

In vitro Measurement of Electrolytes and Nutrients Transport through Intestinal Epithelium during Cholera Toxin Induced Secretion

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ABSTRACT: Cholera toxin and other bacterial toxins can induce electrogenic chloride (Cl⁻) secretion in the small intestine resulting in secretory diarrhoea, when the colonic water reabsorption capacity is overwhelmed. The mechanism underlying this phenomena is that, these toxins increase intracellular cGMP and/or cAMP level through activation of guanylyl and adenylyl cyclase leading to the phosphorylation of the apical chloride channel (CFTR) and electrogenic Cl⁻ secretion as revealed *in vitro* by an increase in short-circuit current reflecting an increase in electrolyte transport in the intestine. The aim of the study was to investigate the effects of D-(+) glucose on water and electrolyte movements across rat jejunum after challenging with cholera toxin and dbcAMP (a lipophilic analog of cAMP which readily crosses the basolateral membrane of small intestinal cells); and also to investigate whether the magnitude of response to D-(+) glucose was related to the extent of secretion induced by dbcAMP. The measurement of the ion transport across the unstripped rat jejunum was carried out using Ussing chambers. The response to D-(+) glucose was studied in both CT-treated and untreated tissue; the results showed no significant difference between I_{sc} responses to D-(+) glucose in unstimulated and CT-stimulated rat jejunum ($\Delta I_{sc} = 45.3 \pm 12.9 \mu A/cm^2$ versus $38.9 \pm 13.9 \mu A/cm^2$, n = 8), whereas, in the studies where tissues treated with dbcAMP instead of CT, results showed a small but significant difference in D-(+) glucose response affected by dbcAMP ($\Delta I_{sc} = 12.9 \pm 4.7 \mu A/cm^2$ versus $24.0 \pm 4.3 \mu A/cm^2$, n = 8).

Key words: Electrolytes, Cholera toxin, Transport

INTRODUCTION

Acute diarrhoeal disease is one of the major causes of child death particularly in our country. Cholera toxin (CT) and other bacterial toxins can induce electrogenic chloride secretion in the small intestine resulting in secretory diarrhoea. The mechanism underlying this phenomena is that, these toxins increase intracellular cAMP level through the activation of adenylyl cyclase leading to electrogenic Cl⁻ secretion as revealed *in vitro* by an increase in short-circuit current reflecting the increase in electrolyte transport in the intestine.^{1,2} A variety of

extracellular mediators are capable of stimulating electrolyte and fluid secretion by both the small and large intestine. The effects of many secretagogues (e.g. CT and *E. coli* heat labile toxin) are mediated by increases in intracellular cyclic AMP that may serve as intracellular messengers of a secretory stimulus.^{1,3,4}

Many studies have reported the effect of cholera toxin, which induced the secretion of fluid along with chloride. The effect of CT has been known to act through the activation of cAMP.^{5,6} These observations have great importance from the clinical point of view. In secretory diarrhoeal disease induced by toxin-producing pathogens, the use of oral

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rehydration solution (ORS) containing glucose is beneficial.⁶ The fact that glucose induced increase in Isc (short circuit current) may suggest that, this component in ORS is essential to allow rehydration during diarrhoea.

The amino acids like alanine, glutamine, lysine, leucine, and aspartic acid are stable and highly soluble. These are co-transported with Na⁺ across the intestinal membrane and hence they might have great importance in improving absorption efficiency of oral rehydration solution.^{7,15,16} The epithelium is histologically arranged so that net absorption of sodium and water can be energetically coupled to amino acid uptake. Studies conducted in animal models have suggested that several amino acids like glutamine and alanine enhance the absorption of sodium and water from the intestine independent of glucose through amino acid-dependent co-transport of sodium.^{8,9,15,16}

Many water soluble organic compounds such as D-hexoses, different amino acids, dipeptides and tripeptides of neutral amino acids can enhance the absorption of sodium from the small intestine.¹⁰ This leads to develop an improved formula of ORS by introducing different amino acids in the formulation that could successfully and potentially replace the lacking of salts and water in diarrhoea. It can also actively induce the reabsorption of endogenous intestinal secretion and thus reduce the volume and duration of diarrhea.^{10,11} Further more, this amino acid containing formulation exerts a nutritional benefit to the patients,^{15,16} primarily because the reduced severity and duration of diarrhea will make it possible to introduce more effective feeding regime earlier.

One approach to developing an improved ORS formulation was based on adding neutral amino acids or their dipeptides to WHO-ORS. Therefore, the transport and absorption efficiency of these amino acids will be measured by using 1) the *in vitro* models of isolated intestinal tissues (in Ussing chamber) of rabbits, and 2) by *in vivo* studies in small intestine of rabbits through a series of perfusion studies which may help to develop an improved ORS

formulation.^{10,12} The main objective of the present study is to assess the transport characteristics of water and electrolytes across the rat intestine reflected by change in Isc after challenging the intestinal tissue with cholera toxin by *in vitro* Ussing chamber technique.

The proposed study is specially designed to develop an improved ORS by using amino acids and short chain fatty acids which would be useful in the treatment of secretory diarrhoea including cholera, *E. coli* infections etc. The organic substrates used in the study are expected to have a favourable absorption promoting effect¹⁶ which may prove to be beneficial for clinical trial of the new ORS formulation. The result of the proposed study will generate new information and aspects which will be beneficial to the society by reducing the risk of child death caused by diarrhoea. The findings of this study will also generate new knowledge of understanding the pathophysiology of secretory diarrhea and help in developing an improved ORS formulation on the basis of the result of these studies.

MATERIALS AND METHODS

The investigation of the studies was done in the Laboratory of Biology, Conservatoire National des Arts et Metiers (CNAM), Paris, France as a part of a training program. The chemicals used in the study CT (approx. 95%), 300,000 units/mg protein from dbcAMP (N⁶, 2-O-dibutylryl adenosine 3',5' cyclic monophosphate), Phloridzin (Phloretin-2-β-D-glucoside), Bumetanid (3-[Aminosulfonyl]-5-[butylamino]-4 phenoxy benzoic acid) were purchased from Sigma Chemical Co., (USA). D-(+) Glucose monohydrate was purchased from Merck (Germany). Automatic voltage clamp system (DVC-1000 Dual Current/Voltage Clamp) was purchased from WPI, USA.

Animals and Tissue preparation. Mature, male Sprague-Dawley rats, SPF (specific pathogen free), weighing from 180-280 gm were obtained from Iffa Credo (France). For fasted rats, food was withdrawn 18 hours prior to the experiment but drinking water

was made ad libitum. Rats were anaesthetized by injecting subcutaneous 6% w/v sodium pentobarbital (Sanofi Sante Animale, France). Then they were sacrificed by decapitation. Segments of proximal jejunum were removed and rinsed by flushing ice-cold Ringer solution. These segments were cut into small pieces (15-20 mm long) and opened along the mesenteric border and mounted immediately into Ussing chambers.

In vitro measurement of ion flux. For *in vitro* experiment, isolated intestinal tissue of healthy rat was used in Ussing & Zerahn apparatus. The chambers were exposed to 0.5 cm² surface area for rat intestinal epithelium. Each of the compartment contained 10 ml circulation Ringer solution, which was kept at 37 °C and gassed with 95% O₂ and 5% CO₂ to maintain the pH 7.4 and for a thorough mixing of chamber liquid. The isotonic Ringer solution used throughout the experiments contained: 115 mM NaCl, 25 mM NaHCO₃, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 2.4 mM K₂HPO₄ and 0.2 mM KH₂PO₄. Pieces of unstriped tissue were mounted as flat sheets between the two halves of Ussing chambers and bathed on both the mucosal and serosal sides with 10 ml of Ringer solution. The spontaneous transmural electrical potential difference (PD), reflecting the asymmetry of electrical charges between the mucosal and serosal membranes was measured through agar bridges (4% w/v agar in 3M KCl solution) which was placed on both sides of the tissue and adapted to calomel electrodes connected with a voltmeter; PD was short-circuited throughout the experiment by a short-circuit current (I_{sc}) through agar bridges in each reservoir which was adapted to Ag/AgCl electrodes and connected with an automatic voltage clamp system. Delivered I_{sc}, corrected for fluid resistance was recorded continuously in all experiments. PD was checked at 10 minute time-interval. Cholera toxin and cyclic AMP was added in mucosal and serosal compartment respectively to exert specific effect on absorption or secretion. Any effect on I_{sc} was measured as peak changes from the baseline values. Different nutrients like several amino

acids and glucose were introduced to measure the change in the I_{sc} value.

Plots of the I_{sc} response to varying concentrations of substrates (amino acids, glucose) was determined by measuring response of tissue I_{sc} to progressively increasing concentrations of substrates, added at 20 minute intervals to both the serosal and mucosal sides of the membrane. Differences between maximal I_{sc} and basal I_{sc} (i.e. prior to the addition of substrate) at each concentration were determined.

Data analysis. The major comparison was done between test and control solution treated groups. The water and electrolytes absorption mediated by different organic solutes will be compared by using appropriate statistical tests including ANOVA, student's T-test, and chi square tests. Significance of difference would be assessed at 5% level.

RESULTS AND DISCUSSION

Electrical parameters and effect of various substrates. Cholera toxin (final concentration 0.5 µg/ml) elicited no significant change in electrical parameters of rat jejunum after exposure for 1 hour (Table 1). In rat jejunum, D-(+) Glucose-stimulated I_{sc} by 40 µA/cm² in both CT-exposed and non-exposed tissues. The stimulation of I_{sc} was inhibited by Phloridzin and Bumetanide added to the mucosal and serosal side respectively. The extent of decrease in I_{sc} in presence of these inhibitors was of comparable magnitude in both the control and CT-treated tissues.

Results are mean ± SEM of (n) jejunal segments from 4 rats. The variation in short-circuit current (ΔI_{sc}), conductance (ΔG) and potential difference (ΔPD) is shown in the table for both CT-treated and untreated tissue. CT was added to the mucosal bath at a concentration of 0.5 µg/ml at t = 0. D-(+) Glucose (10 mM) was added at 60 min after CT to both tissues. 0.5 mM Phloridzin was introduced to the mucosal bath at time 80 min followed by 0.05 mM Bumetanide in the serosal bath at time 100 min, difference from control at p = 0.05. For ΔPD, a

positive value denotes a decrease in transmural potential difference.

dbcAMPc induced a significant rise in Isc after 1 hour exposure, with no alteration of conductance and potential difference (Table 2). Although response to D-(+) Glucose was nearly double in cAMP exposed intestinal segments compared to controls, 0.05 level of significance was not reached. As expected,

Phloridzin-induced inhibition of Isc was more marked in cAMP-treated tissue than in the untreated one, without reaching the level of statistical significance. Decrease in Isc caused by Bumetanide was significantly greater when tissues were pre-stimulated by cAMP than in absence of pre-stimulation.

Table 1. Variation in short-circuit current (Δ Isc), conductance (Δ G) and potential difference (Δ PD) in response to D-(+) Glucose, Phloridzin and Bumetanide in control and CT-exposed rat jejunum.

Reagents	n	Δ Isc (μ A/cm ²)	Δ G (mS/cm ²)	Δ PD (mV)
Ringer Control	8	5.73 \pm 4.68	5.0 \pm 0.68	0.23 \pm 0.21
CT	8	-0.21 \pm 5.06	5.25 \pm 0.68	0.64 \pm 0.23
Glucose Control	8	45.34 \pm 12.9	4.0 \pm 0.91	-0.67 \pm 0.29
CT	8	38.93 \pm 13.95	1.5 \pm 0.91	-0.53 \pm 0.36
Phloridzin control	8	-36.66 \pm 10.68	0.5 \pm 0.93	0.6 \pm 0.26
CT	8	-34.27 \pm 11.55	0.75 \pm 0.93	0.53 \pm 0.33
Bumetanide control	7	-24.0 \pm 3.29	1.39 \pm 1.25	0.89 \pm 0.28
CT	8	-22.95 \pm 3.78	3.54 \pm 1.93	0.81 \pm 0.29

Table 2. Variation in short-circuit current (Δ Isc), conductance (Δ G) and potential difference (Δ PD) in response to D-(+) Glucose, Phloridzin and Bumetanide in control and dbcAMP-exposed rat jejunum.

Reagents	n	Δ Isc (μ A/cm ²)	Δ G (mS/cm ²)	Δ PD (mV)
Ringer Control	8	1.95 \pm 3.81	11.15 \pm 3.77	0.69 \pm 0.20
dbcAMP/cAMP	8	20.79 \pm 4.13	12.28 \pm 3.44	0.39 \pm 0.19
Glucose Control	8	12.91 \pm 4.67	-0.35 \pm 1.27	-0.51 \pm 0.071
cAMP	8	24.0 \pm 4.3	2.5 \pm 1.16	-0.54 \pm 0.06
Phloridzin control	8	-6.18 \pm 3.41	1.90 \pm 1.39	0.31 \pm 0.05
cAMP	8	-17.34 \pm 3.7	-2.13 \pm 1.27	0.34 \pm 0.04
Bumetanide control	8	-16.85 \pm 4.49	6.9 \pm 2.94	0.92 \pm 0.13
cAMP	8	-31.75 \pm 3.79	0.63 \pm 2.69	1.1 \pm 0.12

Results are mean \pm SEM of (n) jejunal segments from 4 rats. The variation in Isc, G and PD is shown in the table for both dbcAMP-treated and untreated tissue. dbcAMP was added to the serosal side at the concentration of 1mM. D-(+) Glucose (10 mM) was added at 60 min to both tissues. Phloridzin (0.5 mM) was introduced to the mucosal bath at time 80 min followed by (0.05 mM) Bumetanide in the serosal bath at time 100 min, difference from control at $p = 0.05$.

The results of the study showed that, there was no difference in the response of glucose-induced increase in short-circuit current (Isc) after stimulation of rat jejunal tissue with cholera toxin (CT). In addition, it is also found that, the effect of glucose on Isc was more prominent when the tissue was pre-stimulated by dbcAMP.

Many studies have reported the effect of CT which induced the secretion of fluid along with chloride.^{6,14} The effect of CT has been known to act through the activation of cAMP.^{13,14} The amplitude of

the effect of CT in intestine depends on many parameters that we have to consider, like the animal (rat to rat variation) and their status (fasting or nonfasting) and in few cases, on the origin of chemical used (lot to lot variation). In our study there was no significant effect of CT in basal Isc although the fact that we used only one lot of CT. From the first set of experiment, we did not find any augmentation of Isc due to glucose; the cause underlying this was cholera toxin, which did not able to produce any stimulation of Isc by increasing Cl⁻ secretion in the experiment. The reason may be that it was an *in vitro* experiment and cholera toxin increased Isc in most *in vivo* tests^{1,14} and also may be CT did not exist long time (60 min).

This study also concluded that the effect of glucose was more significant after stimulation of intestinal tissue with dbcAMP. Glucose and dbcAMP induce increase in Isc by two different mechanisms. The increase in Isc due to glucose reflect the increase in absorption, where as the augmentation of Isc due to dbcAMP is a result of the increase in secretion. The results of the second set of experiment revealed a significant increase in Isc induced by dbcAMP, though glucose-dependent Isc was not augmented significantly, it showed a tendency to rise in Isc value, i.e. increased glucose uptake. Consequently, Phloridzin did not produce a significant decrease in Isc by inhibiting Na⁺/glucose co-transport. But Bumetanide exhibited significant decrease in Isc by blocking Cl⁻ secretion.

These observations and findings have great importance from clinical point of view. In secretory diarrhoeal disease induced by cholera toxin, the use of oral rehydration solution (ORS) containing glucose is usually used. The fact that glucose-induced increase in Isc may suggest that, this component in ORS is essential to allow rehydration during diarrhoea.

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