Vagus nerve modulates secretin binding sites in the rat forestomach

HYEOK Y. KWON, TA-MIN CHANG, KAE Y. LEE, AND WILLIAM Y. CHEY
Konar Center for Digestive and Liver Diseases, University of Rochester School of Medicine and Dentistry, Rochester, New York 14624

Kwon, Hyeok Y., Ta-Min Chang, Kae Y. Lee, and William Y. Chey. Vagus nerve modulates secretin binding sites in the rat forestomach. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1052–G1058, 1999.—Secretin is well known for its inhibitory action on gastric motility. It has been reported that secretin in a physiological dose inhibits gastric motility through mediation by the vagal afferent pathway. Secretin also elicited relaxation of carbachol-stimulated rat forestomach muscle strips by binding to its receptors, suggesting a direct action on this peripheral tissue. We hypothesized that vagal input may affect the action of secretin by modulating the level of secretin receptor in the forestomach. Several treatments, including vagal ligation, vagotomy, perivaginal application of capsaicin or colchicine, intravenous infusion of tetrodotoxin, and intraperitoneal injection of atropine, were performed to investigate their effects on secretin receptor binding to forestomach membranes. Specific binding of 125I-labeled secretin to forestomach membranes was significantly decreased (45%) by vagal ligation, vagotomy (50%), or perivagal colchicine treatment (40%). On the contrary, specific binding of 125I-secretin was not affected by perivagal capsaicin treatment, intravenous infusion of tetrodotoxin, or intraperitoneal injection of atropine. By Scatchard analysis of the binding data, the capacity of the high-affinity binding sites in forestomach membranes was found to decrease significantly after vagal ligation compared with membranes from the sham-operated group. However, the affinity at the high-affinity binding sites, the binding parameters of the low-affinity binding sites, and binding specificity were not changed. Vagal ligation but not perivagal capsaicin treatment reduced the inhibitory effect of secretin on carbachol-stimulated contraction of isolated forestomach muscle strips, causing a right shift in the dose-response curve. These results suggest that vagal input through axonal transport plays a significant role on secretin action by modulating the capacity of secretin binding sites (but not affinity or specificity), at least in rat forestomach.

secretin receptor; receptor modulation; vagal ligation; capsaicin

SECRETIN AS A HORMONE exerts physiological actions on the exocrine pancreas by stimulating secretion of fluid rich in bicarbonate and on the stomach by inhibiting secretion of gastric acid, release of gastrin, and gastric emptying (6, 29). In recent years, it has become evident that physiological actions of secretin are mediated by the nervous system. It has been reported that vagotomy or atropine significantly inhibits pancreatic secretion in response to a physiological dose of exogenous secretin in humans (30) and dogs (7, 24). We have found that both the release of secretin elicited by a luminal secretin-releasing factor and the action of secretin in a physiological dose on the exocrine pancreas are also mediated through a capsaicin-sensitive vagal afferent pathway (14).

Secretin is also an important modulator of gastric motility. It has been reported that secretin receptors are localized on the longitudinal and circular muscle layers of the rat forestomach (fundus or nonglandular portion of the stomach). These receptors have been proposed to mediate the inhibitory effect of secretin on gastric motor function (25). Furthermore, administration of secretin at physiological doses has been shown to delay gastric emptying and to inhibit gastric motility via a vagal afferent pathway originating from the gastroduodenal mucosa (17, 21). The peripheral tissues, secretin immunoreactivity and secretin receptor have been reported to be distributed in the central nervous system (4, 9, 20). Recently, the presence of secretin receptors on vagal afferent fibers has been demonstrated by using an autoradiographic visualization (26). However, little is known about neural regulation of secretin receptors, which mediate the physiological effects of secretin in the peripheral tissues.

We hypothesized that the vagus nerve may also affect the action of secretin by modulating the level of secretin binding sites in the peripheral tissues such as rat forestomach. In the present study, we determined the effects of various treatments, including vagal ligation, vagotomy, peripheral vagal application of capsaicin and colchicine, intravenous injection of tetrodotoxin, and intraperitoneal injection of atropine, on the level of secretin receptor in isolated rat forestomach membranes. Our results indicated that the vagus nerve plays an important role in regulating the level of the secretin binding site in rat forestomach.

MATERIALS AND METHODS

Peptides and Chemicals

Synthetic porcine secretin and pituitary adenylate cyclase-activating polypeptide (PACAP)-27 were generous gifts from Dr. David H. Coy (Tulane University Medical Center, Tulane, LA). Natural porcine vasoactive intestinal peptide (VIP) and synthetic peptide histidine isoleucine (PHI) were obtained from Dr. V. Mutt (Karolinska Institute, Stockholm, Sweden) and Dr. H. Yajima (Kyoto University, Kyoto, Japan), respectively. Glucagon was obtained from Peninsula Laboratories (Belmont, CA). 125I-labeled secretin was prepared using the procedure published previously (5). HEPES, bacitracin, tetrodotoxin (TTX), 5'-guanylylimidodiphosphate (GMP-PNP), colchicine, capsaicin, and Tween 80 were purchased from Sigma Chemical (St. Louis, MO). Atropine sulfate was obtained from

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
American Regent (Shirley, NY). Bethanechol and ampicillin were purchased from Merck (West Point, PA) and Marsam Pharmaceuticals (Cherry Hill, NJ), respectively.

Animal Preparations

To investigate the role of the vagus nerve on the level of secretin receptor binding sites in rat forestomach, animal operations were performed. Next, forestomach membranes for secretin receptor binding were prepared from each group of the operated or sham-operated rats. Male Sprague-Dawley rats weighing between 250 and 300 g were fasted for 18 h with free access to tap water before surgery. Nembutal (6.5 mg/kg body wt ip) was used for anesthesia.

Bilateral subdiaphragmatic vagal ligation or vagotomy. Under anesthesia, a midline laparotomy was performed, and the stomach was carefully manipulated to expose the esophagus. Both anterior and posterior subdiaphragmatic vagal trunks were exposed, and the proximal parts were ligated with silk or transected (sham operated). After recovery from anesthesia, rats were continually fasted. Forestomach membranes were prepared from each group 1 day after surgery.

Perivagal application of capsaicin. Capsaicin was applied perivagally to investigate the role of capsaicin-sensitive vagal sensory nerves on the level of secretin binding sites in the forestomach membranes. Capsaicin was dissolved in Tween 80-ethanol-saline (1:1.8, vol/vol/vol) at the concentration of 10 mg/ml. Both anterior and posterior subdiaphragmatic vagal trunks were exposed and wrapped with a piece of 0.5 cm2 Kimwipe soaked with 30 ml of capsaicin solution or vehicle for 30 min. A piece of parafilm was placed under the esophagus to protect the other organs from capsaicin. All other exposed surfaces were covered with saline-soaked gauze. Thirty minutes later, the vagal trunks were washed with isotonic saline, and the abdominal incision was closed. Forestomach membranes were prepared from each group 2 wk after surgery.

Perivagal application of colchicine. The effect of colchicine, an alkaloid believed to block axonal transport, on the level of secretin binding sites was examined next. Both vagal trunks were exposed and were surrounded by a 1 × 0.5 cm2 Kimwipe, which was soaked in 5 ml of colchicine solution (10 mg/ml in saline) or vehicle for 30 min. A piece of parafilm was placed under the esophagus to protect the other organs from colchicine. All other exposed surfaces were covered with saline-soaked gauze. Thirty minutes later, the vagal trunks were washed with isotonic saline, and the abdominal incision was closed. Forestomach membranes were prepared from each group 1 day after surgery.

Administration of TTX. TTX, a nonspecific blocker of neuronal transmission, was injected (2 mg/kg iv) in a bolus followed by continuous infusion (2 mg·kg−1·h−1) for 1.5 h to determine if there is a neural influence on the level of secretin binding sites. The same dose of TTX was administered again 22 h after initial infusion of TTX. Forestomach membranes were prepared immediately after the second administration of TTX.

Injection of atropine. To determine whether the muscarinic cholinergic pathway would affect the level of secretin binding sites, atropine was injected intraperitoneally (1 mg/kg). Forestomach membranes were prepared 1 day after atropine injection.

Preparation of Rat Forestomach Membranes

Stomachs were removed, opened along the lesser curvature, and rinsed thoroughly in cold saline. Forestomach regions were dissected, minced, and homogenized in 20 vol (wt/vol) of 10 mM HEPES, pH 7.4, and 1 mM MgCl2. The homogenate was centrifuged at 200 g for 10 min, and the resulting supernatant solution was collected and further centrifuged at 13,200 g for 30 min to obtain a membrane pellet. The supernatant solution was removed, and the membrane pellet was stored at −70°C before use.

Secretin Binding Assay

Forestomach membrane pellets were resuspended in 10 mM HEPES, pH 7.4, containing 1% BSA, 1 mg/ml bacitracin, and 1 mM MgCl2. An aliquot of the membrane suspension containing 100 mg of protein was incubated in triplicate at 37°C for 60 min with 80 fmol of 125I-secretin in the presence or absence of unlabeled secretin (~10 pM–1 mM) or other peptides of the secretin/VIP superfamily. Membrane-bound 125I-secretin was separated from the free peptide by centrifugation for 2 min in a Beckman Microfuge B. After centrifugation, the supernatant solution was removed, and the bottom of the tube containing the pellet was cut off and then counted in a LKB model 1261 Multigamma counter (Wallac, Gaithersburg, MD). Saturable binding was calculated by subtracting nonspecific binding (binding in the presence of 1 mM unlabeled secretin) from the total binding. The binding parameters were calculated using LIGAND, a computerized curvefitting program (19).

Muscle Tension Study

Forestomach muscle strips (1 × 0.3 cm2, ~20–30 mg), cut along the circular axis of the stomach, were mounted in an Ussing chamber containing 5 ml Krebs solution at 37°C bubbled with a mixture of 95% O2 and 5% CO2. The Krebs solution had the following composition (in mM): 131.1 NaCl, 5.6 KCl, 1.9 CaCl2, 1.0 NaH2PO4, 25.0 NaHCO3, 1.0 MgCl2, and 5.0 dextrose at pH 7.4. One end of the muscle strip was fixed to a hook at the bottom of the chamber, and the other end was attached to an isometric force-displacement transducer (Grass FT03). The contractile response was measured isometrically and was recorded on a Hewlett-Packard polygraph. Preparations were allowed to equilibrate for 30 min before study. The effect of secretin (~10 nM–1 mM) on the bethanechol (10 mM)-stimulated muscle contraction was studied. Secretin was added to the bath when bethanechol-stimulated muscle contraction was at a steady state.

Statistical Analysis

All of the results were expressed as means ± SE. Student’s t-test for paired data was used to evaluate the statistical significance of differences between the two groups using a computer program. P values <0.05 were considered statistically significant.

RESULTS

Characterization of Secretin Binding Sites

When forestomach membranes were incubated with 80 pM of 125I-secretin in the presence and absence of 1 mM unlabeled secretin at 37°C, saturable binding was observed. Specific binding was one-half maximal after 5 min and maximal at 60 min. The maximal specific binding averaged 5.27 ± 0.75% of total radioactivity/100 mg forestomach membrane protein (n = 6), and nonspecific binding averaged 0.14 ± 0.02% of total radioactivity/100 mg of membrane protein.
To examine the specificity of binding of $^{125}\text{I}}$-secretin to forestomach membranes, secretin/VIP family peptides were tested for their abilities to inhibit binding of $^{125}\text{I}}$-secretin (Fig. 1). Secretin, VIP, PHI, and PACAP-27 inhibited binding of $^{125}\text{I}}$-secretin to forestomach membranes. Secretin was the most potent inhibitor, with a detectable inhibition observed at 0.1 nM, IC$_{50}$ at 1.4 nM, and complete inhibition at 1 mM. IC$_{50}$ of VIP, PHI, and PACAP-27 were observed at 0.19, 0.40, and 1 mM, respectively. Therefore, VIP was 136-fold less potent than secretin, and PACAP-27 and PHI were 5-fold and 12.5-fold less potent than VIP, respectively. Therefore, the binding sites were highly selective for secretin.

Guanine nucleotides negatively modulate binding of polypeptides to receptors that are coupled to adenylate cyclase (23). GMPPNP, the relatively nonmetabolizable analog of GTP, was reported to be the most potent guanine nucleotide inhibiting $^{125}\text{I}}$-secretin to rat brain membranes (9). To characterize the secretin receptors in the rat forestomach, we examined the effect of GMPPNP on the binding of $^{125}\text{I}}$-secretin. Specific binding of $^{125}\text{I}}$-secretin was decreased in a dose-dependent manner by 1–100 mM of GMPPNP (Fig. 2A). As shown in Fig. 2B, the dose-dependent inhibition curve of unlabeled secretin determined in the presence of 20 mM of GMPPNP was shifted to the right of the control curve, indicating a reduction in receptor affinity for secretin. The IC$_{50}$ was 1.26 nM in the absence and 6.31 nM in the presence of 20 mM GMPPNP.

Effects of Vagal Ligation, Vagotomy, Perivagal Treatment with Capsaicin or Colchicine, and Neuronal Blockers on the Level of Secretin Binding Sites

As shown in Fig. 3, bilateral vagal ligation or vagotomy at the truncal level resulted in a significant decrease in specific binding of $^{125}\text{I}}$-secretin to rat forestomach membranes. Specific $^{125}\text{I}}$-secretin binding was 5.48 ± 0.33% of total radioactivity/100 mg protein in the sham-operated group. After vagal ligation or vagotomy, the level of specific $^{125}\text{I}}$-secretin binding was significantly decreased to the value of 3.02 ± 0.25 or 2.72 ± 0.25% of total radioactivity/100 mg protein. The effects of perivagal application of capsaicin and colchicine on specific $^{125}\text{I}}$-secretin binding were significantly different from each other. There was no significant change in specific binding by perivagal capsaicin treatment. On the contrary, perivagal colchicine treatment significantly decreased specific $^{125}\text{I}}$-secretin binding from 5.22 ± 0.69% of total radioactivity/100 mg protein (vehicle group) to 3.15 ± 0.48% of total radioactivity/100 mg protein (colchicine-treated group), corresponding to a decrease of 40%. Both TTX and atropine had no effect on specific $^{125}\text{I}}$-secretin binding.

To determine the observed inhibition of $^{125}\text{I}}$-secretin binding that is due to a decrease in affinity or capacity of binding sites, Scatchard analysis of equilibrium binding data was performed. The binding parameters obtained from membranes prepared from the sham-operated group and vagal-ligated group of rats were...
then compared. The binding data of these membranes fitted a two-sites receptor binding model the best (Fig. 4). The membranes of the sham-operated group had a high-affinity dissociation constant of 1.06 $\pm$ 0.05 nM and a low-affinity dissociation constant of 0.48 $\pm$ 0.22 mM (Table 1). As summarized in Table 1, vagal ligation caused a significant decrease in the number of the high-affinity binding sites from 180.0 $\pm$ 9.4 fmol/mg protein (sham-operated group) to 80.6 $\pm$ 5.7 fmol/mg protein (vagal-ligated group). However, vagal ligation did not affect the affinity at the high-affinity binding sites or the affinity and number of low-affinity binding sites. In addition, the binding specificity among secretin and other peptides of the secretin/VIP family was not altered by vagal ligation (data not shown).

**Effect of Vagal Ligation on Secretin Action in Forestomach Muscle Strips**

To determine if reduction in the capacity of high-affinity secretin binding sites after vagal ligation was functionally related to the action of secretin, the effect of secretin on bethanechol-stimulated contraction of forestomach muscle strips was studied. Bethanechol (10 mM) strongly induced contraction of forestomach muscle strips (0.13 $\pm$ 0.01 g of contractile force/mg muscle strip, n = 10). Bethanechol-stimulated muscle contraction was reduced by secretin in a dose-dependent manner (Fig. 5A). After vagal ligation, the inhibitory effect of secretin on bethanechol-stimulated muscle contraction was significantly reduced at the doses of 50 and 100 nM of secretin (Fig. 5A), resulting in a right shift in the dose-dependent inhibition curve. Consequently, a fourfold increase in the half-maximal dose from 25 nM (sham-operated group) to 100 nM (vagal-ligated group) was detected. On the other hand, the inhibitory effect of secretin on bethanechol-stimulated muscle strip contraction was not altered after perivagal treatment of capsaicin (Fig. 5B). These results agreed well with the above data of the secretin binding assay in which vagal ligation, but not perivagal capsaicin treatment, reduced the number of secretin binding sites.

**Table 1. Effect of vagal ligation on the number and binding affinity of secretin binding sites in the rat forestomach**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>High-Affinity Binding Sites</th>
<th>Low-Affinity Binding Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$, nM</td>
<td>$N_x$, fmol/mg protein</td>
</tr>
<tr>
<td>Sham operation</td>
<td>1.06 $\pm$ 0.05</td>
<td>180.0 $\pm$ 9.4</td>
</tr>
<tr>
<td>Vagal ligation</td>
<td>1.09 $\pm$ 0.05</td>
<td>80.6 $\pm$ 5.7*</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE from 5 separate experiments performed in triplicate. $K_d$, dissociation constant; $N_x$, no. of secretin binding sites. *P < 0.01 vs. sham-operated group.
DISCUSSION

The results of the present study have demonstrated that rat forestomach membranes contain a class of high-affinity binding sites that is highly specific for secretin and is sensitive to the GTP analog GMPPNP and thus is characteristic for a G protein-coupled receptor. Although secretin receptor has been previously examined by quantitative densitometry of an autoradiogram in rat forestomach (25), secretin binding to forestomach membranes has not been reported. In the present study, we found that the binding of $^{125}$I-secretin to forestomach membranes was rapid, saturable, and time dependent. These features were similar to those reported for secretin receptors found in the membranes of rat pancreas (3) and brain (9). By Scatchard analysis, rat forestomach membranes exhibited two classes of secretin binding sites with a high-affinity dissociation constant of 1.06 nM and a low-affinity dissociation constant of 0.48 mM. A previous study by Steiner et al. (25) demonstrated that $^{125}$I-secretin bound to a single class of high-affinity binding sites in rat forestomach with a dissociation constant of 0.6 nM, which was similar to our high-affinity binding sites. It is likely that the autoradiographic method failed to detect the low-affinity binding sites.

A recent report has indicated that physiological action and the release of secretin are closely related to the nervous system, although the relationship between secretin and the nervous system is still unclear (14). Therefore, it is important to characterize the neural regulation of secretin at the receptor level for understanding its physiological actions. Humoral regulation of the secretin receptor has been reported, but little is known about neural regulation of the secretin receptor. Iwakawa et al. (11) have reported that gastrin directly interacts with the secretin binding sites on plasma membranes of rat gastric mucosa, although the exact mechanism of this interaction is unclear. In the pancreas, it has been reported that cholecystokinin decreases the specific binding of $^{125}$I-secretin by 51% in rat pancreatic acini through a postreceptor process (12). On the other hand, it is known that gene expression of secretin receptor in rat cholangiocytes is increased by bile duct ligation (1).

The results of the present study have strongly suggested that secretin receptor level in rat forestomach is modulated by a vagal input. Our results showed that 1) vagal ligation, truncal vagotony, or perivagal treatment with colchicine, but not perivagal capsaicin treatment, reduced the level of secretin receptor in the rat forestomach membranes; 2) vagal ligation reduced the number of high-affinity secretin receptors without affecting its affinity or specificity, and the low-affinity binding sites were not affected by vagal ligation; 3) TTX or atropine had no effect on secretin binding sites. These results suggest that a vagal input to the forestomach modulates the level of the secretin receptor in the rat. Because treatments with capsaicin, TTX, and atropine were ineffective in affecting secretin receptor level in the forestomach, it is unlikely that capsaicin-sensitive vagal afferent fibers, and TTX-sensitive Na$^{+}$ channel-dependent conduction or muscarinic transmission of vagus nerve, are involved in vagal modulation of secretin receptors. As might be expected from the results of these binding studies, vagal ligation but not perivagal capsaicin treatment reduced the inhibitory effect of secretin on bethanechol-stimulated contraction of rat forestomach strips, causing a shift to the right in the dose-response curve. Thus it appears that a vagal input independent of TTX-sensitive and muscarinic neurotransmissions plays a significant role on secretin action by modulating the capacity of high-affinity secretin binding sites in the rat forestomach. This observation is supported by the previous observations that secretin receptors are present on the longitudinal and circular muscle layers of the rat forestomach (25) and that secretin reduces the tone in muscle strips isolated from rat forestomach.

The mechanism through which the vagal input modulates the concentration of secretin receptor in the forestomach is not clear at present. We have attempted to identify the fraction of forestomach membranes that contained secretin receptors by density gradient centrifugation. We observed that the secretin binding sites were always associated with a fraction that contained...
both 5'-nucleotidase activity and binding activity for \( \beta \)-[\( ^{3} \)H]lysitoxin (data not shown). It has been reported that 5'-nucleotidase activity is bound to the external surface of the neuronal membranes of nodose ganglia (13) and is associated with the isolated axolema (28). Therefore, secretin receptors in the forestomach membranes might be derived from vagal nerve terminals or in the myenteric plexus terminated by vagal nerve fibers. The effect of colchicine (a blocker of axonal transport) on secretin binding sites in rat forestomach membranes suggested that secretin receptors may be axonally transported and accumulated in vagus nerve fibers that terminate in the forestomach. This argument is supported by the observations that receptors for neuropeptides and neurotransmitters are present in peripheral nerve and that a fraction of these receptors is undergoing axonal transport that is selectively blocked by colchicine (8, 10, 16, 18, 31, 32). The identification of secretin receptors within the vagus nerve and central nervous system supports the possibility that secretin receptor may be transported through the vagus nerve (9, 26) to reach its termini innervating the smooth muscle layers. A recent study has reported that, in the rat, most of the vagal afferent fibers derived from nodose ganglia that terminate at the stomach and the upper portion of the duodenum are capsaicin-resistant (2). In the forestomach, these capsaicin-resistant nerve terminals form intraganglionic laminar endings (70% resistant) in the myenteric plexus and intramuscular endings or arrays (57% resistant) in the muscle layer. Thus, if secretin receptors were accumulated in these nerve terminals through axonal transport, their number would be expected to decrease by treatment with colchicine but not by capsaicin as we have observed. However, it remains to be elucidated whether such anterogradely transported secretin receptors are able to affect muscle contraction, particularly in the isolated muscle strips as we have observed in the present study. An alternative explanation is that the vagal input may provide, via axonal transport through these capsaicin-resistant nerve terminals, a yet unidentified factor that is vital for expression of secretin receptor on the smooth muscle membrane. This question may be answered by future studies on gene expression and the exact localization of secretin receptors in rat forestomach.

It should be mentioned that, according to our data, capsaicin-sensitive afferent fibers do not play a significant role in vagal modulation of secretin receptor in rat forestomach. This observation does not appear to correlate well with our previous finding that actions of secretin in a physiological dose on exocrine pancreatic (14) and gastric acid secretions (15) are mediated through a capsaicin-sensitive vagal afferent pathway. It also has been demonstrated that secretin inhibits gastric emptying in part via an action on the vagal capsaicin-sensitive afferent pathway (21, 22). Furthermore, transport of secretin receptors through capsaicin-sensitive vagal afferent fibers has been reported through vagal ligation and autoradiographic visualization (26). These receptors were found to accumulate at the proximal site of vagal ligation, suggesting the occurrence of an anterograde axonal transport of the receptor toward the peripheral terminals. It is likely that physiological actions of secretin are mediated in part via secretin receptors in capsaicin-sensitive afferent fibers through a vago-vagal reflex mechanism (14, 15, 21, 22) or via a reflex mechanism through collaterals branching out from these afferent fibers (27). The secretin receptors in the forestomach membranes may function by providing an auxiliary signal to that produced by the afferent reflex to produce an amplified response. Thus it is possible that secretin acts both indirectly by way of its receptors in the vagal afferent pathway and directly on the receptors in the peripheral tissues such as the forestomach to affect gastric motility.

In summary, vagal input plays a significant role in secretin action by modulating the capacity of secretin binding sites, not affinity and specificity, at least in the rat forestomach. Vagal modulation of secretin receptor in rat forestomach appears to be mediated through axonal transport of the receptor or a factor essential for expression of the receptor in the forestomach that is independent of TTX-sensitive, muscarinic cholinergic neural transmission or the capsaicin-sensitive vagal afferent pathway.

Address for reprint requests and other correspondence: W. Y. Chey, Univ. of Rochester Medical Center, PO Box 646, 601 Elmwood Ave., Rochester, NY 14642 (E-mail: williamy_chey@urmc.rochester.edu). Received 27 August 1998; accepted in final form 8 January 1999.

REFERENCES


