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β-Adrenergic Stimulation of Short-Circuit Currents in Human Intestinal Epithelial Caco-2 Cells

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Abstract: To investigate the effects of epinephrine on ion transport of intestinal epithelia, short-circuit currents (I_{SC}) in monolayers of Caco-2 cells grown on permeable membrane supports were measured in the Ussing chamber. The application of epinephrine to the basolateral solution produced a transient increase of I_{SC} (maximal currents induced by 5 μM and more of epinephrine). The value of the peak current was 3.4 μA/cm^2. Isoproterenol (a β-agonist) increased I_{SC} in a manner analogous to epinephrine application, but phenylephrine (an α_1-agonist) induced no I_{SC} change. Clonidine (an α_2-agonist) transiently decreased Isc by about 0.8 μA/cm^2 at a high dose of 100 μM. The I_{SC} increase induced by epinephrine or isoproterenol was mimicked by a membrane-permeable cyclic AMP analogue, dibutyryl cyclic AMP, or a calcium ionophore, A 23187. Verapamil, a calcium channel blocker, eliminated the epinephrine-induced I_{SC} increase, and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a chloride channel blocker, reduced the I_{SC} increase. These observations suggest that in Caco-2 epithelia (i) epinephrine induces I_{SC} increase via β-adrenergic receptors, and (ii) the epinephrine-induced I_{SC} responses have at least two components, namely, verapamil-sensitive Ca^{2+} and NPPB-sensitive Cl^- transports.

Key words: Caco-2 cell, epinephrine, short-circuit-current, ion transport, cAMP, NPPB, verapamil, A23187

INTRODUCTION

Caco-2 cells derived from a human colonic adenocarcinoma[6] have been widely used as a model system to investigate the properties of the intestinal epithelia. In spite of the origin, Caco-2 cells differentiate into a polarized monolayer with the characteristics of the small intestinal epithelial cells rather than normal colonic cells after confluence under standard culture conditions[5,15]. Namely, Caco-2 cells after confluence are accompanied with domes and tight junctions, possessing brush-border microvilli in the apical membranes[15,18]. The activities of several kinds of nutrient transporters, such as the sodium-dependent phosphate transporter[12], the sodium-dependent glucose transporter[17], the proton-dependent dipeptide transporter[22], and the proton-dependent amino acid transporter[23], all of which are present in the apical membranes in the small intestine, have been found in confluent Caco-2 cells.

There have been few model cells for the small intestine to apply short-circuit-current techniques which detect ion flow through epithelia, because the epithelia of small intestine are loose membranes with very low transepithelial electrical resistances. Since Caco-2 cells after confluence ob-

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tain a relatively high transepithelial electrical resistance, the short-circuit-current techniques have been applied to monolayers of Caco-2 cells to examine ion or ion-coupled nutrient transports[7,8,17,23].

There has been a report which shows that epinephrine transiently increased short-circuit currents (I_{SC}) in the monolayers of Caco-2 cells as well as vasoactive intestinal peptide and dibutyryl cyclic AMP[8]. The report has also shown that dibutyryl cyclic AMP induces Cl^- flux from the basolateral to the apical solution. But it was not shown which type of adrenergic receptors was involved in the phenomenon.

In this study, we examined the effects of adrenergic agonists on ion transports in the monolayer of Caco-2 cells, using the short-circuit-current techniques. This study suggests that epinephrine induces I_{SC} by stimulating Ca^{2+} influx through the apical membranes by the elevation of cyclic AMP mediated by β-adrenergic receptors, and Cl^- efflux activated by intracellular Ca^{2+} increase. Portions of this study have been previously presented in abstract form[10].

MATERIALS AND METHODS

Cell culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). For stock cultures, the cells were grown on plastic dishes in Dulbecco's modified Eagle's minimal essential medium (DMEM) with high glucose concentration (25 mM), supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 21 mM $NaHCO_3$, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 ℃. The culture media were changed every two days. For Ussing chamber experiments, Caco-2 cells were seeded on collagen-coated membrane filters (Transwell-COL, 2.4 cm in diameter, 0.4 μm in pore size; Costar, Cambridge, Mass, USA), and used after 23 to 28-day culture.

Solutions

The bath solution for Ussing chamber experiments contained (in mM) 110 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 24 $NaHCO_3$, and 5.6 glucose. To maintain pH 7.4 during Ussing chamber experiments the solution was bubbled with 95% O_2 — 5% CO_2.

Transepithelial electrical measurements

Transepithelial electrical parameters of monolayers of Caco-2 cells were measured with a short-circuit-current amplifier (CEZ-9100, Nihon Kohden, Tokyo, Japan). A cell-culture insert (Transwell-COL) with the monolayer of Caco-2 cells was mounted in an Ussing chamber designed by us to hold the cell-culture insert. Transepithelial potential differences (PD) were measured with a pair of calomel electrodes immersed in saturated KCl and bridged to the Ussing chamber by a pair of polyethylene tubules filled with 3% agar containing the bath solution. A pair of Ag-AgCl electrodes were used as current-passing electrodes. The experiments were carried out under current-clamp conditions, in which the potential difference (PD) between apical and basolateral solutions was continuously recorded, except measuring short-circuit currents (I_{SC}) every twenty seconds under voltage-clamp conditions (about 1 s duration). Current pulses (10 μA, 1 s duration) were applied at 30-s intervals and transepithelial resistances were calculated by Ohm's law. Changes in I_{SC} and PD were shown as difference currents and voltages, respectively, between the values in response phases and their respective baseline values. During the experiments, the temperature of the solution was kept at 37 ℃ by circulating the water at the same temperature through the water jacket around the chamber, and the pHs of both apical and basolateral solutions (60 ml in each compartment) were continuously monitored with micro probes of F100 ISFET pH meter (Beckman, Fuller-

ton, CA, USA). In Ussing chamber experiments, the pHs were not changed by the application of chemicals.

Chemicals

All chemicals used were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), excepting dibutyryl cAMP (DBcAMP) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) obtained from Sigma (St Louis, Mo., USA) and Research Biochemicals International (Natick, MA, USA), respectively. Verapamil and NPPB were prepared as 1000-fold stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO (0.1 %) had little effects on cellular function or viability. The other reagents were prepared on the day of use.

Statistical analysis

All data are expressed as mean±SEM, and *n* indicates the number of experiments. Statistical analysis was performed using Student's *t*-test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

After 23 days or more in culture, Caco-2 cells grown on the permeable collagen-coated membrane filter appeared as a columnar epithelial monolayer with their basolateral membranes firmly attached to collagen-coated surface, and their apical membranes facing the medium. In Ussing chamber experiments reported here, these monolayers maintained transepithelial potential resistance of 687±105 Ωcm², and the values of basal I_{SC} and PD were 7.5±1.2 μA/cm² and 5.1±1.1 mV (n=10), respectively.

Epinephrine-induced electrical responses

The application of epinephrine to the basolateral solution induced transient I_{SC} and PD increases in Caco-2 epithelia (Fig. 1A, B), while apical epinephrine addition had no effects on the electrical properties. Both I_{SC} and PD reached a peak about 2 min after the addition of epinephrine. Thereafter, they slowly decreased and reached a plateau around 10 min. Transepithelial resistances (R_t) were altered almost inversely to I_{SC} and PD changes (Fig. 1C). The I_{SC} and PD responses at the peak increased dose-dependently. Epinephrine at 0.1 μM induced detectable responses in both I_{SC} (Fig. 2A) and PD (Fig. 2B). Epinephrine at the concentration of 5 μM induced nearly maximal reactions ($\triangle I_{SC}$, 3.3 ±0.5 μA/cm²; △PD, 1.2 ±0.2 mV). Therefore 10 μM epinephrine was used in all fol-

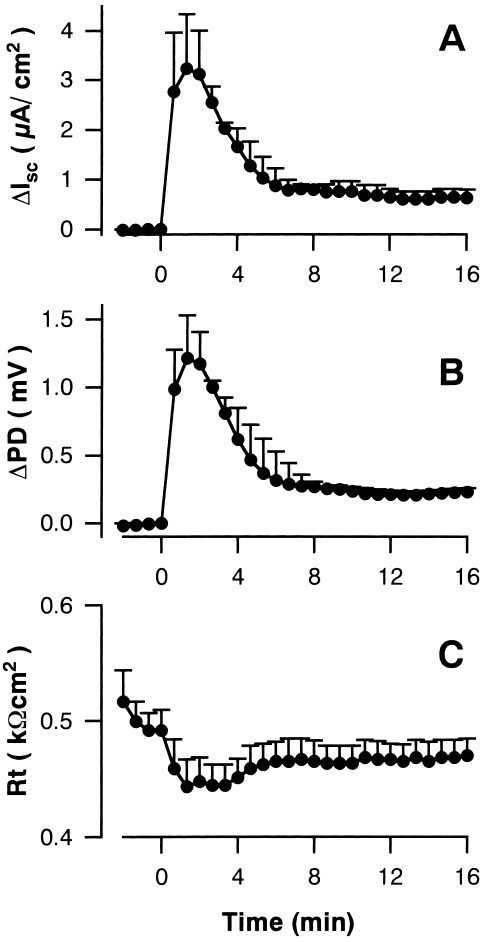

Fig. 1. Effects of epinephrine on: A, short-circuit currents ($\triangle I_{SC}$); B, transepithelial potential differences (△PD); and C, transepithelial resistances (R_t) of monolayers of Caco-2 cells. Epinephrine (10 μM) was added to the basolateral solutions at time 0 (n=3).

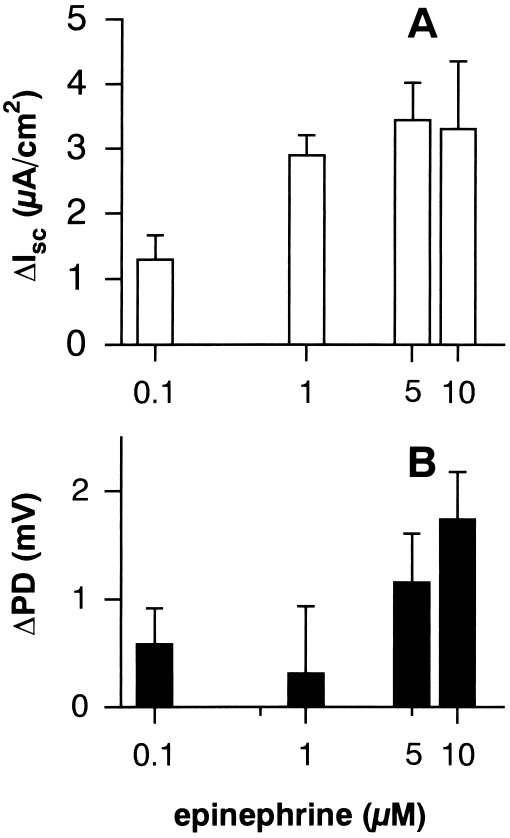

Fig. 2. Dose-dependent effects of epinephrine on A, I_{SC} and B, PD. Epinephrine (0.1-10 μM) was administered to basolateral solutions, and I_{SC} and PD were measured at the peak time (about 2 min after the addition of epinephrine, *n*=3-4).

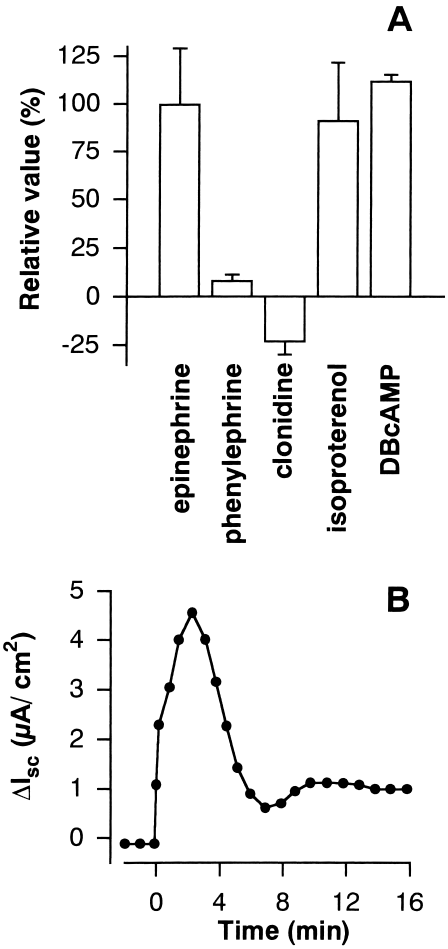

Fig. 3. Effects of adrenergic agonists and intracellular cAMP modulators on I_{SC}. A: Epinephrine (10 μM, *n*=3), phenylephrine (100 μM, *n*=2), clonidine (100 μM, *n*=2), isoproterenol (10 μM, *n*=3) or dibutyryl cyclic AMP (DBcAMP, 200 μM, *n*=3) was added to the basolateral solution. The value of each column represents the relative value of I_{SC} response referred to the I_{SC} value with epinephrine (10 μM) as 100%. **B**: A representative case of changes of $\triangle I_{SC}$ induced by isoproterenol. Isoproterenol (10 μM) was added to the basolateral solutions at time 0.

lowing experiments. Since time courses and dose-response properties of I_{SC} and PD changes induced by epinephrine were almost same not only in the above experiments but in the following experiments, only I_{SC} responses are shown in the following experiments.

β-adrenergic stimulation involved in the epinephrine effects

We examined which type of epinephrine receptors is involved in the I_{SC} increase induced by epinephrine (Fig. 3A). An α_1-agonist, phenylephrine (10-100 μM), induced no I_{SC} change, while an α_2-agonist, clonidine, transiently decreased I_{SC} by about 0.8 $\mu A/cm^2$ in a high dose of 100 μM. A β-agonist, isoproterenol (10 μM), increased Isc in a manner analogous to epinephrine application (Fig. 3A, B). The action of epinephrine was completely blocked by propranolol pretreatment (a β-antagonist) (Fig. 4).

We next used reagents which elevate intracellular cyclic AMP, because cyclic AMP is known as the second messenger of the β-adrenergic receptor. Basolateral administration of dibutyryl cyclic AMP (100 μM) which is one of the membrane per-

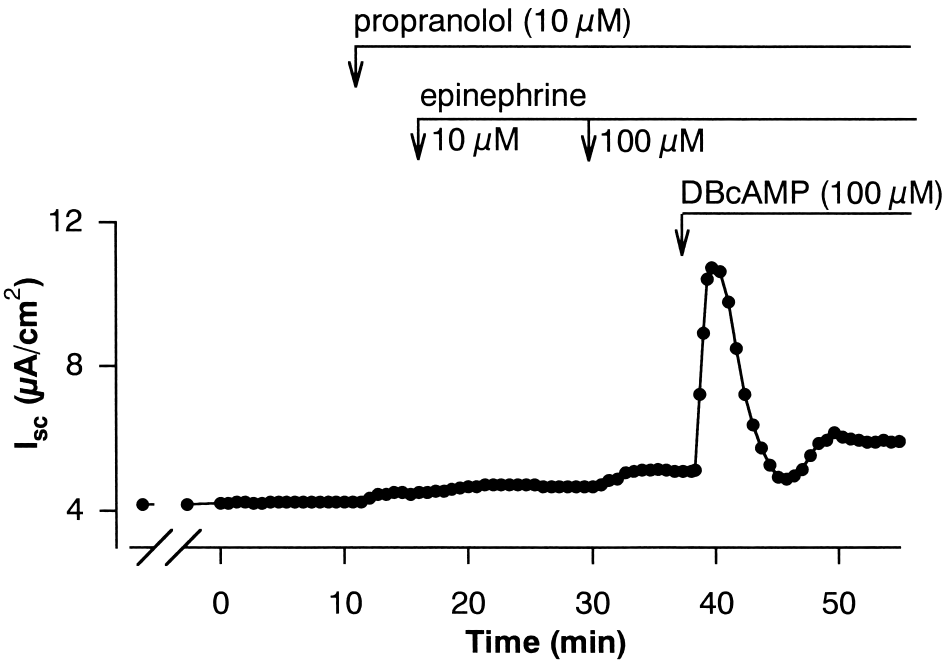

Fig. 4. Effect of propranolol on epinephrine-induced I_{SC}. Propranolol (a β-adrenoceptor antagonist, 10 μM) was added to the basolateral solutions at time 10 min, and 10 μM and 100 μM epinephrine were added to the basolateral solutions at time 15 and 30 min, respectively. Epinephrine did not increase I_{SC} after the pretreatment with propranolol. DBcAMP (100 μM) added at time 38 min induced I_{SC} response even in the presence of propranolol.

meable analogues of cyclic AMP stimulated the I_{SC} response to the similar extent to the epinephrine effects (Fig. 3A). Dibutyryl cyclic AMP could induce the I_{SC} response even when the basolateral membrane was pretreated with propranolol which completely eliminated the effects of epinephrine on I_{SC} (Fig. 4). Basolateral application of forskolin (10 μM), an adenylyl cyclase activator, also induced similar responses of I_{SC} to the administration of epinephrine (data not shown).

These observations reveal that the epinephrine-induced electrical responses in Caco-2 epithelia are induced by the elevation of intracellular cyclic AMP via β-adrenergic receptors.

Inhibition of isoproterenol-induced I_{SC} by verapamil and NPPB

Since I_{SC} increase induced by isoproterenol (β-agonist) application represents a net flow of cation from the apical to basolateral solutions, or a net flow of anion from the basolateral to apical solutions, we examined effects of several ion channel blockers on isoproterenol-induced I_{SC} responses (Fig. 5).

An epithelial sodium channel blocker, amiloride (100 μM) was added to the apical solution before isoproterenol application, but amiloride showed little effect on the epinephrine-induced I_{SC} responses. Since there have been reports showing that the elevation of intracellular cyclic AMP activates L-type calcium currents in cardiac myocytes[9,24], we next examined effects of verapamil, known as an L-type calcium channel blocker. The addition of verapamil (1 mM) to the apical solution before isoproterenol application almost completely inhibited I_{SC} responses to isoproterenol. Further, the application of a calcium ionophore, A23187 (1 μM), to the apical solution largely increased Isc in the similar manner to isoproterenol. The results suggest that isoproterenol activates calcium chan-

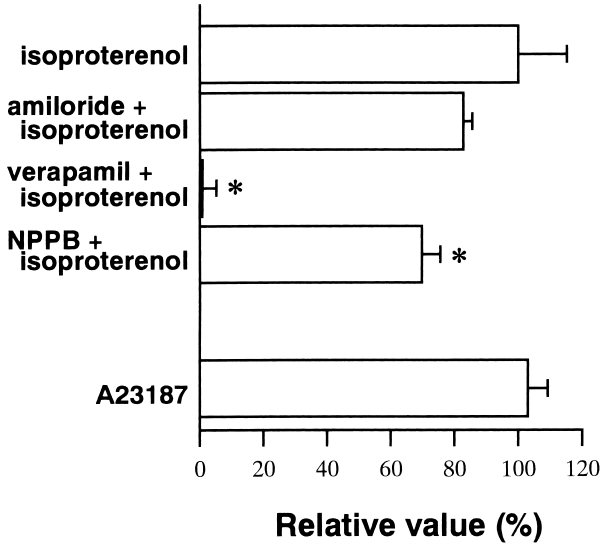

Fig. 5. Effects of ion-channel blockers on isoproterenol-induced I_{SC}, and A23187-induced I_{SC} response. Amiloride (100 μM, n=2), verapamil (1 mM, n=2) or 5-nitro-2- (3-phenylpropylamino) benzoic acid (NPPB, 100 μM, n=5) was added to the apical solutions about 15 min before isoproterenol (10 μM) administration to the basolateral solutions. Verapamil and NPPB significantly reduced isoproterenol-induced I_{SC} responses.
$*p<0.05$ vs. isoproterenol.
The application of calcium ionophore, A 23187 (1 μM), to the apical solution stimulated I_{sc} at the similar extent to the control (10 μM isoproterenol, n=2).

nels in the apical membranes through intracellular cyclic AMP elevation.

The application of NPPB (100 μM), a potent chloride channel blocker, to the apical solution before isoproterenol administration, significantly decreased the Isc response (Fig. 5). The magnitude of Isc change with NPPB pretreatment was about 70 % of the response in isoproterenol experiments. These results suggest that the activation of NPPB-sensitive, Ca^{2+}-activated Cl^- channels in the apical membranes are involved in the isoproterenol-induced Isc responses.

Taken together, the above results suggest that isoproterenol-induced I_{SC} response has two components through the apical membranes; (1) calcium influx activated by cyclic AMP elevation and (2) chloride efflux activated by intracellular calcium increase.

DISCUSSION

It was previously shown that epinephrine transiently increased short-circuit currents (I_{SC}) in the monolayers of Caco-2 cells as well as vasoactive intestinal peptide and dibutyryl cyclic AMP[8]. The present study clarified that epinephrine induces I_{SC} responses via β-adrenergic receptors. Isoproterenol (β-agonist) induced a similar response to epinephrine which was eliminated by propranolol (β-antagonist), but phenylephrine (α_1-agonist) has no effects and clonidine (α_2-agonist) stimulated an inverse I_{SC} change only at high concentration. In other kinds of epithelial cells, there have been several reports which shows epinephrine induces ion transports via α- or β-adrenergic receptors. In rabbit tracheal epithelia, α-adrenergic stimulation activates Na^+-K^+-$2Cl^-$ cotransport, resulting Cl^- secretion[11]. In fetal sheep alveolar epithelia, epinephrine stimulates amiloride-sensitive Na^+ absorption and bumetanide-sensitive Cl^- secretion, mediated by β-adrenergic receptors[21]. In mouse endometrial epithelium, Cl^- secretion is activated by β-adrenergic stimulation[2]. In rabbit proximal colon, α_2-agonist clonidine increases net Na^+ and Cl^- absorption, but α_1-agonist phenylephrine and β-agonist isoproterenol did not induce ion transports[19]. In guinea-pig distal colon, epinephrine stimulates bumetanide-sensitive, K^+ secretory currents[16].

It is suggested in the present work that the elevation of intracellular cyclic AMP concentration activates Ca^{2+}-permeable channels in the apical membranes, resulting in Ca^{2+} influx through the apical membranes which is one component of epinephrine-induced I_{SC} increase. This consideration is supported by the results that a calcium channel blocker, verapamil, almost completely inhibited the epinephrine-induced I_{SC} increase.

There have been reports which show that Ca^{2+} channel activation is induced by the elevation of intracellular cyclic AMP. β-adrenergic agonist activates Ca^{2+} currents through phosphorylation-dependent mechanisms in frog ventricle myocytes[9]. Vasoactive intestinal peptide, and secretin also activate L-type Ca^{2+} currents via the increase of intracellular cyclic AMP[24]. In Caco-2 cells, though an L-type calcium channel blocker, verapamil, almost completely inhibited isoproterenol-induced I_{SC} responses, it still remains to be investigated whether the same type of calcium channels are present in the apical membranes, or verapamil-sensitive, another type of calcium-permeable cation channels are involved in the reaction.

As shown in the present study, in Caco-2 cells isoproterenol-induced I_{SC} increase has a component of NPPB-sensitive Cl^- transport, due to the activation of Cl^- channels in the apical membranes. NPPB reduced the A23187-induced I_{SC} response by about 30% (data not shown), suggesting that the NPPB-blockable Cl^- channels are activated by the increase of intracellular Ca^{2+} concentration. There have been reports which reveal similar mechanisms in epithelial Cl^- transport[4,14]. In the human colonic carcinoma cells, T_{84} which has been widely used as a model cell for investigating the properties of colonic epithelia, carbachol stimulates I_{SC} and Cl^- flux through increasing intracellular Ca^{2+} [4]. We also applied carbachol to the monolayers of Caco-2 cells, but carbachol did not stimulate I_{SC} responses (data not shown), suggesting that Caco-2 cells differentiate diverse from colonic cells. Also in renal epithelial A6 cells, a portion of Cl^- transport activated by a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) depends on intracellular Ca^{2+} increase[14].

The present study suggests that epinephrine activates Ca^{2+}-sensitive Cl^- channels in the apical membrane of Caco-2 cells. Caco-2 cells differentiate after confluence under standard culture conditions into a polarized monolayer with the properties of small intestinal epithelial cells[5,15], and express CFTR (cystic fibrosis transemembrane conductance regulator) Cl^- channels which are activated by the increase of intracellular Ca^{2+} [3]. In human small intestine, CFTR expression is high in the mucosal epithelium[20]. Taken together these facts, epinephrine may activate CFTR Cl^- channels in the mucosal epithelium of small intestine through the elevation of intracellular Ca^{2+}.

The result that verapamil almost completely blocked the isoproterenol-induced Isc increase suggests that isoproterenol-induced, NPPB-sensitive Cl^- transport was activated by the elevation of intracellular Ca^{2+} concentration. However, it has been reported, using patch-clamp, single-channel recording techniques, that verapamil directly blocks outwardly rectifying Cl^- channels in a human colonic cell line, HT29D4[1], and in pig small intestinal enterocytes[13]. In Caco-2 cells, from the facts that a calcium ionophore, A23187, stimulated similar I_{SC} responses to isoproterenol and that NPPB reduced A23187-induced I_{SC} increase, Ca^{2+}-sensitive Cl^- transport should be involved in the isoproterenol-induced I_{SC}. Whether verapamil-sensitive Cl^- channels are present in the apical membranes of Caco-2 cells remains to be clarified by patch-clamping.

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ヒト腸管上皮細胞 Caco-2における短絡電流に対するβアゴニストの作用

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腸管粘膜上皮のイオン輸送に対するepinephrineの効果を明らかにするために，透過性膜上に単層培養したCaco-2細胞を用いて，Ussingチャンバー内で短絡電流を測定した．epinephrineを基底膜側に投与すると短絡電流は一過性に増加し，5μM以上の濃度で短絡電流は最大値となった．ピーク時の短絡電流の値は3.4μA/㎠であった．Isoproterenol（βアゴニスト）はepinephrineと同様の反応を引き起こしたが，phenylephrine（α_1アゴニスト）は短絡電流に全く影響を与えず，clonidine（α_2アゴニスト）は100μMの高濃度で，短絡電流を一過性に約0.8μA/㎠低下させた．サイクリックAMPの細胞膜透過性誘導体であるDBcAMP，あるいはカルシウムイオノフォアであるA23187でもepinephrineと同様の反応が見られた．Ca^{2+}チャネル阻害剤であるverapamilは，epinephrineによる短絡電流の増加を消失させた．また，Cl^-チャネルの阻害剤であるNPPBは，epinephrineで引き起こされる短絡電流の増加を減少させた．

以上の結果より次のように推察された．(i)Caco-2細胞において，epinephrineはβ作用により短絡電流の増加を引き起こす．(ii)epinephrineによる短絡電流の増加は，少なくとも二つの要素，すなわちverapamil感受性のCa^{2+}輸送系とNPPB感受性のCl^-輸送系とによるものである．