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Ussing chamber technique a tool for studying antisecretory and barrier protective effects of plant extracts

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Abstract

Ussing chamber technique is widely used to study the transport of electrolytes, drugs, and other molecules across various epithelial tissues. The short circuit current (I_{sc}) in Ussing chamber studies is a measure of electrogenic ion movement and the ion movement at the resting stage is considered the basal I_{sc}. Diarrhea results from increased anion secretion. Thus to study anion secretion using Ussing chamber technique, it is important to identify the nature of basal I_{sc} in intestinal tissues of Swiss albino male mice in Ussing chamber studies. In Ussing chamber studies, the conductance is a measure of the viability of intestinal tissues. The change in conductance with time is essential to standardize the time for Ussing chamber experiments. These studies confirmed that the I_{sc} in intestinal tissues of Swiss albino mice was mostly contributed by anion secretion. The increased I_{sc} and G with time in Ussing chamber studies can be used to study the antisecretory and barrier protective functions of plant extracts.

Ussing chamber system, an electro-physiological technique developed by Hans Ussing is widely used to study the transport of electrolytes, drugs, and other molecules across various epithelial tissues including intestinal tissues *ex vivo* while maintaining the viability of tissues[2,15,18]. Ussing chamber technique works based on Ohm's law and measures the transmembrane short circuit current (I_{sc}) and conductance (G) across various epithelial tissues. Paracellular ion movements driven by the passive forces of transepithelial concentration and osmotic and hydrostatic gradients were eliminated by continuously clamping the potential to zero with an external current passed across the epithelium. Thereafter, the measured short-circuit current (I_{sc}) is equivalent to the algebraic sum of electrogenic ion movement by active transport across the epithelium.

The I_{sc} recorded in small intestinal-tissues at the resting stage is considered as the basal I_{sc}. Previous studies have shown that

murine small intestine does not exhibit electrogenic sodium absorption in the absence of glucose or amino acids, the measured I_{sc} under basal conditions is mostly anion secretory current[2]. However, amiloride-sensitive electrogenic sodium channel (ENaC) was shown to be present in rat small intestine following proctocolectomy[5]. In our experimental setup, the studies were done in the absence of both glucose and amino acids. Experiments were therefore undertaken to establish the nature of the I_{sc} measured in the small intestinal tissues of swiss albino male mice in the absence of glucose or amino acids. Identifying the nature of I_{sc} (cation or anion current) in mouse intestinal tissues in Ussing chamber studies is essential to use as an effective tool for studying the antisecretory effect of test agents. Any agent which inhibits the anion current under basal conditions can be considered as a lead agent to cure the secretory effect associated with diarrhea.

Transepithelial conductance (G), reciprocal of transepithelial resistance (R) is the measure of the integrity of the epithelial cell barrier, which is calculated from I_{sc} and voltage using Ohm's law[2]. The quality and integrity of intestinal preparation is an important factor for accurately measuring the transepithelial ion transport. Thus, increased G in Ussing chamber studies can also be used as a tool, and any agent which reduces the G may possess barrier protective properties.

Our studies have shown that basal I_{sc} in the intestinal tissues of Swiss albino male mice is mostly contributed by the anion channels and a decrease in I_{sc} and G in presence of plant extracts showed their antisecretory and barrier protective properties respectively.

Materials : NPPB (Sigma, Germany), Amiloride (Sigma, Germany), and Phloridzin (Sigma, Germany), were used in this study. All other common chemicals used were of analytical grade.

Animals : Swiss albino male mice of eight weeks old were used for the entire study. All experimental protocols were approved by the Institutional Animal Ethical Committee, SCTIMST (SCT/IAEC-242/AUGUST/2017/94).

Electrophysiological studies in Ussing chambers: The small intestine collected after sacrificing the mice by CO_2 euthanasia was opened longitudinally and cut into eight pieces. The intestinal tissues were mounted in Ussing chambers (VCC MC-8; Physiologic Instruments, USA) and were continuously voltage-clamped at zero. Apical and basolateral sides of the tissues were bathed in physiological ringer solution (140 mM of Na^+, 119.8 mM of Cl^-, 5.2 mM of K^+, 2.4 mM of HPO_4^-, 0.4 mM of $H_2PO_4^-$, 1.2 mM of Mg^{2+}, 1.2 mM of Ca^{2+}, and 25 mM of HCO_3^-) at pH 7.4 and osmolarity of 292-300 m Osms. The tissues were bubbled with 95% O_2 and 5% CO_2 and incubated at 37°C throughout the experimental process. Tissues were allowed to stabilize in the chamber before treating with test agents. The I_{sc} and G were recorded by an automated DI 720 (Dataq instruments, USA) for real-time data capture and analyzed using Acquire and analyze software system rev. II (Physiologic Instruments, USA).

Time-dependent changes in short circuit current and conductance in intestinal tissues mounted in Ussing chambers :

To determine the time-dependent changes in I_{sc} and G, the tissues were mounted in the Ussing chambers and observed for a period of two and half hours[7,13,14]. After euthanasia, the intestine is exteriorized, and serosa and muscular layers were removed by stripping through the submucosal plane to obtain mucosal sheets devoid of myenteric and Meissner's plexus. The intestinal sheets were then mounted in Ussing chambers and additional nutrients were not provided. After these procedures, time was given to the tissue s to acclimatize in the chamber. These experiments will help to determine the change in I_{sc} and G with time and to standardize the duration of Ussing chamber experiments.

Contribution of cation transport on short circuit current measured in mouse small intestine in the absence of glucose or amino acids :

The intestinal tissues absorb sodium-coupled with Cl- and are mediated through NHE3 and PAT1 and/or DRA, respectively. Because of the coupling of NHE3 with anion exchanger, salt absorption through NHE3 is electroneutral. So, inhibition of NHE3 does not directly affect I_{sc} in Ussing chamber technique. To determine the effect of NHE3 inhibition on basal I_{sc}, 10 µM hexamethyl amiloride, a potent inhibitor of NHE1 and NHE3 was added in the apical side of the Ussing chambers and incubated for half an hour. Since NHE1 is not expressed on the apical membrane, any effect of HMA will be because of its effect on NHE3. The change in I_{sc} and G were measured and compared with that of control tissues and data were represented as ΔI_{sc} or ΔG.

The majority of the literature suggests that ENaC was not expressed in small intestinal epithelial cells under normal conditions. However, the conflicting reports for the presence of electrogenic epithelial sodium channel in the small intestinal epithelium were mostly observed only during large bowel resection. To determine the role for ENaC if any in the I_{sc} measure in mouse small intestine, 50 µM amiloride, a specific ENaC blocker was added to the apical side of the tissues and incubated for half an hour. The change in I_{sc} and G were measured and compared with that of control tissues and data were represented as ΔI_{sc} or ΔG.

Even though the intestinal lumen is flushed with regular ringer solution on both apical and basolateral sides, there may be some residual glucose in the unstirred layer. To rule out the possibility for the glucose in the unstirred layer contributing to the basal short circuit current, phloridzin (1 mM), a potent inhibitor of SGLT1 was added in the apical side of the Ussing chamber and incubated for half an hour. The data were represented as ΔI_{sc} or ΔG.

Contribution of anion transport on measured short circuit current in mouse small intestine in the absence of glucose or amino acids :

To determine the contribution of anion secretion to the measured I_{sc}, 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (100 µM), a non-specific anion channel blocker was added to the apical side of the Ussing chambers. The ΔI_{sc} and ΔG method was used to represent the data.

Short circuit current and conductance increased with time in mouse intestinal tissues in Ussing chambers :

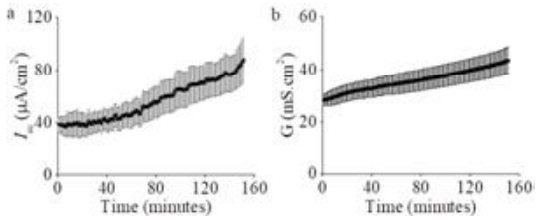


Figure 1: Change in **a)** short circuit current and **b)** conductance with time in mouse intestinal tissues in Ussing chambers. Data represents mean±SEM (n=6).

The changes in I_{sc} and G observed during the experiment opened a method of selection of plant extracts for secretion and barrier defect. The intestinal tissues took 30 minutes for getting stable I_{sc} and G that lasted for approximately 15 minutes (Fig.1). However, there was a progressive increase in I_{sc} (47.2±9.9 µA/cm^2) and G (11.4±2.5 mS.cm^2) after the initial stabilization period. Since I_{sc} and G are continuously increasing in the chamber, the time intervals 30 minutes, 45 minutes and 1 hr can be selected to evaluate the effect of active agents and these time intervals will help to determine how fast an agent can decrease I_{sc} and G.

Electrogenic cation absorption did not contribute to basal short circuit current:

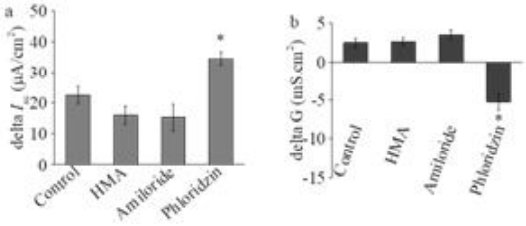


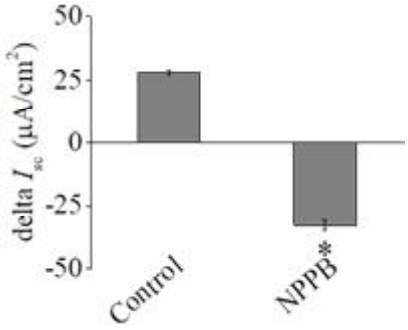
Figure 2 **a)** Electrogenic short circuit current (I_{sc}) sensitive to HMA, Amiloride and Phloridzin **b)** Change in conductance in presence of HMA, Amiloride, and Phloridzin. Each bar represents mean ± SEM (n = 4).
* represents P < 0.05.

Addition of HMA did not significantly decrease both I_{sc} (15.9±3 µA/cm^2 vs 22.5±2.8 µA/cm^2) and G (2.6±0.5 mS.cm^2 vs 2.5±0.6 mS.cm^2) compared to control group. The data confirmed that the electroneutral transport did not contribute to the I_{sc} in Ussing chamber studies and the inhibition of NHE3 was not influencing the I_{sc} and G.

Addition of amiloride did not significantly reduce both I_{sc} (15.4±4.3 µA/cm^2 vs22.5±2.8 µA/cm^2) and G (3.5±0.6 mS.cm^2 vs 2.5±0.6 mS.cm^2) compared to control group. The data suggests that in healthy mouse small intestine, ENaC did not contribute to basal sodium absorption.

The phloridzin incubating tissues did not decrease the short circuit current as expected which eliminates the possibility of sodium-coupled glucose transport under basal conditions and its significant contribution to I_{sc} in Ussing chamber studies. But phloridzin significantly increased I_{sc} (34.5±2 µA/cm^2 vs 22.5±2.8 µA/cm^2, P < 0.01) and decreased G (-5.3±1.1 mS.cm^2 vs 2.5±0.6 mS.cm^2, P < 0.01) compared to control group.

Basal short circuit current in Ussing chamber studies mostly contributed by anion channels:



To determine the contribution of anion channels in the mouse small intestine, a non-specific anion channel blocker was used. Addition of NPPB significantly decreased I_{sc} (-33±2 μA/cm^2 vs28±1 μA/cm^2, $P < 0.01$) compared to control group in Ussing chamber studies. This data suggesting that the basal I_{sc} in mouse intestinal tissues in Ussing chamber is mostly contributed by the secretion through anion channels. The basal I_{sc} inhibited within one and half hours and the conductance was progressively increasing. Thus incubation time for NPPB is determined as one and half hours for future experiments.

Ussing chamber studies are widely used to study the transport of nutrients, ions, and drugs across epithelial cell membrane[2]. The activities of transporters and channels are studied using specific blockers and stimulators[6]. The studies used intestinal tissues of a Swiss albino male mouse, a murine model. The short circuit current across the intestinal epithelium is mostly contributed by the anion channels and a small contribution is made from potassium channel[2]. Studies reported that the anion secretion is mainly mediated through CFTR channels[2]. Hence an agent that reduces short circuit current may work by inhibiting CFTR or influencing the signaling pathway that leads to the anion secretion through CFTR. Thus, this Ussing chamber tool can be used for preliminary screening of agents for determining their antisecretory properties[19]. Tissue integrity is maintained by the activity of tight junctional proteins that includes claudins, occludens, and E-cadherins[4, 11]. And a reduction in the rate of increasing transepithelial resistance may be mediated through maintaining tight junction proteins. Thus, an agent that decreases conductance may have barrier protective properties and any agent that increases the conductance in the chamber may have toxic effect and it further decreases tissue integrity and viability. So Ussing chamber technique can be used as a tool for studying both antisecretory and barrier protective properties and toxicity of test agents.

The I_{sc} in intestinal tissues mounted in Ussing chamber is a measure of net electrogenic ion movement[1]. NHE3 works together with PAT1 or DRA, helps to absorb NaCl in an electroneutral manner[20]. The addition of HMA did not influence the I_{sc} in Ussing chamber studies. An insignificant change in I_{sc} in presence of amiloride eliminated the possibility of ENaC expression in the small intestine and confirmed that ENaC mediated sodium absorption is not contributed to I_{sc} in intestinal tissues mounted in Ussing chamber studies. The addition of phloridzin significantly increased I_{sc} in Ussing chamber studies. The possible mechanism can be explained by the relation between N_a^+/K^+ ATPase and its role in maintaining resting membrane potential[9]. The inhibition of SGLT1 relatively hyperpolarized the cell which increases I_{sc} in Ussing chamber studies[3]. The increased I_{sc} may be mostly contributed by the outward potassium current and is electrogenic. These studies showed that the I_{sc} in mouse small intestine in Ussing chamber is not influenced by NHE3, ENaC, and glucose in the unstirred layer under basal conditions.

Basal I_{sc} was not decreased in presence of phloridzin, but it significantly decreased basal G[1, 8]. Increased SGLT1 activity is associated with intrajunctional dilatations and perijunctional cytoskeletal condensation[8] and actomyosin

contraction and these reports may be associated with increased paracellular permeability[16,17].

The NPPB decreased basal I_{sc} in the mouse small intestine. Thus, these studies established that the I_{sc} in the mouse small intestine is mostly mediated through anion channels. Experiments were done to find out the contribution of CFTR and Anoctamin 1 on basal I_{sc} in Ussing chamber, but the CFTR-inhibitor 172 and CaCC blockers were not working in the selected animal model. Previous studies reported that in the murine intestine, the anion secretion is mostly contributed by CFTR and which is followed by Calcium-activated chloride channels like Anoctamin 1[10]. The presence of other anion channels like SLC26A9 was also reported in mouse small intestine[12].

Diarrhea results from increased anion secretion from secretory channels in presence of secretagogues like cAMP, cGMP, and Calcium. Plant extracts that could decrease or which do not increase basal anion secretion may be considered as lead molecules for diarrhea. Similarly, plant extracts that could decrease or which do not increase conductance may be effective in reducing paracellular permeability. Thus, this study demonstrated Ussing chambers can be used as a tool for screening and selection of plant extracts that inhibit anion secretion and conductance.

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