reichert-Jung Ultracut E ultramicrotome and collected on Formvar-coated copper slot grids. Sections were examined on a JEOL 100CX electron microscope.

Evans Blue-labeled Albumin. In order to determine whether a blood-tissue barrier, similar to the blood-brain barrier and blood-myenteric barrier, does exist in the gallbladder, Evans blue-labeled albumin was given intravascularly and tissue was examined by fluorescence microscopy, as previously described (Gershon and Bursztajn, 1978; Olsson and Reese, 1971). Bovine serum albumin (Sigma Chemical Company) was dissolved in normal saline (5 g/100 ml). Evans blue was then added to this solution (1 g/100 ml) and mixed for 20 minutes; these conditions provide complete coupling of Evans blue to the albumin (Olsson and Reese, 1971). Guinea pigs were anesthetized (50 mg/kg Ketamine; 5 mg/kg Xylazine) and 1.0 ml of the Evans blue-albumin solution or saline (control) was slowly injected into the femoral vein. The animals were killed at intervals of 10 and 30 minutes following injection of the tracer. The gallbladders were quickly removed from the animals, pinned flat, and fixed for 1 hour with a solution of 4% formaldehyde in 0.1 M phosphate buffer. The preparations were then cryoprotected for 24 hr in a solution of 30% sucrose in 0.1 M phosphate buffer, and sectioned at 15–20 µm on a cryostat. Immunohistochemistry for substance P (SP) was conducted with FITC-labeled secondary antisera so that ganglia could be readily localized in the preparations. The preparations

TABLE 1. Primary antisera

Antigen	Type	Host	Dilution	Source
ChAT	polyclonal	rabbit	1:2000	Dr. Michael Schemann (Hannover, Germany)
SP	monoclonal	rat	1:500	Fitzgerald Industries Intl., Inc
VIP	polyclonal (No. 7193)	rabbit	1:1000	Dr. John Walsh UCLA, Los Angeles
CGRP	polyclonal	rabbit	1:4000	Zeneca/CRB
NOS	monoclonal	mouse	1:150	Transduction Laboratories
MAP 2	monoclonal	mouse	1:1000	Boehringer Mannheim

ChAT, choline acetyltransferase; SP, substance P; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; NOS, nitric oxide synthase; MAP, microtubule associated protein.

were examined with a fluorescence microscope; Evans blue-albumin was visualized with the rhodamine filter set and SP was visualized with the fluorescein filter set.

RESULTS AND DISCUSSION

STRUCTURE OF THE GANGLIONATED PLEXUS OF THE GUINEA PIG GALLBLADDER

A number of studies have been conducted to determine the structural properties of the ganglionated and axonal plexuses of the guinea pig gallbladder (Cai and Gabella, 1983; Cornbrooks et al., 1992; Mawe and Gershon, 1989; Talmage et al., 1992). The wall of the guinea pig gallbladder is made up of three principle layers: the mucosa, the muscularis, and a layer of thick subperitoneal connective tissue, the serosa. Each of these layers is innervated by a plexus of nerve fibers. The most prominent of these is a ganglionated network that is located at the interface between the muscularis and serosal layers. This ganglionated plexus is comprised of an array of small, irregularly shaped ganglia, interconnected by bundles of unmyelinated axons, arranged in no discernible order. The ganglionated plexus is contiguous with a perivascular plexus that parallels blood vessels in the serosa. The neural plexus of the muscularis is primarily comprised of nerve fibers that travel parallel to the direction of muscle bundles. A rich network of nerve fibers pass singly or in bundles in the mucosal layer where they are often located in close association with the epithelium.

Results of ultrastructural studies indicate that the ganglionated plexus of the guinea pig gallbladder shares properties that are characteristic of both enteric and non-enteric autonomic plexuses (Cornbrooks et al., 1992). Like enteric ganglia, gallbladder ganglia are devoid of intercellular spaces, capillaries, or connective tissue elements such as collagen and basal laminae. These ganglia are surrounded by a single, continuous basal lamina that is enclosed within a fibroblast and collagen capsule. This arrangement is also evidenced by light microscopic immunohistochemical findings that gallbladder ganglia lack laminin, although a laminin-rich capsule surrounds each cluster of neurons (Mawe and Gershon, 1989). Within ganglia, neuronal elements are insulated by the processes of cells that resemble the astrocyte-like glia of enteric ganglia. Although few classical synapses are present, sites of direct apposition are commonly observed between vesicle-rich profiles and processes of gallbladder neurons. Vesiculated profiles in these ganglia contain small clear vesicles and large dense core vesicles.

As seen in non-enteric peripheral autonomic systems, unmyelinated axons within interganglionic connectives are insulated from one another by processes of glia that resemble Schwann cells. Within these fiber bundles, direct appositions between vesicle-rich profiles and the connective-limiting basal laminae can be found. Furthermore, unlike the ganglia, these fiber bundles are rich in collagen filament bundles.

STRUCTURE OF INDIVIDUAL NEURONS IN GUINEA PIG GALLBLADDER GANGLIA

The shapes of individual gallbladder neurons have been demonstrated by intracellular injection of ganglion cells with horseradish peroxidase (HRP) or neurobiotin (Cornbrooks et al., 1992; Mawe, 1990). These results show that gallbladder neurons are more simple in structure than enteric neurons. Moreover, they are somewhat similar to neurons of parasympathetic ganglia (Snider, 1987). Guinea pig gallbladder neurons consist of a cell body and one or two long processes, with no appreciable dendritic arborization. The long processes of these cells appear to be largely confined to the ganglionated plexus. The processes pass from their ganglion of origin into interganglionic nerve bundles where they often pass for some distance and terminate. Frequently, these processes exhibit large varicosities as they pass through one or more adjacent ganglia, indicating that interganglionic communication may exist in the ganglionated plexus of the gallbladder. When the muscularis was left intact, labeled processes were sometimes observed passing from the ganglionated plexus and terminating in the muscularis of the guinea pig gallbladder. Since the mucosa was always removed in these preparations, to enable the visualization of ganglia, projections from the ganglion cells to the subepithelial plexus have not been documented.

PHYSIOLOGICAL PROPERTIES OF GUINEA PIG GALLBLADDER NEURONS Membrane Properties of Guinea Pig Gallbladder Neurons

Two types of cells have been identified electrophysiologically in guinea pig gallbladder ganglia (Mawe, 1990). One type of cell is capable of generating action potentials, has a resting membrane potential of -50 mV and an input resistance of 80 M Ω . These cells, which are the gallbladder neurons, are rather inexcitable in their resting state. They fire only one to a few (usually one) action potentials at the onset of a prolonged depolarizing current pulse (Fig. 1), regardless of the amplitude or duration of the current pulse, and spontaneous activity is only rarely observed. The other type of cell has a resting membrane potential of about -70 mV, a low input resistance, does not fire action potentials in response to intracellular injection of large current pulses, and does not receive synaptic input. Cells with these properties have been injected intracellularly with HRP and have been shown to be glial cells (Cornbrooks et al., 1992).

The action potentials of guinea pig gallbladder neurons involve a rapid upstroke with an overshoot of 5 – 10 mV, a rapid repolarization phase, and an afterhyperpolarization (AHP) that lasts about 170 milliseconds (Fig. 1B; Mawe, 1990). An inward Na^+ conductance is largely responsible for the upstroke of the action potential,

since it can be reversibly blocked by TTX. However, there also appears to be an influx of Ca^{2+} associated with the spike since a Ca^{2+} -dependent spike is unmasked in the presence of TTX and tetraethylammonium (TEA).

The action potentials are followed by an AHP that involves two sequential Ca^{2+} -dependent K^+ events (Mawe, 1990). The early phase of the AHP, which results in the initial repolarization of the spike, is abolished in the presence of TEA or Ca^{2+} -free Krebs solution, and is likely to result from the activation of BK channels. The late phase of the AHP is diminished in the presence of apamin or Ca^{2+} -free Krebs solution, but persists in the presence of TEA and/or 3,4-diaminopyridine. These properties are characteristic of the Ca^{2+} -activated K^+ conductance that is associated with SK channels (see Latorre, 1989). The late phase of the AHP may limit the ability of gallbladder neurons to fire repetitively, since in the presence of apamin, depolarizing current pulses result in repetitive action potentials throughout their duration (Fig. 1C).

Synaptic Inputs to Guinea Pig Gallbladder Neurons

Every gallbladder neuron that has been studied in this laboratory ($n > 800$) has received synaptic input resulting in nicotinic fast excitatory postsynaptic potentials (EPSPs) (Fig. 2A). These synaptic responses can be elicited by single stimuli of the interganglionic fiber tracts, are usually suprathreshold, and do not exhibit amplitude rundown at stimulus frequencies of up to 20 Hz (Mawe, 1990). They are reversibly eliminated when the tissue is bathed in a Ca^{2+} -free/ 10 mM Mg^{2+} Krebs solution. Furthermore, they are reversibly suppressed by hexamethonium chloride (100 μM). Recent evidence suggests that, although interganglionic communication in the form of nicotinic EPSPs is likely to exist in these ganglia, the major source of nicotinic input to gallbladder neurons is from vagal preganglionic nerve fibers (Mawe et al., 1994).

Slow EPSPs can be elicited in approximately one-third of the neurons in guinea pig gallbladder ganglia by high frequency fiber tract stimulation (Fig. 2B) (Mawe, 1990). These events are Ca^{2+} -dependent, and are associated with a decrease in input resistance. The slow EPSP is likely to involve an activation of a non-selective cation conductance since its amplitude decreases as cells are depolarized and increases when the membrane is hyperpolarized, with an estimated reversal potential near 0 mV. Recent evidence suggests that tachykinins are a mediator of slow EPSPs in these ganglia (Mawe, 1995) (see below).

No inhibitory postsynaptic potentials have been observed in guinea pig gallbladder ganglia.

NEUROACTIVE COMPOUNDS THAT ARE EXPRESSED BY GUINEA PIG GALLBLADDER NEURONS

The level of transmitter diversity in the gallbladder is not nearly as extensive as that seen in the bowel. However, the ganglionated plexus of the guinea pig gallbladder appears to express a number of neurotransmitters, including acetylcholine (ACh), catecholamines (norepinephrine and dopamine), substance P (SP), neuropeptide Y (NPY), somatostatin (SOM), vasoactive

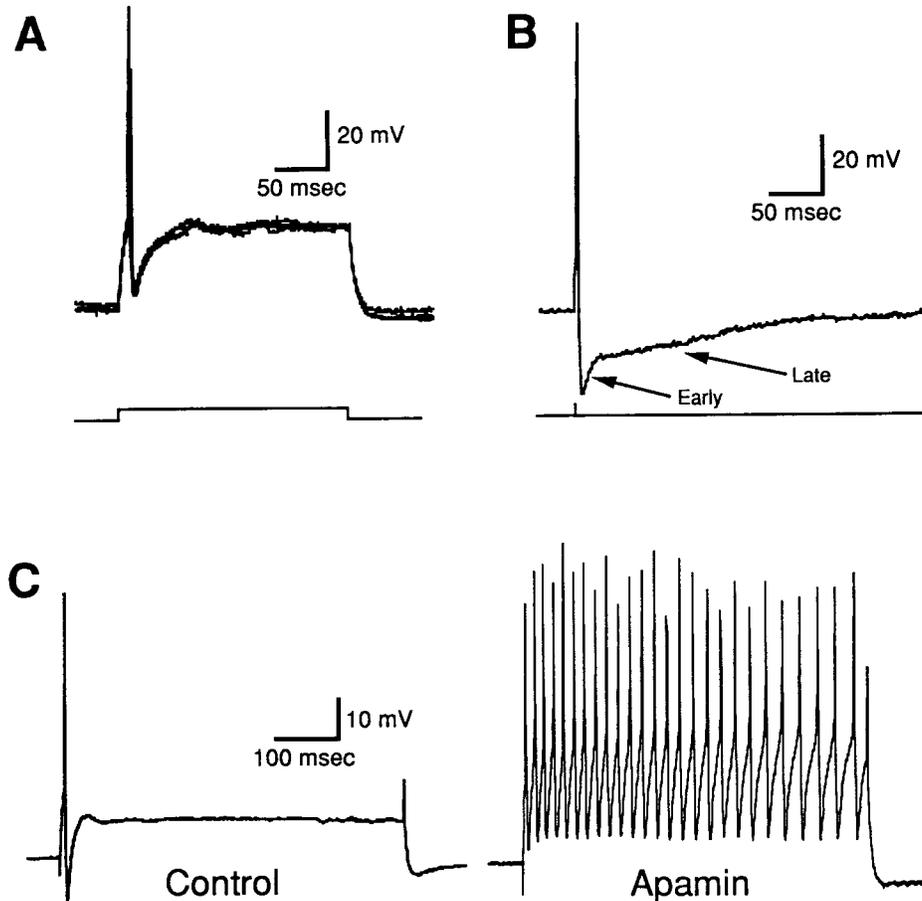


Fig. 1. Active electrical properties of guinea pig gallbladder neurons. **A:** Four consecutive overlapping traces showing the response of a gallbladder neuron to a prolonged depolarizing current pulse (250 msec, 0.2 nA). As is typical of guinea pig gallbladder neurons, the cell generated only one action potential at the onset of each current pulse. **B:** Response of a gallbladder neuron to a brief depolarizing current pulse (2 msec, 0.2 nA) illustrating a typical afterhyperpolarization (AHP). The AHP of gallbladder neurons is composed of early and late

phases, indicated by arrows, that are sensitive to tetraethyl ammonium and apamin, respectively. **C:** Elimination of the late phase of the AHP, by application of apamin (100 nM) to the bath, converts gallbladder neurons from a phasic state, firing one action potential in response to a depolarizing current pulse, to a tonic state, generating a burst of action potentials throughout the duration of a depolarizing current pulse. Resting membrane potentials: A, -49 mV; B, -52 mV; C, -52 mV.

intestinal peptide (VIP), nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), galanin, and serotonin. Compounds that are expressed by gallbladder ganglion cells are described below.

Acetylcholine

Cholinergic nerves in the ganglionated plexus of the gallbladder are likely to arise from intrinsic and extrinsic sources. Although it is not an absolute indicator of the presence of acetylcholine within a given neuron, the catabolic enzyme acetylcholinesterase is plentiful in the neurons and nerve fibers of the guinea pig gallbladder (Cai and Gabella, 1983; Mawe and Gershon, 1989).

Efforts to identify the cholinergic neurons in the ganglionated plexus of the gallbladder have failed until recently. This was because antisera that could recognize the synthetic enzyme choline acetyltransferase (ChAT) in the peripheral nervous system were not available. Recently, Schemann et al. (1995) have generated a polyclonal antiserum that can be used to localize ChAT immunoreactivity in the periphery. With this

antiserum we have been able to successfully immunostain for ChAT in the ganglionated plexus of the guinea pig gallbladder. Immunoreactivity for ChAT was observed in nerve fibers in the paravascular plexus and in the ganglionated plexus. It appeared that all gallbladder neurons were immunoreactive for ChAT. To evaluate this further, preparations were double labeled for the presence of immunoreactivities for ChAT and microtubule associated protein 2 (MAP2). This was done because immunoreactivity for MAP2 is expressed by all gallbladder neurons (Mawe and Gershon, 1989). Results of these experiments indicated that all MAP2 immunoreactive neurons in the gallbladder were also immunoreactive for ChAT (Fig. 3).

Neuroactive Peptides

To evaluate the expression of neuroactive peptides by guinea pig gallbladder ganglion cells, we have performed immunohistochemistry on colchicine-treated preparations (Talmage and Mawe, 1993; Talmage et al., 1992). Throughout these studies we have concentrated

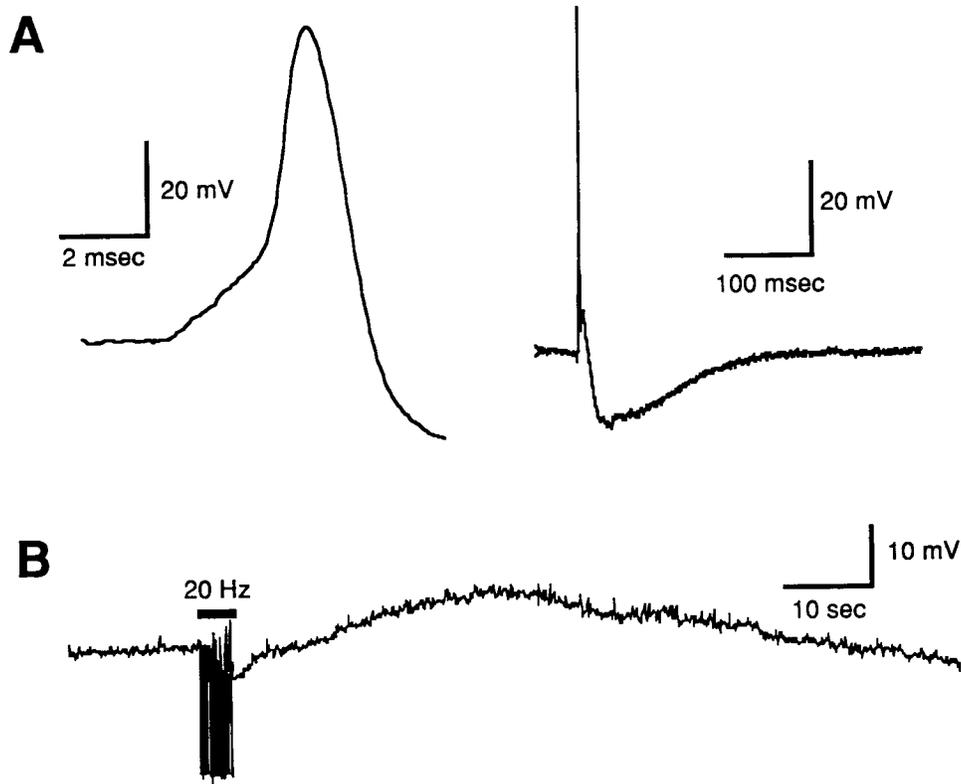


Fig. 2. Two types of synaptic inputs, fast and slow excitatory postsynaptic potentials (EPSPs), can be detected in gallbladder ganglia. These events are elicited in response to electrical stimulation of interganglionic fiber bundles, or the cystic nerves that pass to the gallbladder along the cystic duct. **A:** High and low speed traces illustrating suprathreshold fast EPSPs that were elicited by brief (0.4

msec) stimulation of the cystic nerve. Cholinergic inputs that travel along the cystic nerves originate from the vagus nerves since they are eliminated following vagotomy. **B:** A slow EPSP that was elicited by 20 Hz stimulation of an interganglionic fiber bundle. Resting membrane potentials: A, -50 mV; B, -51 mV.

our efforts on neuroactive compounds for which immunoreactivity had been established to be present without colchicine treatment. Most gallbladder neurons are immunoreactive for SP, NPY, and SOM, whereas immunoreactivity for VIP is present in a small percentage of the gallbladder neurons which do not express SP immunoreactivity. From these data we have concluded that a majority of guinea pig gallbladder neurons express SP, NPY, and SOM. Furthermore, a small, distinct population of neurons in guinea pig gallbladder ganglia expresses VIP.

Nitric Oxide Synthase

We and others have demonstrated that guinea pig neurons stain positively for NADPH-diaphorase (NADPH-DA) activity (Siou et al., 1994; Talmage and Mawe, 1993). These findings indicate that nitric oxide may be synthesized by gallbladder neurons since NADPH-diaphorase has been shown to be present in neurons that are immunoreactive for nitric oxide synthase (Bredt et al., 1991; Hope et al., 1991). In support of this view, nitric oxide synthase (NOS) immunoreactivity has been demonstrated in a subset of gallbladder neurons (Grozdanovic et al., 1994).

Recently, we have conducted experiments to establish whether NOS immunoreactivity is expressed by the same neurons that exhibit NADPH-DA activity. In

these studies, we first processed whole mount preparations for NOS immunoreactivity and documented the results photographically, then we processed the tissue for NADPH-DA activity and re-localized ganglia that contained neurons with NOS-immunoreactive neurons. We found that there was a one-to-one correspondence between NOS immunoreactivity in a given neuron and expression of NADPH-DA activity (Fig. 4, upper micrographs). Therefore, it appears that histochemical staining for NADPH-DA activity is a valid approach to identifying NOS-positive neurons in the guinea pig gallbladder. In our original study of NADPH-DA expression in gallbladder neurons, we found that the NADPH-DA-positive neurons were immunoreactive for VIP, but not for NPY (Talmage and Mawe, 1993). We now report that when colchicine-treated whole mount preparations were double labeled with a rabbit anti-VIP polyclonal antiserum and a mouse anti-NOS monoclonal antiserum, all labeled neurons were immunoreactive for both VIP and NOS (Fig. 4, lower micrographs).

Summary of Coexpression Patterns of Guinea Pig Gallbladder Neurons

When considering the previous results along with the novel results reported here on ChAT and NOS immunoreactivities, it is clear that guinea pig gallbladder ganglia contain at least two types of neurons, based on

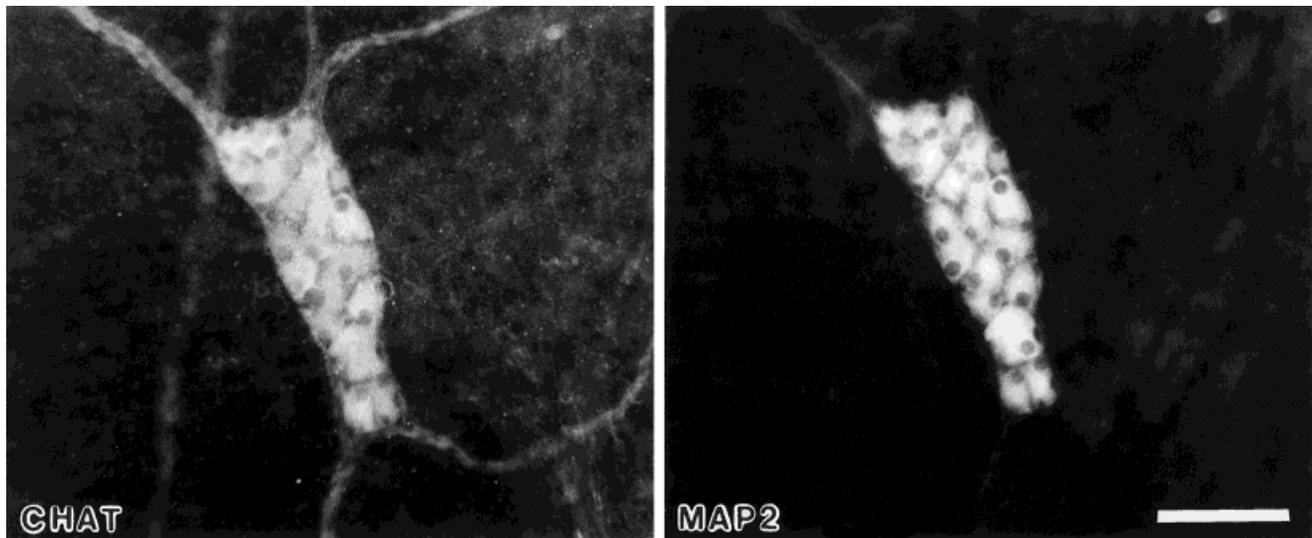


Fig. 3. All neurons in guinea pig gallbladder ganglia are immunoreactive for choline acetyltransferase. Two fluorescence photomicrographs of the same field demonstrating immunoreactivity for choline acetyltransferase (ChAT) and microtubule associated protein 2 (MAP2). ChAT immunoreactivity was visualized with a Cy3-labeled secondary

antiserum and a rhodamine filter cube. MAP2 was visualized with a fluorescein-labeled secondary antiserum and a narrow band fluorescein filter cube. MAP2 immunoreactivity is expressed by all gallbladder neurons. Note that all of the neurons in the ganglion are also immunoreactive for ChAT. Scale bar = 100 μ m.

chemical coding (Fig. 5). All neurons appear to be cholinergic since they express ChAT immunoreactivity. The majority of the neurons express immunoreactivities for ChAT, SP, NPY, and SOM, and a separate group of neurons express ChAT, NOS, and VIP immunoreactivities. The issue of whether these represent two sets of neurons with different targets, or neurons with divergent inputs to the same targets, remains to be resolved.

Catecholamines in SIF Cells

Another type of cell that is present in a subset of guinea pig gallbladder ganglia is a small catecholamine containing cell, which is similar to the small intensely fluorescent (SIF) cells of sympathetic ganglia (Cai and Gabella, 1983, 1984; Mawe and Gershon, 1989). Although essentially nothing is known about their function, the presence of these cells has been demonstrated with both histochemical and immunohistochemical techniques. They can be identified with catecholamine histofluorescence techniques within the ganglia of the gallbladder where they exist either singly or in clusters. They are immunoreactive for the biosynthetic enzyme, tyrosine hydroxylase (TH), but not dopamine- β -hydroxylase (D β H) (Mawe and Gershon, 1989). Therefore, the catecholamine that they express is likely to be dopamine.

INPUTS TO GUINEA PIG GALLBLADDER GANGLIA

A summary of the actions of inputs to guinea pig gallbladder ganglia is provided in Table 2.

Vagal Preganglionic Fibers

The gallbladder receives cholinergic parasympathetic preganglionic input from the vagus nerves. Injection of axonal tracers in the wall of the gallbladder results in bilateral retrograde labelling of neurons in the dorsal motor nucleus of the vagus nerve (Mawe and

Gershon, 1989). Furthermore, vagotomy results in the complete elimination of extrinsic nicotinic inputs, which are normally received by all gallbladder neurons (Mawe et al., 1994). In wholemount preparations of the guinea pig gallbladder that are immunostained for ChAT, immunoreactive fibers are located in the paravascular plexus. Therefore, unlike the ganglion cells in the bowel, all gallbladder neurons receive direct input from the central nervous system. The fibers that provide this input are likely to be the ChAT-immunoreactive nerve fibers that follow the blood vessels into the wall of the organ along the paravascular plexus.

Sympathetic Postganglionic Fibers

In addition to receiving input from vagal preganglionic fibers, the ganglionated plexus of the gallbladder contains sympathetic postganglionic fibers (Cai and Gabella, 1983, 1984; Mawe and Gershon, 1989). Injection of axonal tracers in the wall of the gallbladder results in bilateral retrograde labelling of neurons in the celiac ganglia (Mawe and Gershon, 1989). Furthermore, TH/D β H-immunoreactive nerve fibers are abundant in the ganglionated plexus, and in the perivascular plexus, of the guinea pig gallbladder. These fibers are probably noradrenergic since they express both of these enzymes, and since catecholamine histofluorescent nerve fibers are abundant in gallbladder ganglia (Cai and Gabella, 1983, 1984; Mawe and Gershon, 1989). The TH/D β H-immunoreactive nerve fibers that are located in the perivascular plexus of the guinea pig are also immunoreactive for NPY (Mawe and Gershon, 1989).

Within gallbladder ganglia, the sympathetic postganglionic fibers have a presynaptic inhibitory effect on the release of ACh from vagal preganglionic terminals (Mawe, 1993; Mawe et al., 1994). Norepinephrine decreases the amplitude of fast EPSPs in a concentration dependent manner, with an EC₅₀ of 280 nM (Mawe, 1993). Norepinephrine mediates this effect by acting on

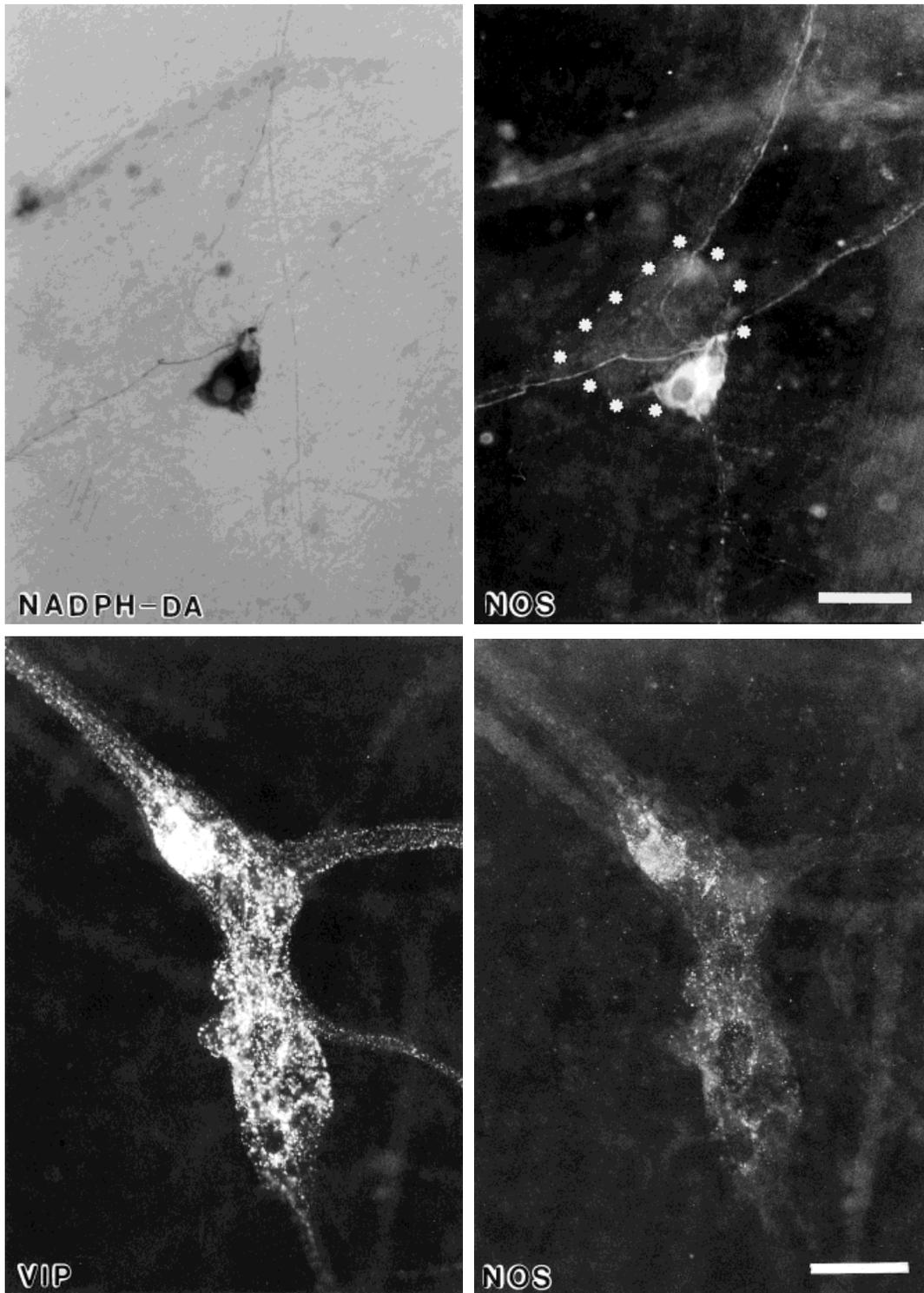


Fig. 4. In guinea pig gallbladder ganglia, neurons that express NADPH-diaphorase (NADPH-DA) also express nitric oxide synthase (NOS) immunoreactivity, and these neurons also immunoreactive for vasoactive intestinal peptide (VIP). The top two photomicrographs demonstrate a neuron that stained positively for NADPH-DA, and was

immunoreactive for NOS. The outline of the ganglion that contains this neuron is indicated by asterisks in the fluorescence photomicrograph. The bottom two photomicrographs demonstrate a neuron that was immunoreactive for both VIP and NOS. Scale bars = 50 μ m.

α_2 adrenoreceptors. The action of norepinephrine is mimicked by the α_2 adrenoreceptor agonist clonidine ($EC_{50} = 30$ nM), and is antagonized by the α_2 adrenore-

ceptor antagonist, yohimbine ($K_B = 1.6$ nM). Release of endogenous catecholamine stores, by tyramine application or by electrical stimulation of the vascular plexus,

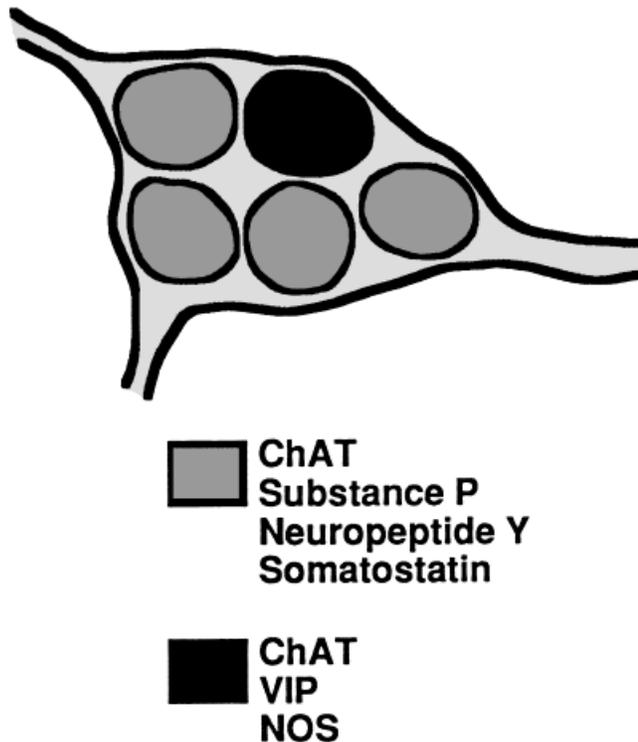


Fig. 5. Schematic diagram showing the two types of neurons that have been identified in guinea pig gallbladder ganglia, based on differential expression of immunoreactivities for neuroactive compounds or associated synthetic enzymes. ChAT, choline acetyltransferase; VIP, vasoactive intestinal peptide; NOS, nitric oxide synthase.

TABLE 2. Actions of inputs to guinea pig gallbladder ganglia

Compound	Site of action	Effect	Receptor	Reference
ACh	postsynaptic	+	nicotinic	Mawe, 1990 Mawe et al., 1994
CCK	presynaptic	+	CCK-A	Mawe, 1991
NE	presynaptic	-	α_2	Mawe, 1993
Tachykinins	postsynaptic	+	NK-3	Mawe, 1995
CGRP	presynaptic	+	unidentified	Mawe and Gokin, 1994
	postsynaptic	+	unidentified	

CCK, cholecystokinin; NE, norepinephrine; CGRP, calcitonin gene-related peptide.

also causes a yohimbine-sensitive decrease in fast synaptic activity. Therefore, the decrease in gallbladder tone that can be elicited by stimulation of the splanchnic nerves (Pallin and Skoglund, 1964; Persson, 1971, 1972, 1973; Yamasato and Nakayama, 1990) may be the result of a presynaptic inhibitory effect of sympathetic nerves on the vagal terminals in gallbladder ganglia.

Sensory Fibers

The ganglionated plexus of the gallbladder is rich in nerve fibers that are immunoreactive for both substance P and CGRP (Goehler et al., 1988; Mawe and Gershon, 1989). These SP/CGRP nerve fibers are abundant in ganglia, interganglionic fiber bundles, and in the paravascular plexus (Fig. 6). Within the ganglia, numerous varicosities are present along the nerve fibers as they appear to surround the ganglion cells.

These fibers must arise exclusively from outside of the gallbladder since gallbladder neurons do not express CGRP immunoreactivity. They are likely to be projections of sensory neurons located in thoracic dorsal root ganglia, which have been shown to project to the gallbladder (Mawe and Gershon, 1989).

It is possible that SP/CGRP-immunoreactive sensory fibers act as the afferent limb of a local axon reflex circuit in the wall of the gallbladder. As noted above, these fibers exhibit varicosities within the ganglia. Also, CGRP and SP are released when gallbladder preparations are exposed to capsaicin (Maggi et al., 1989). We recently investigated the actions of tachykinins and CGRP in guinea pig gallbladder ganglia and found that they both have an excitatory effect (Mawe, 1995; Gokin et al., 1996). Both tachykinins (Fig. 7) and CGRP depolarize gallbladder neurons by activating a non-selective cation conductance. Furthermore, the excitability of gallbladder neurons is increased following exposure to either tachykinins (Fig. 7) or CGRP.

Since potent pharmacological tools are available to study neurokinin receptor-mediated events we have studied the tachykinin-mediated depolarization with receptor-specific agonists and antagonists (Mawe, 1995). The tachykinin-induced depolarization is mediated by neurokinin-3 receptors. Of the receptor-specific agonists tested, only the neurokinin-3 receptor agonist, Senktide, depolarized gallbladder neurons. Moreover, the neurokinin-3 antagonist, [Trp⁷, β -Ala⁸]-NKA (4-10), inhibited the responsiveness of gallbladder neurons to substance P with a K_B of 49 nM, and depressed both capsaicin-induced depolarizations and stimulus-evoked slow excitatory postsynaptic potentials. It is possible that in response to inflammation or high intraluminal pressure, tachykinins and CGRP may be released within ganglia by sensory fibers and act directly on intrinsic neurons to facilitate ganglionic transmission.

Hormonal Cholecystokinin

Cholecystokinin (CCK) was named for its ability to cause gallbladder contractions, an action that has been recognized for over 60 years (Ivy and Oldberg, 1928). However, the exact site(s) of CCK's action in the gallbladder has been debated. Cholecystokinin may act directly on smooth muscle cells to cause gallbladder contractions, or it may act through a neural mechanism to facilitate the excitatory output of gallbladder ganglia. One of the difficulties in accounting for a direct action of CCK on the muscle of the gallbladder is that the concentrations of CCK that are required to contract gallbladder muscle strips, which are in the nanomolar range, are higher than the picomolar serum concentrations of CCK following a meal.

Evidence for a neural mechanism of CCK's action on gallbladder tone comes from studies showing that in the presence of atropine or TTX, CCK-induced gallbladder contractions are reduced (Behar and Biancani, 1980, 1987; Fisher et al., 1985; Grossman, 1975; Gullo et al., 1984; Hanyu et al., 1990; Marzio et al., 1985; Pozo et al., 1989; Strah et al., 1985, 1986; Takahashi et al., 1982). Furthermore, CCK causes the release of ACh from gallbladder preparations (Rakovska et al., 1989; Takahashi et al., 1987; Yamamura et al., 1986; Yau and

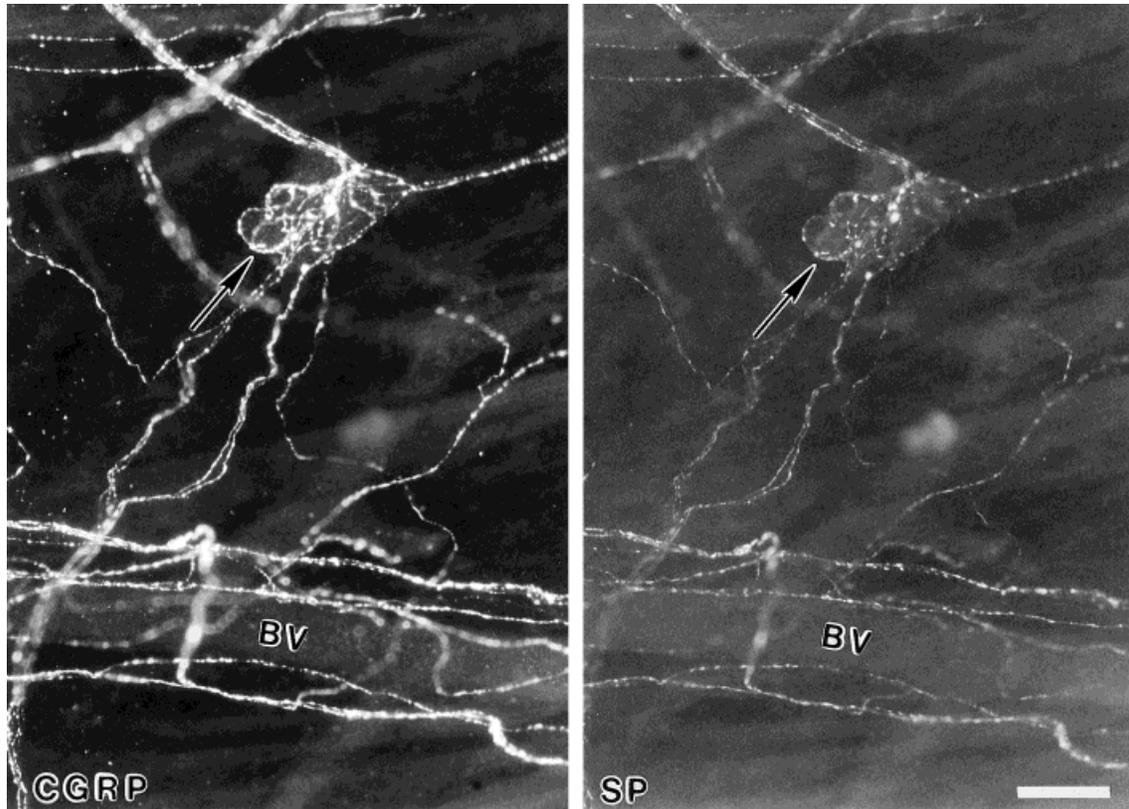


Fig. 6. In the wall of the guinea pig gallbladder, there is an abundance of extrinsic nerve fibers that are immunoreactive for both calcitonin gene-related peptide (CGRP) and substance P (SP). The two photomicrographs demonstrate immunoreactivities for CGRP and SP in the same field. CGRP immunoreactivity was visualized with a Cy3-labeled secondary antiserum, and the SP was visualized with a

fluorescein-labeled secondary antiserum. Note the abundance of double labeled varicose nerve fibers in the ganglion. Double-labeled immunoreactive fibers are found in the paravascular plexus the passes along a blood vessel (BV) in the bottom of the field. These fibers are believed to be extrinsic sensory fibers since CGRP immunoreactivity has never been demonstrated in gallbladder neurons. Scale bars = 50 μ m.

Yother, 1984). These results led to the proposal that CCK receptors on cholinergic neurons participate in the mediation of the contractile effect of CCK on the gallbladder. There is also evidence for the idea that CCK works by acting on a class of neurons or terminals other than the gallbladder cholinergic neurons themselves. Numerous studies of the effects of vagotomy on gallbladder responsiveness indicate that CCK-induced contractions are lessened or delayed following disruption of the vagal input to the gallbladder (Fried et al., 1983; Isaza et al., 1971; Masclee et al., 1990; Pitt et al., 1983; Takahashi et al., 1991; Tinker and Cox, 1969). These data indicate that CCK may act on CCK receptors that are located on vagal afferent and/or efferent nerves.

Over the past several years, studies involving *in vivo* preparations have provided support for the view that the principle physiological effect of CCK in the gallbladder involves a neural mechanism (Hanyu et al., 1990; Pozo et al., 1989; Takahashi et al., 1991). These studies demonstrated that gallbladder contractions elicited by feeding, or by intravascular injections of physiological post-feeding concentrations of CCK, were disrupted by atropine, hexamethonium, or cold block of the vagus nerves. It is plausible that the neural effect that was described resulted from actions of CCK on vagal affer-

ent fibers and/or within gallbladder ganglia. Electrophysiological studies of gallbladder ganglia indicate that ganglia of the gallbladder are a likely site of CCK's action, although a facilitory effect on vagal afferents may also occur.

The actions of CCK in gallbladder ganglia have been studied in the guinea pig (Mawe, 1991; Mawe et al., 1994) and in the opossum (Bauer et al., 1991) using intracellular electrophysiological recording techniques. In both species, CCK has a presynaptic site of action. It increases the amplitude of cholinergic fast EPSPs, usually converting subthreshold EPSPs to suprathreshold EPSPs (Bauer et al., 1991; Mawe, 1991). Recently, we have demonstrated that the nerve terminals that are sensitive to CCK are from the vagus nerve (Mawe et al., 1994).

The pharmacological properties of the presynaptic effect of CCK in the gallbladder have been most thoroughly studied in the guinea pig (Mawe, 1991). In guinea pig gallbladder ganglia, CCK increases the quantal content by threefold without altering quantal size. Furthermore, it has been shown that CCK is quite potent in its ability to increase the amplitude of postsynaptic currents in a concentration dependent manner. This effect is maximal at 1.0 nM, and has a half-

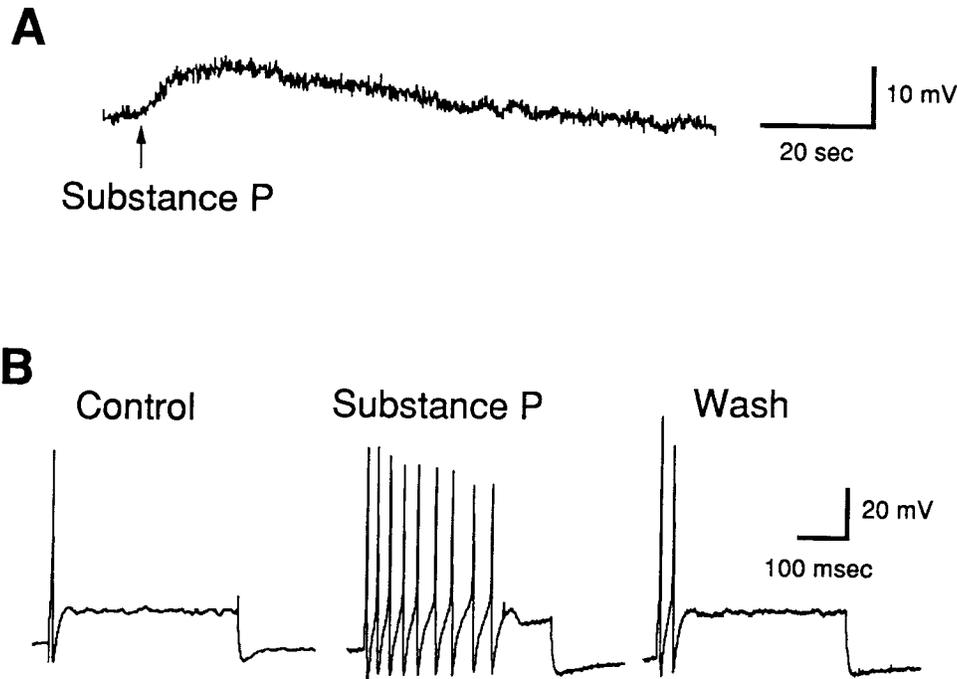


Fig. 7. Substance P causes a prolonged depolarization of guinea pig gallbladder neurons that is associated with an increase in excitability. A: Response of a gallbladder neuron to a brief pressure microinjection of substance P (0.1 mM; 500 msec; 10 PSI). Note the similarity between this response and the slow excitatory postsynaptic potential

shown in Figure 2B. B: Following application of substance P, gallbladder neurons exhibit an increase in excitability which is demonstrated by the generation of a burst of action potentials during a depolarizing current pulse. Resting membrane potentials: A, -54 mV; B, -52 mV.

maximal effective concentration (EC_{50}) of 33 pM, which is well within the range of serum levels of CCK following a meal (Takahashi et al., 1991). The action of CCK in gallbladder ganglia appears to be mediated by CCK-A receptors, since it is diminished by the CCK-A antagonist MK-329 (formerly L-364,718), but it is unaffected by the CCK-B antagonist L-365,260. From these results, it appears that CCK is capable of facilitating ganglionic output by enhancing the release of ACh from nerve terminals, possibly the terminals of vagal preganglionic fibers.

Immunoreactivity for CCK has not been detected in the ganglionated plexus of the guinea pig gallbladder (Mawe, 1991); therefore, if CCK does have a physiological action in these ganglia it probably involves hormonal CCK rather than CCK released from nerve terminals. This leads to the question of whether circulating CCK can indeed gain access to the neuropil of ganglia to exert its proposed presynaptic action. The results of *in vivo* studies that were described above indicate that CCK can gain access to ganglia to exert its neural effect. However, studies of the enteric nervous system indicate that a blood-tissue barrier exists in the intestine that restricts the access of macromolecules to the myenteric plexus (Gershon and Bursztajn, 1978). As is true of the blood-brain barrier, this enteric blood-tissue barrier was localized to the endothelial cells of specialized capillaries supplying the neural tissue. Because circulating hormones have such a profound effect on gallbladder function, and because the neuronal component of the overall effect of hormones is not understood, it is important to determine whether such

molecules would have access to neuronal membranes in the ganglionated plexus.

Two lines of evidence indicate that a blood-plexus barrier does not exist between the capillaries and ganglia of the gallbladder. One of these involved standard transmission electron microscopy and the other involved intravascular injections of Evans blue-labeled albumin. In the process of evaluating the ultrastructural characteristics of gallbladder ganglia, we observed that the capillaries in the region of the interface between the muscularis and the serosa, where the ganglionated plexus is located, were fenestrated (Fig. 8). This feature, which has also been demonstrated in capillaries of the human gallbladder (Ebe and Kobayashi, 1972), is associated with a local vascular system that promotes, rather than inhibits, the transendothelial flow of macromolecules.

The second set of data indicating the absence of a blood plexus barrier in the gallbladder involved intravascular injection of Evans blue-labeled albumin. For these experiments, we followed the protocol used by Gershon and Bursztajn (1978). Double labeling for SP immunoreactivity was done in order to localize the ganglia in the tissue sections. The Evans blue-labeled albumin gained access to the interior of gallbladder ganglia at the shortest experimental time point (10 minutes) following injection into the femoral vein (Fig. 9). Together, these data and the finding that gallbladder capillaries are fenestrated are strong indicators that hormonal CCK can readily access the vagal preganglionic terminals that lie within gallbladder ganglia and

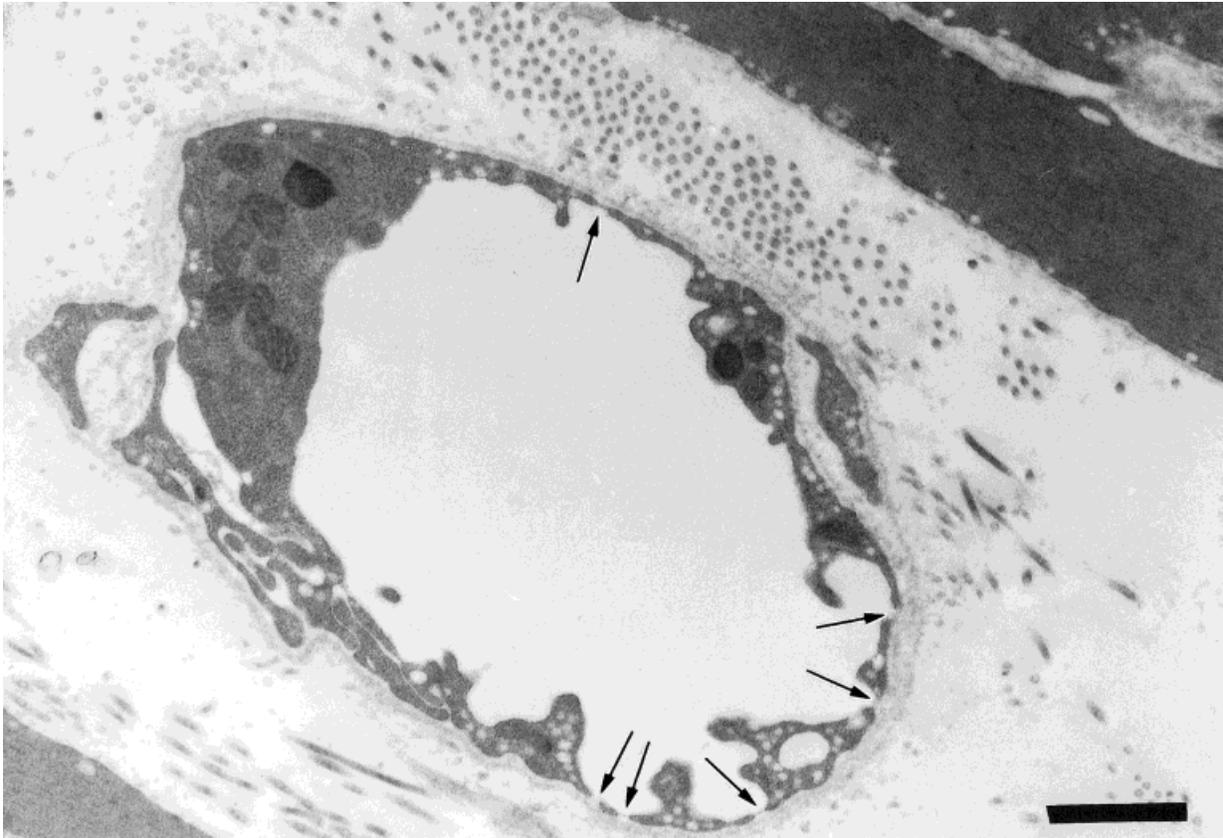


Fig. 8. Capillaries in the wall of the guinea pig gallbladder, near the interface between the muscularis and the serosa, are fenestrated. The electron micrograph shows a capillary near the outer edge of the muscularis. Arrows indicate fenestrations. Scale bar = 1.0 μ m.

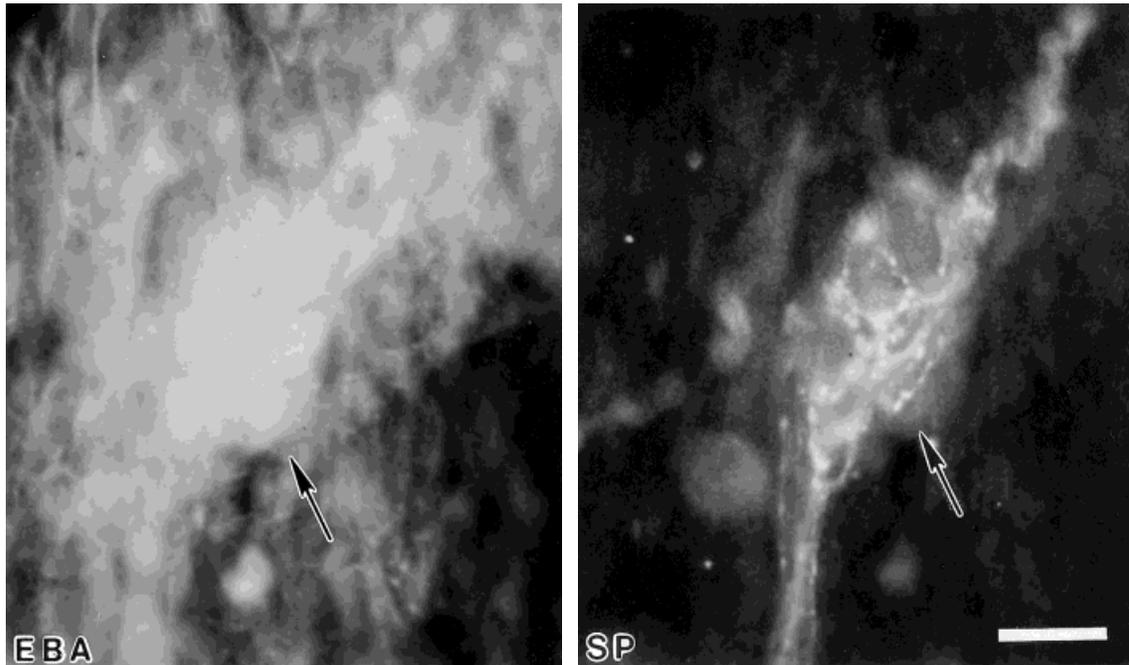


Fig. 9. Evans blue-labeled albumin, injected intravenously, can exit capillaries and gain access to gallbladder ganglia. The photomicrograph on the left demonstrates Evans blue-labeled albumin, which was illuminated with a rhodamine filter cube. The photomicrograph on the right demonstrates substance P immunoreactivity in the same

field shown on the right. The arrows are located in the same position within the field in each micrograph, and indicate the location of a ganglion. In this experiment, the guinea pig was killed 10 minutes after the injection of Evans blue-labeled albumin into the femoral vein. Scale bar = 50 μ m.

exert the presynaptic facilitatory effect that was measured electrophysiologically.

Unidentified Extrinsic Fibers

Nerve fibers that are immunoreactive for serotonin (Mawe and Gershon, 1989) and galanin (Siou et al., 1992) have also been demonstrated in the ganglionated plexus of the guinea pig gallbladder. At this time the source of these nerve fibers has not been determined. However, they may arise from neurons in the myenteric plexus of the duodenum, located near the choledochoduodenal junction. Neurons in this region have been shown to project to the gallbladder (Mawe and Gershon, 1989), and subsets of the neurons in this area are serotonergic and galanin-immunoreactive.

CONCLUDING REMARKS

During the past 15 years or so, neurobiologists have become more appreciative of the complexity associated with the local neural regulation of organ function. No longer are autonomic ganglia thought of as simple conveyors of impulses from the brain to the effector tissues. Autonomic ganglia are now recognized as the destinations of various modulatory inputs to a given organ from several sources. In the case of the gallbladder, the findings described here show that inputs to ganglia can come from several sources, including: the brain, sensory fibers, sympathetic ganglia, enteric ganglia, intrinsic ganglia, and circulating hormones. Another likely source of modulatory inputs to gallbladder ganglia, which has yet to be explored, is inflammatory cells that release various agents such as prostaglandins, during cholecystitis. The potential output signals that are communicated by individual gallbladder neurons can also be diverse. In the guinea pig, two groups of neurons exist, based on their distinct expressions of neuroactive compounds. Most guinea pig gallbladder neurons express substance P, NPY and somatostatin, while others appear to be capable of releasing nitric oxide and VIP. The precise targets of each of these groups of neurons and their net effects on those tissues remain to be explored.

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