CALCIUM AND ACTIVE TRANSPORT IN INTESTINE OF THE WINTER FLOUNDER, PSEUDOPLEURONECTES AMERICANUS

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Calcium has been shown to be involved in the regulation of active intestinal electrolyte transport. In rabbit ileum, the calcium ionophore A23187 decreased Na and Cl absorption and increased Cl secretion (Bolton and Field, J. Memb. Biol. 35:159, 1977). In contrast, reduction of intracellular calcium via exposure to the calcium channel blocker verapamil, increased Na and Cl absorption (Donowitz, et al., Clin. Res. 28:274A, 1980). Whether calcium is involved in regulation of intestinal transport in the Winter flounder, Pseudopleuronectes americanus, is not known. Regulation of intestinal transport is thought to be at least partially under control of the numerous neurohumoral substances found in the intestine. Again based on studies of rabbit intestine, neurohumoral substances appear to act through two major intracellular second messengers - cyclic AMP and calcium.

The purpose of the current studies was to determine whether calcium is involved in regulation of intestinal ion transport in the flounder and whether the flounder intestine responds to neurohumoral substances.

Methods

Winter flounder, fasted approximately 48 hours, were sacrificed and the intestine removed, rinsed, stripped of the serosa and muscle layers. Segments of mucosa were studied in Ussing chambers under short-circuited conditions using an automatic voltage clamp.

In some experiments unidirectional mucosal-to-serosal and serosal-to-mucosal Cl fluxes were studied with ^{36}Cl using mucosal segments differing in conductance by less than 25%.

To assess structural viability representative tissues were prepared for histology at the end of the experimental period. Tissues were quickly removed from Ussing chamgers and submerged in 2% glutaraldehyde, 2.5% paraformaldehyde in cacodylate buffer, at pH 7.4 at 4°C. Two to four 1 x 2mm tissue blocks were then removed from the center of the mucosal sheet, fixed for two hours in the above solution, post-fixed in 1% osmium, dehydrated and embedded in Epon. One μm well-oriented sections were then obtained and stained with toluidene blue.

Results

Calcium Ionophore A23187: With addition of calcium ionophore A23187 solubilized in absolute ethanol, the short-circuit current (Isc) decreased in absolute magnitude (became less negative) and conductance (G) decreased within 10 min. This effect occurred with concentrations of ionophore $\geq 10^{-7}$M. The ionophore-induced change in Isc occurred with addition of ionophore to the serosal but not the mucosal surface; while serosal addition of the same amount of ethanol needed to dissolve the ionophore did not significantly change Isc. The effect on Isc of 10^{-6}M calcium ionophore lasted 15-30 min; thereafter Isc gradually increased and approached baseline values in 60-90 min. The effects of 10^{-6}M ionophore on active electrolyte transport are summarized in Table 1. Ionophore caused a significant decrease in Isc and PD and a slight but not significant increase in G. Ionophore significantly decreased J^{Cl}_{net} to a value significantly less than zero. This was explained by an increase in J^{Cl}_{sm} with no significant change in J^{Cl}_{ms}. Although only one fish of five randomly chosen for flux studies failed to respond to ionophore with a change in Isc, PD or G, 8 of 19 fish exposed to Ca ionophore failed to respond. By light microscopy there were no structural features which permitted separation of responders and non-responders and Ca ionophore did not cause histologic changes.

Table 1.--Effect of Calcium Ionophore A23187 (10^{-6}M) on Active Ion Transport in Flounder Intestine*

Time	Addition	Isc	PD	G	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}
(A) 20-60 min	0	-67.5 ± 8.4	2.4 ± 0.2	27.9 ± 3.0	6.54 ± 0.65	3.95 ± 0.74	2.59 ± 1.38
(B) 70-110 min	Calcium Ionophore	-24.2 ± 5.9	0.9 ± 0.3	31.8 ± 6.1	6.89 ± 0.68	9.39 ± 0.51	-2.50 ± 0.81
	ΔPeriod B-A	43.5 ± 14.1	-1.5 ± 0.4	3.9 ± 3.2	0.35 ± 0.44	5.44 ± 0.31	-5.09 ± 0.55
	p	<0.05	<0.05	ns	ns	<0.001	<0.001

*Units are: Isc, uAmps/cm^2; PD, mvolts; G, mS/cm^2; Cl fluxes, uEq/cm^2-h. Tissue from four fish was studied. p values represent comparison of Period B and A in same tissue (paired t test).

Verapamil: Addition of d,l-verapamil, a calcium channel blocker, to the serosal bathing solution caused a dose-dependent decrease in the absolute magnitude of Isc without significantly changing the conductance (10^{-6}M: 3.3 ± 9.1 μAmps/cm^2 (N=4); 5×10^{-6}M: -9.4 ± 3.6 (N=5); 10^{-5}M: -28.3 ± 8.9 (N=4).

Trifluoperazine: The effect of the phenothiazine, calcium-calmodulin inhibitor trifluoperazine was studied at a single concentration. While addition of trifluoperazine (2×10^{-4}M, N=2) to the mucosal bathing solution did not significantly alter Isc, serosal addition caused an increase in Isc (+14 μAmps/cm^2) which was short-lived (15-20 min) and associated with an increase in conductance (+11%).

Neurohumoral substances: The effects of several neurohumoral substances on Isc, Pd and G were studied. In rabbit ileum, these agents are thought to alter active intestinal transport either by activating the adenylate cyclase-cAMP system (vasoactive intestinal polypeptide); by increasing intracellular calcium (carbamylcholine, serotonin, neurotensin and substance P); or by as yet unidentified mechanisms (bombesin). To assess the role of increased intracellular calcium in electrical responses to the agents, the effects of the neurohumoral substances were compared in the presence and absence of 5×10^{-6}M verapamil in the serosal bathing solution. Exposure to VIP, serotonin, substance P, bombesin and neurotensin all significantly decreased the absolute magnitude of the Isc (Table 2). Of the neurohumoral substances studied only carbamylcholine did not significantly affect intestinal ion transport. A single experiment with parathormone (10^{-5}M) also did not significantly alter Isc following exposure for 40 min. As demonstrated in Table 2, the effect on Isc of cyclic AMP (5mM theophylline plus 0.2 mM 8-Br-cyclic AMP), VIP and bombesin was not altered by the presence of verapamil in the serosal bathing solution. In contrast, the effects of serotonin, substance P and neurotensin were significantly less in the presence of verapamil.

The additive effect of agents presumably acting through cyclic AMP and calcium was determined. In single experiments, 5mM theophylline plus 0.2mM 8-Br-cyclic AMP decreased Isc by 32 μAmps/cm^2; 10^{-5}M serotonin by 31 μAmps/cm^2; and the simultaneous addition of serotonin plus theophylline plus cyclic AMP by 27 μAmps/cm^2. In addition, sequential addition of cyclic AMP and serotonin did not cause an additive effect on Isc. In contrast, when Isc had returned to baseline after serotonin exposure, addition of cyclic AMP then caused its usual change in Isc, while a second addition of serotonin had no effect.

Table 2.--Effect of Neurohumoral Substances on Flounder Intestinal Short-Circuit Current*

	Maximal Absolute Decrease in Isc (μAmps/cm^2) verapamil absent	verapamil present	P
Cyclic AMP (5mM theophylline + 0.2mM 8-Br-cyclic AMP) N=1	45	44	--
VIP (10^{-7}M) N=4	23.8 $\pm$ 3.7	23.9 $\pm$ 6.6	ns
Serotonin (10^{-4}M) N=6	37.0 $\pm$ 3.4	15.1 $\pm$ 6.3	<0.02
Substance P (10^{-6}M) N=5	25.1 $\pm$ 3.2	10.6 $\pm$ 5.2	<0.05
Carbamylcholine (10^{-5}M) N=4	0.8 $\pm$ 0.9	--	--
Bombesin (10^{-6}M) N=3	19.5 $\pm$ 2.2	17.1 $\pm$ 6.3	ns
Neurotensin (10^{-6}M) N=1	13.0	0	--

*Effect on Isc determined in presence of 5×10^{-6}M verapamil. N refers to number of fish studied. P values represent comparisons of effect of neurohumoral substances in presence and absence of verapamil in tissue from the same fish (paired t test).

The effect of serotonin on active flounder intestinal ion transport was determined as an example of a neurohumoral substance which probably acts through calcium. The dose response of the serotonin effect on Isc showed: no effect at 10^{-8}M; a threshold effect at 10^{-7}M; maximal effect at 10^{-5}M; with a half-maximal effect at approximately 5×10^{-6}M. The effect of 10^{-4}M serotonin on flounder intestinal ion transport was determined. Serosal addition of 10^{-4}M serotonin caused a decrease in Isc which was significant by 5 min and was constant for approximately 40-50 min before gradually increasing. The effect of serotonin during the 10-50 min following addition was compared to transport in the same piece of tissue studied for two 20 min periods before serotonin addition. In addition untreated tissue studied over the same time period was used as a control. As demonstrated in Table 3, control tissue studied 20-60 min after mounting and isotope addition was not significantly different in any parameter from that studied 70-110 min after isotope addition. Serotonin caused a decrease in absolute magnitude of Isc, PD and G. Net Cl transport was also significantly decreased; this was due to a significant decrease in the mucosal-to-serosal flux without a significant change in the serosal-to-mucosal flux. The change in net Cl transport exceeded the change in Isc (1.99 vs 0.78).

Histological examination revealed that mucosal structure was not altered even after study periods up to 4 hours in control tissue or tissue exposed to serotonin.

Discussion

These studies present strong though indirect evidence that calcium has a role as an intracellular regulator of intestinal ion transport in the intestine of the Winter flounder; that neurohumoral substances regulate transport in this intestinal tissue; and that the effect of at least some neurohumoral substances may be mediated by calcium.

Since the flounder intestine is thought to contain only absorptive cells, these results suggest that altering intracellular calcium inhibits ion absorption. Of note is the net Cl secretion seen in tissue exposed to ionophore. This is a different response from that previously reported for cyclic AMP and seen with serotonin in which there was a decrease in net Cl transport but no net secretion. The significance of this difference is not known.

In these studies the flounder small intestine responded to a series of neurohumoral substances in a remarkably similar pattern to that seen in rabbit ileum. As has been postulated in rabbit ileum, serotonin, neurotensin, substance P may alter transport in flounder intestine by increasing intracellular calcium since their effects were inhibited by the presence of the calcium channel blocker verapamil.

Table 3.--Effect of Serotonin (10^{-4}M) on Active Ion Transport in Flounder Intestine*

Time	Addition	Isc	PD	G	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}
(A) 20-60 min	0	-84.2 ± 19.1	3.7 ± 0.6	23.8 ± 5.3	11.00 ± 2.41	5.92 ± 1.38	5.08 ± 1.15
(B) 70-110 min	0	-84.9 ± 15.4	3.4 ± 0.7	25.0 ± 3.1	10.67 ± 1.92	5.89 ± 1.87	4.78 ± 1.08
	ΔPeriod B-A	-1.0 ± 4.5	-0.2 ± 0.1	1.2 ± 1.4	-0.33 ± 0.35	-0.02 ± 0.59	-0.30 ± 0.61
	p	ns	ns	ns	ns	ns	ns
(A) 20-60 min	0	-84.9 ± 7.0	3.7 ± 0.7	23.3 ± 2.0	8.92 ± 0.89	3.10 ± 0.29	5.82 ± 0.76
(B) 70-110 min	Serotonin	-63.7 ± 9.1	2.9 ± 0.6	22.1 ± 1.9	6.83 ± 0.66	3.00 ± 0.43	3.83 ± 0.36
	ΔPeriod B-A	21.2 ± 7.8	-0.8 ± 0.3	-1.2 ± 0.2	-2.09 ± 0.78	-0.10 ± 0.16	-1.99 ± 0.54
	p	<0.05	<0.05	<0.05	<0.02	ns	<0.05
	P	<0.05	ns	ns	0.05	ns	0.05

*Units as in Table 1; tissue from six fish was studied, p values represent comparison of Period B and A in same tissues (paired t test); P values represent comparison of changes in control and serotonin exposed tissues (unpaired t test).

The only qualitative difference in effects of neurohumoral substances on ion transport in the flounder intesting compared to rabbit ileum was the failure of the cholinergic agonist carbamylcholine to alter the Isc. Why this cholinergic agonist failed to alter ion transport is not known through a similar failure of parasympathetic agonists to alter ion transport in the dogfish has previously been observed (Burger, et al., J. Physiol., 35:205, 1962). We are not aware of descriptions of distribution of parasympathetic nerves in the flounder intestine.

Similar to studies in rabbit ileum, calcium and cyclic AMP effects on ion transport were not additive as judged by changes in the Isc (Donowitz, et al., J. Clin. Invest. 66:341, 1980). However, the addition of cyclic AMP to a tissue which had responded to serotonin but in which the Isc returned to baseline did result in a further Isc response. Such a response did not occur with a second exposure to serotonin. This demonstrates that cross desensitization to serotonin and cyclic AMP does not occur; and that serotonin and cyclic AMP exert their effects through independent intracellular mechanisms, each of which can, by itself, saturate the transport process affected.

The effect of ionophore on Cl transport is similar to that previously described for cyclic AMP and primarily consists of an increase in the serosal-to-mucosal Cl flux (Smith, et al., J. Memb. Biol., 55:157, 1980). In contrast, the effect of serotonin was primarily to inhibit the mucosal-to-serosal Cl flux. In addition cyclic

AMP and calcium ionophore A23187 tended to increase while serotonin decreased the tissue conductance. It is postulated that cyclic AMP primarily inhibits Na and Cl influx from the mucosal surface into the tissue and that the increase in the serosal-to-mucosal Cl movement represents increased permeability in the brush border. Why calcium ionophore and serotonin have different effects on unidirectional fluxes is not known. While it is possible that this is related to different magnitudes in level of change in intracellular calcium content it is also possible that the basic effects on brush border or tight junction permeability of serotonin versus ionophore and/or cyclic AMP may differ. These studies were supported by NIH Grants AM26523, AM20700 (MD); AM27972 (JM); and AM17537 (JT). Dr. Donowitz is a recipient of NIH RCDA 1K04-00588.

CORNEAL ULTRASTRUCTURE AND SWELLING PROPERTIES OF THE SCULPIN AND SKATE CORNEA

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The objective of the current study is to describe the ultrastructure of the Longhorn Sculpin Cornea and to compare the swelling properties of the outer and inner stroma to that of the skate corneal stroma. The stromal swelling was compared in deionized water, teleost and elasmobranch Ringers.

For transmission electron microscopy the sculpin corneas were fixed in 3% glutaraldehyde in phosphate buffer at pH 7.2 and an osmolality of 350 mOsm for at least 8 hrs at 4°C (skate at pH 7.2 at 1000 mOsm). The corneas were then embedded, cut and stained according to our previous studies (Exp. Eye Res. 32:133, 1981). For the swelling studies, the eyes from sculpin (Myoxocephalus octodecimspinosus) and skate (Raja erinacea) were enucleated from euthanized animals, the corneas excised and incubated in 2 cc of either deionized H_2O, marine teleost or elasmobranch Ringers at 15°C in a Dubnoff metabolic shaking incubator. Control corneas and corneas following 30, 60 and 120 minutes of incubation were removed, blotted dry, weighed and dried at 120°C for 24 hrs and reweighed. The percent stromal water was calculated.

The ultrastructure of the sculpin cornea is shown in Figure 1. This cornea reveals a distinct epithelium, outer stroma, inner stroma, Descemet's membrane and an endothelium all of which correspond to the optical reflections of the corneal layers observed in the specular microscope by Fischer and Zadunaisky (Exp. Eye Res. 25:149, 1977). A prominent group of cells that contain pigment granules was also observed between the outer and inner stroma. The pigment granules give rise to the yellow pigmentation in the corneas of many teleosts first reported in 1933 by Walls and Judd (Br. J. Ophthalmol. 17:641, 1933). Other reports by Moreland and Lythgoe (Vis. Res. 8:1377, 1968) and Muntz (Vis. Res. 13:2235, 1973) have shown that marine fish corneas have an absorption maximum of 425, 450 and 480 nm. These authors have postulated that the corneal pigment acts as a filter to improve both visual acuity and contrast discrimination through reduction of chromatic aberration, overhead glare and short-wave scatter. The collagen of the inner stroma adjacent to the endothelium is compact with many cells that contain a highly developed endoplasmic reticulum, which contributes to the irridescent nature of the cornea as reported in many teleost corneas by Lythgoe (Proc. R. Soc. Lond. 188:437, 1975).

The swelling properties of the sculpin and skate corneal stroma following incubation in either deionized water or Ringers is shown in Figures 2 and 3.

In the sculpin cornea the anterior and posterior stroma swell markedly (89-92%) in deionized water. By comparison the skate cornea that contains sutural fibers (Smelser, Invest. Ophthalmol. 1:11, 1962) only shows a minimal increase in water content throughout the 120 min in deionized water (75%).