Ca\(^{++}\) regulation of paracellular permeability in the middle intestine of the eel, *Anguilla anguilla*

**Abstract** The role of Ca\(^{++}\) on the regulation of the paracellular pathway permeability of the middle intestine of *Anguilla anguilla* was studied by measuring the transepithelial resistance and the dilution potential, generated when one half of NaCl in the mucosal solution was substituted iso-osmotically with mannitol, in various experimental conditions altering extracellular and/ or intracellular calcium levels. We found that removal of Ca\(^{++}\) in the presence of ethylene glycol-bis(\(\beta\)-aminoethyl ether) (EGTA) from both the mucosal and the serosal side, but not from one side only, reduced both the transepithelial resistance and the magnitude of the dilution potential. The irreversibility of this effect suggests a destruction of the organization of the junction in the nominal absence of Ca\(^{++}\). However a modulatory role of extracellular Ca\(^{++}\) cannot be excluded. The decrease of the intracellular Ca\(^{++}\) activity, produced by using verapamil to block the Ca\(^{++}\) entry into the cell, or by adding 3,4,5-trimethoxybenzoic acid 8-(diethy lamino) octyl ester (hydrochloride) (TMB-8), an inhibitor of Ca\(^{++}\) release from the intracellular stores, reduced both the transepithelial resistance and the magnitude of the dilution potential, indicating a role of cytosolic Ca\(^{++}\) in the modulation of the paracellular permeability. However the rise of calcium activity produced by the Ca\(^{++}\) ionophore calcimycin (A23187) evoked an identical effect, suggesting that any change in physiological intracellular Ca\(^{++}\) activity alters the paracellular permeability.

**Key words** *Anguilla anguilla* · Middle intestine · Paracellular pathway permeability · Ca\(^{++}\)

**Abbreviations** A23187 calcimycin · d.p. dilution potential · DMSO dimethyl sulfoxide · EGTA ethylene glycol-bis(\(\beta\)-aminoethyl ether) · \(I_{sc}\) short-circuit current · \(R_{t}\) transepithelial resistance · TMB-8 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (hydrochloride) · \(V_{t}\) transepithelial potential

**Introduction**

In epithelia the exchange of substances between the outer and inner environments occurs through both the intercellular and paracellular pathways. In the paracellular pathway the tight junctions represent the rate-limiting barrier to diffusion of solutes (Powell DW 1981; Reuss 1991). The tight junctions have long been considered as a static structure but more recently it was demonstrated that they can be finely regulated by various intracellular messengers like cAMP (Dufley et al. 1981), G-protein, protein kinase C, phospholipase C, calmodulin (Balda et al. 1991), and Ca\(^{++}\) (Palant et al. 1983; González et al. 1990; Jovov et al. 1994).

In teleost fishes, the regulation of paracellular pathway by cAMP (Krasny et al. 1983; Bakker and Groot 1984; Rao and Nash 1988) and cGMP (Trischitta et al. 1996) is well documented. In contrast, a regulatory role for Ca\(^{++}\) in the control of paracellular pathway permeability was only proposed in the goby intestine based on the observation that the Ca\(^{++}\)-calmodulin antagonist trifluoperazine decreased the serosal-to-mucosal Cl\(^{-}\) flux and increased transepithelial resistance (Loretz 1987).

The aim of this work was to evaluate whether the paracellular permeability of the intestine of the teleost
fish *Anguilla anguilla* is Ca\(^{2+}\)-dependent and to determine the relative role of extracellular versus intracellular calcium.

In some epithelia, like the cultured human cervical epithelium (Gorodeski et al. 1997) and frog urinary bladder (Lacaz-Vieira 1997), the regulation of the tight junction permeability is independent from cytosolic calcium but is directly dependent on extracellular calcium. However, the tight-junction permeability in *Necturus* gallbladder (Palant et al. 1983) and A6 cells is modulated by intracellular calcium levels (Jovov et al. 1994).

Our study was conducted in the isolated middle intestine of the eel, *A. anguilla* by measuring the trans-epithelial resistance (*R*\(_e\)) and the dilution potential (d.p.), generated when one half of the NaCl in the mucosal solution was replaced iso-osmotically with mannitol, in the control, and in various experimental conditions altering extracellular and/or intracellular Ca\(^{2+}\) levels.

**Materials and methods**

European eels, *A. anguilla*, were used. They were obtained from a commercial pond and were maintained in a large tank containing artificial seawater for at least 3 weeks prior to experimentation. The eels were killed by decapitation and the intestine was rapidly removed. The middle part of the intestine was isolated, stripped of the serosal and muscular layers and vertically mounted in an Ussing chamber having two compartments of 6 ml each and a tissue exposed area of 0.3 cm\(^2\). One tissue per fish was used. The tissues were continuously perfused on both sides by a gravity flow of electrolyte solutions from reservoirs thermostated at 18 °C by water jackets.

The composition of the control experimental solution was (mmol·L\(^{-1}\)): NaCl 133, KCl 3.2, NaHCO\(_3\) 20, MgCl\(_2\) 1.4, CaCl\(_2\) 2.5, KH\(_2\)PO\(_4\) 0.8, glucose 20, osmolality was 315 mosmol·kg\(^{-1}\) and the pH 8.0 when the solution was bubbled with a mixture of 99% O\(_2\) and 1% CO\(_2\).

The trans-epithelial potential (\(V_t\)) and the d.p. were measured by means of a current-voltage clamp (Tecnovision, SH 89, Milano, Italy), with an automatic compensation for the chamber fluid resistance. \(V_t\) was measured by means of two Hg/HgCl\(_2\) electrodes which made contact with the bathing solutions via Ringer agar bridges. \(I_m\) was measured by the passage of sufficient current through Ag/AgCl electrodes to reduce the spontaneous \(V_t\) to zero. \(R_e\) was measured by passing 500-ms current pulses from a battery operated pulse generator (20 μA·cm\(^{-2}\)) through the tissue via Ag/AgCl electrodes placed directly in the bathing solutions. The tissues were kept open-circuited except for a few seconds every 5–10 min to measure \(I_m\). The negative sign of \(V_t\) referred to the serosa with respect to the mucosa (grounded). \(V_o\), \(I_{oc}\) and \(R_e\) were allowed to stabilize before starting any experimental maneuver.

A d.p. was induced by perfusing the mucosal side of the tissue with a solution in which 66.5 mmol·L\(^{-1}\) NaCl was replaced by 133 mmol·L\(^{-1}\) mannitol. The d.p.s were corrected for the measured liquid junction potential (about 1.5 mV).

The dependence of paracellular pathway permeability on extracellular Ca\(^{2+}\) was evaluated by removing Ca\(^{2+}\) and by adding ethylene glycol-bis(β-aminoethylether) (EGTA; 5·10\(^{-4}\) mmol·L\(^{-1}\)) in the serosal and/or mucosal bath. The influence of cellular Ca\(^{2+}\) on the permeability of the paracellular pathway was tested by adding to the luminal and/or serosal solutions: 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (hydrochloride) (TMB-8; 3·10\(^{-4}\) mmol·L\(^{-1}\)), an inhibitor of Ca\(^{2+}\) release from intracellular stores, or calcimycin (A23187; 2·10\(^{-7}\) mmol·L\(^{-1}\)), a Ca\(^{2+}\) ionophore, or (±) Verapamil (hydrochloride), (10\(^{-4}\) mmol·L\(^{-1}\)), a Ca\(^{2+}\)-channel blocker.

A23187 was dissolved in dimethyl sulfoxide (DMSO) to prepare concentrate solutions which were diluted in the experimental solutions. The end concentration of DMSO was 0.1%, which showed no effects on the transepithelial parameters in pivotal experiments. All substances were purchased from Sigma (St. Louis, Mo., USA).

The data are given as means±SE. Statistical analyses were performed using the Student’s t-test for paired samples.

**Results**

Figure 1 shows that in the control conditions the substitution of 66.5 mmol·L\(^{-1}\) NaCl with 133 mmol·L\(^{-1}\) mannitol in the mucosal bath produced a serosa-negative d.p.

To assess the effects of extracellular Ca\(^{2+}\) on the selective permeability of the paracellular pathway, we removed the ion from the mucosal and/or serosal solution and added EGTA (5·10\(^{-4}\) mmol·L\(^{-1}\)) to the Ca\(^{2+}\)-free solutions and measured d.p. in these experimental conditions. As Fig. 2 shows, the Ca\(^{2+}\) removal from either mucosal or serosal bath did not modify either the d.p. or the \(R_e\). However, the bilateral Ca\(^{2+}\) removal significantly reduced both d.p. and \(R_e\). As bilateral Ca\(^{2+}\) removal irreversibly abolished also \(V_t\) and \(I_{oc}\) and hence the transcellular transport (Fig. 3), we measured d.p. after the inhibition of the transcellular transport with bumetanide, a known blocker of Na/K/Cl cotransport. Figure 4 shows that d.p. measured after the mucosal addition of 10\(^{-5}\) mol·L\(^{-1}\) bumetanide had produced its maximal inhibitory effect on \(V_t\) was not reduced with respect to the control value. The experiments were performed in triplicate; the mean values of the amplitude of the d.p. were 14.2±0.6 mV in the control conditions vs. 15.1±0.5 mV in the presence of the loop diuretic.