show a therapeutic window) because we have observed the same phenomenon with other drugs of this class[10]. It remains for future work to clarify the exact mechanisms involved; however, it appears to involve a negative feedback system of some kind – at least that is our current guess. The clinician should be made aware of the therapeutic window evidenced by pramiracetam and other drugs of its class, because they may encounter situations where better therapeutic effects can be achieved only by reducing dosage.

1 Poschel, B.P.H., Marriott, J.G., and Gluckman, M.I., Drugs exp. clin. Res. *IX* (1983) 853.
2 Pugsley, T.A., Poschel, B.P.H., Downs, D.A., Shih, Y-H., and Gluckman, M.I., Psychopharmac. Bull. *19* (1983) 721.
3 Poschel, B.P.H., Ho, P.M., and Ninteman, F.W., Experientia, submitted for publication (1984).
4 Butler, D.E., Nordin, I.C., L'Italien, Y.J., Zweisler, L., Poschel, B.P.H., and Marriott, J.G., J. med. Chem. *27* (1984) 684.
5 Morris, R., Garrud, P., Rawlins, J., and O'Keefe, J., Nature *297* (1982) 681.
6 Surwillo, W.W., Timing of behavior in senescence and the role of the central nervous system, in: Human aging and behavior, p.1. Ed. G.A. Talland. Academic Press, New York 1968.
7 Hughes, J.R., and Cayaffa, J.J. Electroenceph. clin. Neurophysiol. *42* (1977) 776.
8 Obrist, W.D., Electroencephalographic changes in normal aging and dementia, in: Brain function in old age, p. 102. Eds F. Hoffmeister and C. Muller. Springer-Verlag, Berlin 1979.
9 Bunney, B.S., Roth, R.H., and Aghajanian, G.K., Psychopharmac. Com. *1* (1975) 349.
10 Poschel, B.P.H., Ho, P.M., Callahan, M.J., and Ninteman, F.W., unpublished observations (1984).

0014-4754/85/091153-04$1.50 + 0.20/0

Nerve-mediated action of forskolin on guinea pig ileal mucosa

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Summary. The effects of forskolin on myenteric neuronal activity and mucosal function were examined in guinea pig ileum. Forskolin increased the excitability of myenteric neurons, and increased mucosal chloride secretion by stimulating enteric neurons as well as by acting directly on enterocytes.
Key words. Forskolin; intestinal secretion; myenteric neurons, neural stimulation; mucosal transport.

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii,* stimulates adenylate cyclase by activating the catalytic subunit[2,3]. Forskolin has been reported to inhibit sodium absorption and to convert chloride absorption to secretion in the rat descending colon[4]. These effects are similar to the changes in ion transport that have been observed in both ileum and colon for other substances known to alter intracellular cyclic-AMP levels[5,6].

The small intestinal mucosa is innervated by nerve processes that originate in intrinsic ganglia of the submucosal and myenteric plexuses and in extrinsic ganglia[7]. Recent studies in this laboratory have shown that stimulation of enteric nerves evokes chloride secretion in isolated flat sheets of guinea pig ileum[8,9]. This response is mediated, in part, by acetylcholine that is released from enteric cholinergic neurons[8]. Because enteric neurons modulate mucosal function, exogenous substances added to the solutions bathing in vitro tissues could have dual actions, and alter transport function either by acting directly on transporting cells or paracrine cells or by stimulating enteric neurons that innervate enterocytes.

The aim of this study was to compare the action of forskolin on enteric neurons and on the intestinal epithelium of the guinea pig. The results suggest that forskolin stimulates chloride secretion by direct action on enterocytes as well as by activation of the innervation of the enterocytes.

Methods. Non-albino guinea pigs of either sex weighing 315–525 g were stunned by a blow to the head and exsanguinated. The terminal 10 cm of ileum was discarded and a 10 cm segment of the remaining ileum was removed.

Conventional methods, which are described in detail elsewhere, were used to record intracellular electrical activity in myenteric ganglion cells in vitro[10]. Forskolin was applied to the neurons by addition to the superfusion solution (Krebs solution). Ethanol alone was added to the superfusion solution as a control to rule out possible actions of the vehicle in which forskolin was dissolved.

Alterations in neuronal excitability were determined by counting the number of action potentials evoked by intracellular injection of rectangular depolarizing pulses of constant current and duration before, during the presence and after washout of forskolin (fig. 1). A statistically-significant increase in the mean number of spikes evoked per current pulse was interpreted as reflecting an increase in neuronal excitability.

For the mucosal function studies, segments of ileum were stripped of both circular and longitudinal muscles and were mounted as flat sheets in flux chambers. This dissection procedure removed the myenteric ganglia and left intact the submucosal ganglia. The chambers were equipped for measuring transmural electrical potential differences and short-circuit currents (Isc). Warmed and oxygenated solutions of identical composition bathed mucosal and serosal surfaces of the tissues. Under these conditions changes in Isc reflected alterations in active ion transport. Forskolin or ethanol carrier was added to the serosal bathing fluids. Electrical field stimulation was used to assess the effectiveness of neuronal blockade by tetrodotoxin (TTX). Alanine or carbachol was added at the termination of the experiment to determine tissue responsiveness to absorptive or secretory stimuli, respectively.

Results and discussion. In order to determine whether forskolin altered enteric neuronal activity, 0.5–1.0 μM forskolin was added to the superfusion fluid bathing myenteric neurons. Application of forskolin enhanced the excitability of the AH/Type 2 neurons (fig. 1). This was the case for all of 19 cells that were tested in preparations from 14 guinea pigs with one to three trials per cell. The augmented excitability was accompanied by depolarization, increased input resistance, reduction of amplitude and duration of postspike hyperpolarization and by spontaneous spike discharge (fig. 2). Washing with drug-free solution reversed these effects; however, reversal required 5–15 min of washing when the time of exposure to forskolin was 30 sec to 1 min.

Forskolin mimicked the changes in neuronal behavior that oc-

curred during slow synaptic excitation in AH/Type 2 myenteric and submucosal ganglion cells[11,12]. It also produced the same effects as serotonin and substance P, which are putative neurotransmitters for slow synaptic excitation in the enteric nervous system[12,13]. Forskolin stimulates production of large amounts of cyclic AMP in dorsal root ganglion cells[14], and this is probably the case also for the enteric neurons in the present study. This suggests that adenylate cyclase is involved in the production of slow synaptic excitation of enteric neurons and implicates cyclic nucleotides as intracellular mediators of neurotransmitter or hormonal actions on these cells.

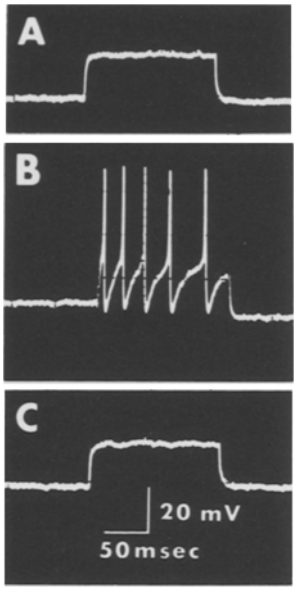

Figure 1. Excitatory effect of forskolin on an AH/Type 2 myenteric neuron in guinea pig small intestine. *A* Intracellular injection of depolarizing current did not evoke discharge of spikes in the control. *B* Multiple spike discharge evoked by depolarizing current in the presence of 1.0 μM forskolin. *C* After washout of forskolin. Strength of constant-current depolarizing pulses was 285 pA.

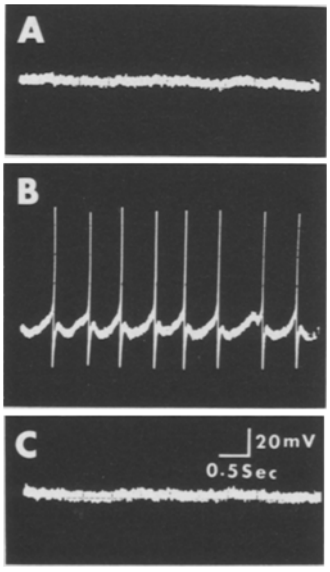

Figure 2. Ongoing spike discharge evoked by forskolin in an AH/Type 2 myenteric neuron of guineapig small intestine. *A* Control. *B* In the presence of 1 μM forskolin. *C* After washout of forskolin.

Since forskolin activated a subset of enteric neurons, the possibility that the forskolin-evoked increase in Isc[4] could have been due to stimulation of enteric neurons that innervate enterocytes was investigated. The effect of forskolin on Isc was compared to its effect when enteric neuronal activity was blocked by TTX. In control tissues, forskolin evoked a dose-dependent increase in Isc (fig. 3A). In the presence of TTX, the increase in Isc evoked by forskolin was significantly reduced at the two lowest concentrations studied (fig. 3A). Neural blockade was complete, because the mucosal response to electrical field stimulation was abolished in the presence of TTX. Since TTX prevents action-potential-dependent release of neurotransmitters, without altering directly Isc, or sodium and chloride transport processes in the guinea pig ileum[9], it is likely that the reduction in the forskolin-induced response is due to inhibition of neural activity. These data suggest that at concentrations less than 1.5 μM, forskolin increases Isc by activating neurons that influence mucosal function.

At concentrations greater than 11.5 μM, the increase in Isc evoked by forskolin was unaltered by the addition of TTX (fix. 3A). This suggests that the increase in Isc is due to a direct effect of forskolin on enterocytes, rather than activation of enteric neurons as was seen at the lower concentrations. At the highest concentrations, the direct action of forskolin drives the secretory epithelium to maximum capacity, and therefore blockade of the neurons by TTX does not result in a significant decrease in Isc. Midway between the highest and lowest concentrations, both neural and direct effects account for the forskolin-evoked increase in Isc.

The effect of forskolin on mucosal function was evaluated in the presence and absence of furosemide in order to determine if the response was due to chloride secretion (fig. 3B). Forskolin produced a dose-dependent increase in Isc (fig. 3A, 3B). Furosemide significantly reduced the forskolin-evoked increase in Isc to values near zero (fig. 3B), suggesting that forskolin stimulates chloride secretion in the guinea pig ileum. This finding is consistent with the effects of other agents known to stimulate cyclic AMP in the intestine[5,6], as well as with the effects of stimulating enteric neurons[8].

The effects of TTX or furosemide on the Isc could not be attributed to adverse effects on the mucosa, because alanine, an amino acid known to stimulate sodium absorption, evoked a 23.1 ± 6.2 μA · cm^{-2} increment in Isc in TTX-treated tissues, and a 20.3 ± 4.8 μA · cm^{-2} increase in furosemide-treated tissues, and

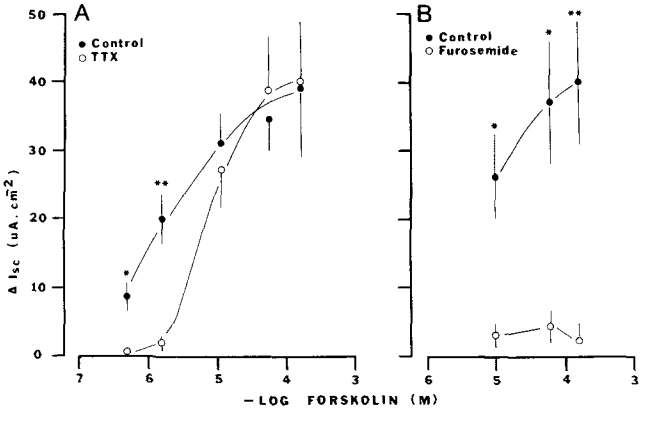

Figure 3. The change in short-circuit current evoked by forskolin in the presence and absence of tetrodotoxin or furosemide in guinea pig ileum. Means ± SE are given for 5–7 animals in A and 7–9 animals in B. *A* Effect of 0.1 μM tetrodotoxin (TTX) on the forskolin-evoked change in short-circuit current (Isc). ● Control; ○ TTX. *B* Effect of 9.5 mM furosemide on the forskolin-evoked change in Isc. ● Control; ○ furosemide. $^*p < 0.05$; $^{**}p < 0.01$.

these values were not significantly different from controls. Carbachol, a chloride secretory stimulus that acts on muscarinic receptors on enterocytes, evoked similar changes in Isc in control (64.5 ± 4.5 $\mu A \cdot cm^{-2}$ or TTX-treated tissues (66.9 ± 4.4 $\mu A \cdot cm^{-2}$), and this lends further support to the idea that TTX did not adversely alter transport characteristics.

The results suggest that forskolin stimulates chloride secretion at low concentrations primarily by activating neurons that influence epithelial function. With increasing concentrations, the proportion of the chloride secretory response that is due to a direct effect of forskolin on the enterocytes increases, so that at the highest concentrations, neural influences become insignificant.

Although the stimulation of chloride secretion by forskolin is consistent with previously reported observations in the rat colon[4], it is not clear from those experiments whether stimulation of enteric neurons was involved in the response. The present experiments cannot distinguish the specific types of neurons that are involved in the secretory response to forskolin; however, direct evidence that forskolin alters activity in one subset of enteric neurons is provided by electrical recording from myenteric ganglion cells.

The results suggest that the use of agents like forskolin in intestinal transport studies should be carried out with the consideration that the agent may have both a direct influence on enterocyte function and an indirect influence mediated by activation of enteric nerves that subsequently alter transport processes through release of neurotransmitters.

1 Acknowledgment. The authors thank E. Martens, S. Bowen for technical assistance and N. Schaad for secretarial assistance. This work was supported by National Institutes of Health Grants, F32-AM07301, K-104-AM1004, RO1-AM29699, RO1-AM26742 and an Eloise Gerry Fellowship.

2 Bhat, S. V., Bajwa, B. S., Dornauer, H., and de Souza, N. J., Tetrahedron Lett. *19* (1977) 1669.
3 Seamon, K. B., Padgett, W., and Daly, J. W., Proc. natn. Acad. Sci. USA *78* (1981) 3363.
4 Bridges, R. J., Rummel, W., and Simon, B., Naunyn-Schmiedebergs Arch. Pharmak. *323* (1983) 355.
5 Binder, H. J., in: Physiology of the Gastrointestinal Tract, vol. 2, p. 1003. Ed. L. R. Johnson. Raven Press, New York 1981.
6 Field, M., in: Physiology of the Gastrointestinal Tract, vol. 2, p. 963. Ed. L. R. Johnson. Raven Press, New York 1981.
7 Furness, J. B., and Costa, M., Neuroscience *5* (1980) 1.
8 Cooke, H. J., Am. J. Physiol. *246* (1984) G263.
9 Cooke, H. J., Shonnard, K., and Wood, J. D., Am. J. Physiol. *245* (1983) G290.
10 Wood, J. D., and Mayer, C. J., Pflügers Arch. *374* (1978) 265.
11 Suprenant, A., J. Physiol., Lond. *351* (1984) 343.
12 Wood, J. D., and Mayer, C. J., Nature, Lond. *276* (1979) 836.
13 Katayama, Y., and North, R. A., Nature, Lond. *274* (1978) 387.
14 Kilmer, S. L., and Carlsen, R. C., Nature, Lond. *307* (1984) 455.

0014-4754/85/091156-03$1.50 + 0.20/0

Free amino acid composition of the intestinal contents, intestinal cells and hemolymph of *Philosamia cynthia* larvae[1]

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Summary. Free amino acid composition of the intestinal contents, intestinal cells and hemolymph has been determined in larvae of the moth *Philosamia cynthia*. From the hemolymph/lumen concentration ratio, an active transport could be inferred for neutral and basic amino acids. The values of cell/lumen and hemolymph/cell ratios suggested that the active step in the transport mechanism could be localized at the luminal pole of the enterocyte for neutral amino acids (except aromatic amino acids) and at the basolateral pole of the enterocyte for basic amino acids (except arginine).

Key words. Amino acid analysis; lepidopteran larvae midgut; free amino acid composition; amino acid transport.

In the gut of lepidopteran larvae, amino acids are both actively absorbed and utilized as the main source of metabolic energy[2,3]. The ratio between the concentration of individual amino acids in the hemolymph and in the lumen can indicate the involvement of the intestinal barrier in the active and selective absorption of each amino acid. Therefore, the analysis of the free amino acid composition of the intestinal contents, intestinal cells and hemolymph can give an indication of the physiological activity of the intestinal wall. Hemolymph composition in lepidopteran larvae and the role of aminoacidemia in osmoregulation of the internal environment, as well as its contribution to the biosynthesis of silk protein, have been well established[4,5]. By contrast, no reports are available about the free amino acid composition of the intestinal cells and lumen contents. The composition of this latter compartment is almost constant since lepidopteran larvae are strictly monophagous.

In previous work, we demonstrated the secondary active transport of some neutral amino acids at the luminal membrane of the enterocytes of *Philosamia cynthia* larvae[2,6-8]. In the present paper, the free amino acid concentrations in the intestinal contents, intestinal cells and hemolymph have been measured in the same experimental substrate. The data obtained give further evidence of the selective nature of the active absorption of amino acids in lepidopteran midgut, and they will allow a more physiological choice of amino acids to be studied in transport experiments.

Materials and methods. Larvae of *P. cynthia* (Lepidoptera, Saturnidae) in the fifth instar, fed on *Ailanthus glandulosa* leaves, were used. The midgut was dissected, deprived of malpighian tubules and intestinal contents, rinsed with cold 100 mM mannitol, 10 mM HEPES-Tris pH 7.4 and rapidly homogenized in cold 0.6 M perchloric acid (4 ml/g fresh weight) with a glass teflon Thomas homogenizer, 9 strokes at 3000 rev/min. The homogenate was kept in an ice bath for 10 min and then centrifuged at 3000 × g for 15 min at 4 °C. The pH of the supernatant was adjusted to 7.0 by the addition of 2.5 M K_2CO_3. After 15 min at 0 °C, the sample was centrifuged as before and the supernatant was collected. Intestinal contents, free from leaf fragments, and hemolymph were treated with perchloric acid and processed as above described. Aliquots of the supernatants were used for glutamine assay according to Lund[9]. The remaining supernatants were diluted 1:3 with 0.1% trifluoroacetic acid:methanol 70:30 and passed through SEP-PAK C_{18} car-