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Stimulation of Cl^- secretion by AlF_4^- and vanadate in T84 cells

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We investigated the mechanism of Cl^- secretion by fluoroaluminate(AlF_4^-) and sodium orthovanadate(vanadate) using the human colonic T84 cell line. T84 cell monolayers grown on collagen-coated filters were mounted in Ussing chambers to measure short circuit current(I_{sc}). Serosal addition of AlF_4^- or vanadate to T84 monolayers produced a sustained increase in I_{sc}. Removal of Ca^{2+} from the serosal bathing solution partially inhibited AlF_4^--and vanadate-induced I_{sc}, and readministration of Ca^{2+} restored AlF_4^--and vanadate-induced I_{sc}. Carbachol application in the presence of forskolin, AlF_4^- or vanadate induced a synergistic increase of I_{sc}. Forskolin and vanadate significantly increased cellular cAMP level, while carbachol and AlF_4^- did not. Carbachol, AlF_4^- and vanadate significantly increased $[Ca^{2+}]_i$. After Na^+ in mucosal bathing solution was replaced with K^+, and the mucosal membrane of T84 cell was permeabilized with amphotericin B, AlF_4^-, vanadate, and carbachol increased K^+ conductance, but forskolin did not. After sodium chloride in serosal bathing solution was replaced with sodium gluconate and the serosal membrane was permeabilized with nystatin, forskolin, AlF_4^-, and vanadate increased Cl^- conductance, but carbachol did not. AlF_4^--induced I_{sc} was remarkably inhibited by the pretreatment of pertussis toxin(2 μg/ml) for 2 hours. These results indicate that AlF_4^- and vanadate can increase Cl^- secretion via simultaneous stimulation of Cl^- channel and K^+ channel in T84 cells. However, the AlF_4^- action is mostly attributed to stimulation of pertussis toxin-sensitive G-proteins, whereas the vanadate action mostly results from G protein-independent mechanisms.

Key Words : *Cl^- secretion, AlF_4^-, Vanadate, T84 cells.*

INTRODUCTION

The physiological functions of many epithelial tissues depend on salt and water secretion that is driven by transepithelial Cl^- transport. Tissues such as intestine and trachea share a common mechanism for Cl^- secretion. Chloride enters secretory cells by the operation of the following three basolateral membrane transport processes : (1) Na-K-2Cl cotransport, which brings Cl^- into the cell ; (2) the Na-K pump, which removes the Na^+ that enters via cotransport ; and (3) K^+channels, which recycle the K^+ that enters via the pump to the serosal solution-

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(Welsh, 1987). Chloride leaves secretory cells by diffusion through apical membrane Cl^- channels, which cAMP and other intracellular messengers control the rate of the secretion(Worrel et al., 1989 ; Cliff and Frizzel, 1990 ; Anderson and Welsh, 1991).

AlF_4^-, which is known to mimic GTP at its binding site to heterotrimeric G proteins, has been used to study the functions of G-proteins in a variety of cells(Casey et al., 1989). Vanadate has been reported as a P-type ATPase inhibitor and a G protein stimulator(Simons, 1979 ; Paris and Pouyssegur, 1987). We previously reported that AlF_4^- and vanadate increase Cl^- secretion in rabbit colon mucosal preparation via an increase in prostaglandin synthesis which is mediated by the increase in $[Ca^{2+}]_i$ (Jung et al., 1992) and Plass et al.(1992) reported the same result using vanadate. However, in mucosal preparations it is difficult to interpret experimental results as direct effects of agents to epithelial cells because of the heterogeneity of cells. Therefore, we have chosen T84 cells, a human colonic carcinoma cell line, which is known as a good model to investigate the signaling mechanism of Cl^- secretion to see direct effects of both agents on epithelial cells. The results from this study reveal that AlF_4^- and vanadate can increase Cl^- secretion in T84 cells via distinct pathways. Vanadate and AlF_4^- increased Cl^- secretion via simultaneous stimulation of Cl^- channel and K^+ channel in T84 cells, but their action mechanisms are different each other.

MATERIALS AND METHODS

Cell culture and measurement of short circuit current(I_{sc})

Growth and maintenance of T84 cells and transepithelial electrolyte transport studies were performed as described previously(Worrel et al., 1989 ; Cliff and Frizzel, 1990 ; Anderson and Welsh, 1991). T84 cells were obtained from American Type Culture Collection and studied from passages 55-75. Cells were grown in a 1 : 1 mixture of Dulbeco's Modified Eagle's Medium(DMEM) and Ham's F-12 medium supplemented with N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid(HEPES) (15mM), penicillin(40 mg/l), ampicillin(8 mg/l), streptomycin(90 mg/l), and fetal calf serum(5%) at 37℃ in a humidified atmosphere of 5% CO2-95% room air. The medium was replaced at 3-4 day intervals. To measure I_{sc}, cells were plated on collagen-coated Millicell-HA filters(12 mm, 0.45 mM ; Millipore Corp., Bedford, MA) at 5×10^5 cells/filter and grown for 6-10 days. Before measurement of I_{sc}, transepithelial resistance was measured with an EVOM epithelial voltohmmeter(World Precision Instruments, Sarasota, FL) to ensure the presence of intact tight junctions. The I_{sc} of monolayers, of which resistance was greater than 800 Ohm$*$cm^2, was measured with a DVC-1000 voltage clamp(World precision Instruments) while clamping transepithelial voltage at 0 mV for 50 sec and 20-40 mV for 1 sec alternatively. A physiological salt solution(PSS) containing(mM) : 140 Na^+, 5 K^+, 1.5 Ca^{2+}, 1.0 Mg^{2+}, 130 Cl^-, 24 HCO_3^-, 1.3 HPO_4^-, 0.3 $H_2PO_4^-$, and 10 glucose, was used for most electrical measurements.

Measurement of serosal K^+ conductance and mucosal Cl^- conductance

The serosal K^+ conductance was measured using a technique developed by German and coworkers in turile colon(German et al., 1986) and used in T84 cells by Wong and coworkers(Wong et al., 1990). NaCl in mucosal bathing solution was replaced by KCl to generate a K^+ concentration gradient from mucosal to serosal surfaces, and then amphotericin B(10 μM) was added to the mucosal side to eliminate the mucosal barrier as rate limiting to transepithelial K^+ movement. A change in I_{sc} represents serosal K^+ conductance because the flux of K^+ across the cell is limited by the serosal K^+ conductance.

The mucosal Cl^- conductance was measured using the method of Anderson and Welsh(1991). NaCl(120 mM) in mucosal bathing solution was replaced by sodium gluconate(120 mM) to generate a Cl^- concentration gradient from serosal to mucosal surfaces, and then nystatin(0.36 mg/ml) was added to the serosal side to eliminate the serosal barrier as rate limiting to transepithelial Cl^- movement. A change in I_{sc} represents mucosal Cl^- conductance because the flux of Cl^- across the cell is limited by the mucosal Cl^- conductance.

Measurement of cellular cAMP and cGMP levels

After cells were grown on collagen-coated Millicell-HA filters as a manner identical to those used in I_{sc} measurements, they were washed and

preincubated in PSS at 37℃ for 1 hour under a mixture gas of CO_2/O_2(5/95). Preincubation buffer was aspirated and replaced with PSS containing the indicated reagents. After 30 min total cAMP was extracted from cells by addition of 2 ml ice-cold ethanol to the filters. The extracts were assayed for cAMP content using a commercial radioimmunoassay kit(New England Nuclear, Boston, MA). cGMP levels were measured exactly as described for cAMP, except that a specific radioimmunoassay kit for cGMP(New England Nuclear, Boston, MA) was used.

Measurement of $[Ca^{2+}]_i$

To measure changes in $[Ca^{2+}]_i$ T84 cells were grown on glass cover slips and were loaded with 5 μM of actoxymethylester of the Ca^{2+}-sensitive dye, fura-2, at room temperature for 30 min. The cover slips were placed in a perfusion chamber at 37℃ and were mounted on the stage of a microscope. Single cell or cells in small islands were alternatively excited at 340 and 380 nm wavelength with emission of 510 nm. The 340/380 ratios were converted to $[Ca^{2+}]_i$ values using the equation of Grykiewicz et al.(1985).

Materials

Fura-2/AM was purchased from Molecular Probes. Nystatin, sodium fluoride, forskolin, carbachol, and amphotericin B were purchased from Sigma Chemical Co.. [^{125}I]cAMP and [^{125}I]cGMP measurement kits were obtained from New England Nuclear. The concentrations of drugs which were solubilized in dimethyl sulfoxide(DMSO) or ethanol never exceeded 0.2%. All drugs were added into serosal bathing solution unless otherwise indicated.

Statistical analysis

Results are represented as mean±SEM. For multiple comparisons, significance was evaluated by analysis of variance. A Scheffe's post hoc test was used to locate significant mean differences. P value less than 0.05 was regarded as significant.

RESULTS

Stimulation of Cl^- secretion across T84 monolayers by AlF_4^- and vanadate

A time dependency of changes in I_{sc} induced by the addition of AlF_4^- or vanadate to the serosal bathing solution is shown in Fig. 1A. AlF_4^- and vanadate increased I_{sc} to a maximum at 8-10 min and 5 min, respectively, which were maintained for over 30 min. AlF_4^-- and vanadate-induced I_{sc} were inhibited by bumetanide and dependent on the presence of Na^+ in the serosal bathing solution(data not shown), indicating that they result from Cl^-

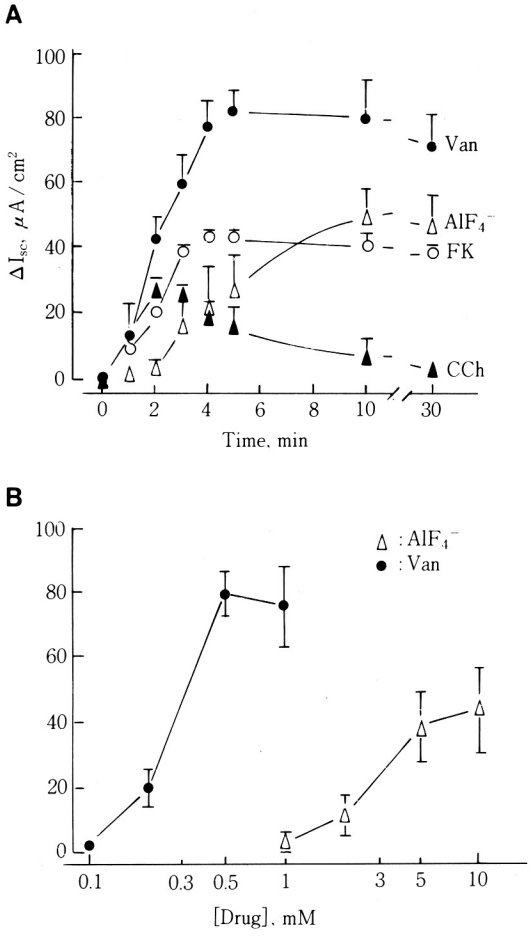

Fig. 1. A : Time course of I_{sc} responses to various secretagogues in T84 monolayers mounted in Ussing chambers. Forskolin(FK, 1 μM), carbachol(CCh, 0.1 mM), vanadate(Van, 0.5 mM) or AlF_4^-(10 mM, NaF 10 mM+20 μM $AlCl_3$) was added into serosal bathing solution. Data are expressed as the means±SEM (n=8-12). B : Dose dependency of effects of vanadate(Van) and AlF_4^- on changes in I_{sc}(ΔI_{sc}) in T84 monolayers. Data are expressed as the means±SEM(n=4).

secretion. The time-dependency of Isc changes by both drugs was similar to that by forskolin(1 μM) which was resulted from stimulation of cAMP activated Cl^- channel(Anderson and Welsh, 1991) rather than that by carbachol(0.1 mM) which was resulted from stimulation of Ca^{2+} mediated K^+ channel(Warhurst et al., 1991 ; Hartmann et al., 1992). The T84 cells responded to AlF_4^- and vanadate in a dose-dependent fashion(Fig. 1B). AlF_4^- and vanadate induced I_{sc} with the threshold stimulation at 1 mM and 0.1 mM, and the maximal response at 10 mM and 0.5 mM, respectively. Maximal concentrations of AlF_4^-(10 mM NaF+20 μM $AlCl_3$) and vanadate(0.5 mM) were used for subsequent studies.

Effect of Ca^{2+} in the AlF_4^- and vanadate induced Cl^- secretion

As in rabbit colon mucosa AlF_4^--and vanadate-induced actions are mediated by changes in intracellular Ca^{2+} concentration(Jung et al., 1992 ; Plass et al., 1992), we examined the effect of Ca^{2+} removal from serosal bathing solution on I_{sc}(Fig. 2).

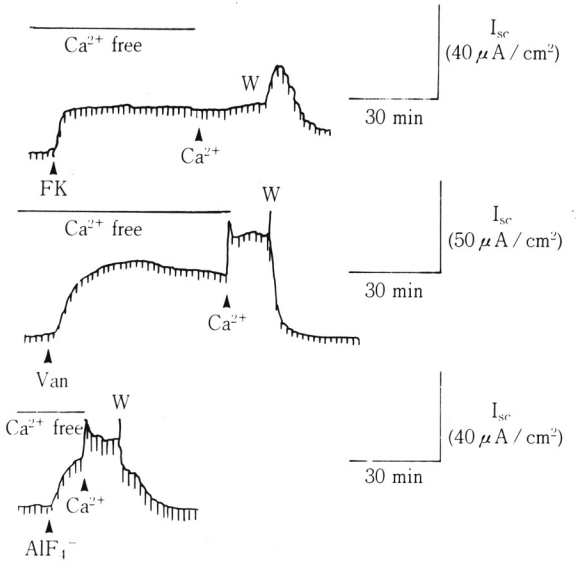

Fig. 2. Effect of Ca^{2+} removal from serosal bathing solution and Ca^{2+} readministration on I_{sc} by forskolin(FK, 1 μM), vanadate(Van, 0.5 mM), and AlF_4^-(10 mM). In Ca-free solution 0.1 mM EGTA was added. Ca^{2+}(1.2 mM) was readminstered to serosal bath during the plateau phase of the response by each agent. "W" indicates washing with physiologic saline solution.

In this experiment 0.1 mM EGTA was also added to the Ca^{2+}-free serosal solution. Removal of Ca^{2+} from the serosal bathing solution partially inhibited the effects of AlF_4^- and vanadate on I_{sc} and readministration of Ca^{2+} restored AlF_4^- - and vanadate-induced I_{sc}. However, forskolin-induced I_{sc} was not affected by Ca^{2+}removal. These results suggest that both Ca^{2+}-dependent and Ca^{2+}-independent pathways are involved in Cl^- secretion by AlF_4^- and vanadate in T84 cells.

Synergism by carbachol in the presence of AlF_4^- or vanadate

A synergistic interaction between carbachol and cyclic nucleotide elevating agonists has been described in a number of studies with T84 cells(Cartwright et al., 1985 ; Lenvine et al., 1991). We ex-

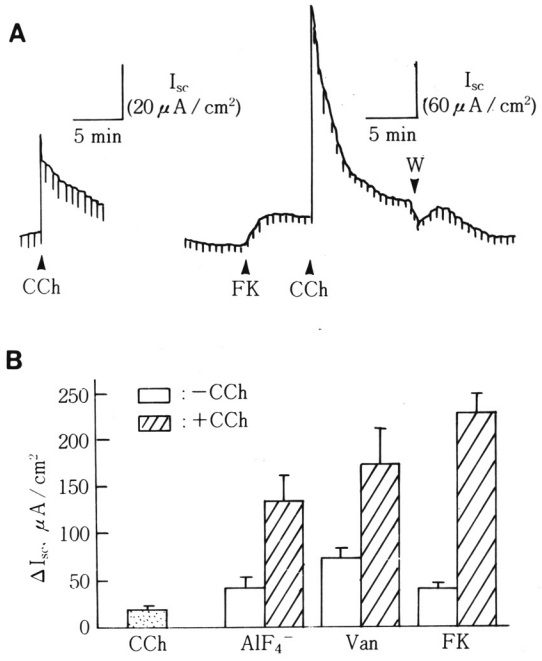

Fig. 3. A : A representative tracing showing the synergistic effect of carbachol(CCh, 0.1 mM) on I_{sc} in the presence of forskolin(1 μM, FK). B : Peak changes in I_{sc}(ΔI_{sc}) by forskolin(FK, 1 μM), vanadate(Van, 0.1 mM) and AlF_4^-(10 mM) in the absence and presence of carbachol(CCh, 0.1 mM). Each reagent was added during the plateau of change in Isc by CCh. The stipled bar represents change in I_{sc} by carbachol alone. Data are expressed as the means±SEM(n=5-8).

amined a change in I_{sc} by carbachol in the presence of AlF_4^- or vanadate(Fig. 3). Carbachol was added during the plateau phase of responses of the forskolin, AlF_4^- or vanadate. As shown in Fig. 3 A, sequential addition of forskolin and carbachol resulted in a synergistic response. Qualitatively, the I_{sc} increase by carbachol in the presence of AlF_4^- or vanadate was similar to that in the presence of forskolin : a rapid spike followed by a plateau and gradual decrease. Mean peak I_{sc} values of synergism were represented in Fig. 3B. In contrast to the synergism by carbachol in the presence of forskolin, AlF_4^- or vanadate, simple additive effects on I_{sc} were observed by combination of forskolin, AlF_4^- and vanadate(n=3, data not shown).

Measurement of secondary messengers in AlF_4^- and vanadate response

Previous studies have shown that a variety of secretagogues activate chloride secretion across T84 cells by increasing one of three secondary messengers : cAMP, cGMP, or free cytosolic calcium. We therefore measured intracellular levels of these secondary messengers in AlF_4^-- or vanadate-treated T84 cells. The results are summarized in Table 1. Forskolin and vanadate significantly increased cellular cAMP level, while carbachol and AlF_4^- had no effect on it. Both AlF_4^- and vanadate had no effect on cellular cGMP level. Carbachol, AlF_4^-, and vanadate but not forskolin significantly increased $[Ca^{2+}]_i$. The carbachol-induced changes in $[Ca^{2+}]_i$ were clearly biphasic, but AlF_4^-- or vanadate-induced change in $[Ca^{2+}]_i$ was monophasic without initial spike. A representative tracing of $[Ca^{2+}]_i$ change induced by each agent is shown in Fig. 4.

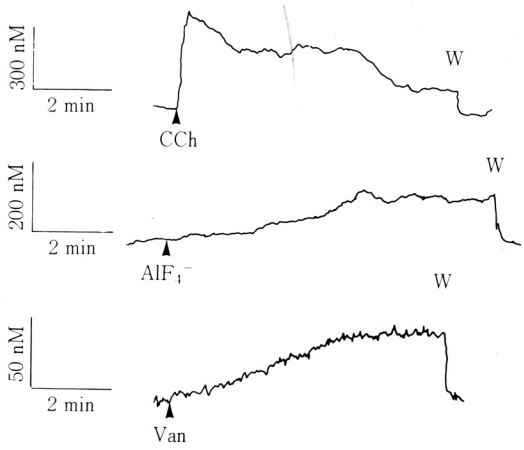

Fig. 4. Representative tracings of changes in $[Ca^{2+}]_i$ to carbachol(CCh, 0.1 mM), vanadate(0.5 mM) and AlF_4^-(10 mM) in T84 cells.

Measurement of serosal K^+ conductance and mucosal Cl^- conductance

Changes in serosal K^+ and mucosal Cl^- conductance by each secretagogue were measured(Fig. 5). The mucosal side was permeabilized by the addition of amphotericin B(10 μM) as described in the MATERIALS AND METHODS. After NaCl in mucosal bathing solution was replaced by KCl and amphotericin B was added to the same side, I_{sc} was remarkably increased for 30 min and remained stable(I_{sc}=54.5±12.4 μA/cm², n=8). Tissue conductance was also increased by 3-4 times after 30 min. The addition of AlF_4^-, vanadate, and carbachol, but not forskolin, increased I_{sc} in the preparations. When the serosal side was permeabilized

Table 1. Changes in cyclic nucleotides levels and $[Ca^{2+}]_i$ to various secretagogues.
Cyclic nucleotides were extracted with ethanol after T84 monolayers were treated for 30 min by each agent on the serosal side. The results of cyclic nucleotides level are expressed as means±SEM for 5 experiments in each group. Changes in $[Ca^{2+}]_i$ were measured using calcium-binding fluorescent probe fura-2. The data of $[Ca^{2+}]_i$ are peak responses for carbachol and maximal plateau values for AlF_4^- and vanadate(see Fig. 4) and were means±SEM for 3-4 experiments in each group.

	cAMP, pmol/filter	cGMP, pmol/filter	$[Ca^{2+}]_i$, nM
Control	17.3±1.2	2.1±0.2	121.5±15.7
Carbachol(0.1 mM)	20.3±1.7	ND	647.0±54.5*
Forskolin(1 μM)	260.1±61.2*	ND	122.4±23.6
AlF_4^-(10 mM)	15.7±2.8	1.2±0.1	207.6±28.3*
Vanadate(0.5 mM)	34.5±4.7*	1.4±0.1	177.2±12.5*

*value different from the control value($p<0.01$)
ND, not determined

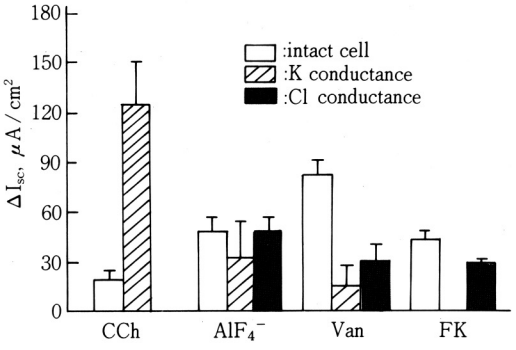

Fig. 5. Effects of carbachol(CCh, 0.1 mM), forskolin(1 μM), vanadate(0.5 mM) and AlF_4^- on serosal K^+ and mucosal Cl^- conductance. Peak change in I_{sc}(ΔI_{sc}) by each secretagogue after serosal and mucosal permeabilization was demonstrated as "Cl^- conductance" and "K^+ conductacne", respectively. Data are expressed as the means±SEM(n=5).

with nystatin(0.36 mg/ml) for 30 min after addition of sodium gluconate solution into mucosal side, tissue conductance and I_{sc} were simultaneously increased by 3-4 times and 62.5±21.4 μA/cm^2(n=9), respectively. After maximum current was reached, the addition of forskolin, AlF_4^-, or vanadate but not carbachol increased I_{sc}.

Effect of pertussis toxin

Because AlF_4^- stimulates G protein in various tissues(Casey et al., 1989 ; Jung et al., 1992) and induces G protein-mediated Cl^- secretion in intestinal cells(Tilly et al., 1991), we examined effect of pertussis toxin on AlF_4^- and vanadate induced Cl^- secretion in intact T84 cells. As shown in Fig. 6, AlF_4^--induced I_{sc} was remarkably inhibited by the pretreatment of pertussis toxin(2 μg/ml) for 2 hours, whereas vanadate-induced I_{sc} was not(Fig. 6).

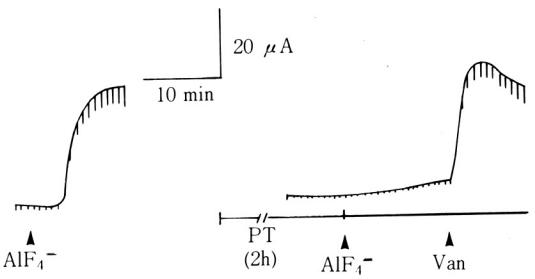

Fig. 6. Effect of pertussis toxin(PT, 2 μg/ml for 2 hours) on the vanadate(0.5 mM) and AlF_4^-(10 mM)-induced I_{sc}. These tracings are one of 2 experiments. Pertussis toxin was added to mucosal and serosal bathing solution.

DISCUSSION

This study provides the first demonstration that AlF_4^- and vanadate stimulate Cl^- secretion in T84 cells grown on permeable supports(Fig. 1). Several studies have established that changes in intracellular cAMP and Ca^{2+} levels affect on chloride secretion in this cell line. It is well known that cAMP stimulates apical Cl^- channel, but the mechanism of Ca^{2+}-dependent Cl^- secretion is still not completely solved.

We previously reported that AlF_4^- and vanadate stimulated Cl^- secretion via increasing in $[Ca^{2+}]_i$ and subsequent synthesis of prostaglandin in rabbit colon(Jung et al., 1992). Although in the present study the vanadate and AlF_4^- actions are partly dependent on changes in cellular Ca^{2+} level(Fig. 2), prostaglandin-mediated action cannot be applied to T84 cells because they lack cyclooxygenase and lipoxygenase(Dharmsathaphorn et al.. 1989). Therefore, the Ca^{2+} dependency of AlF_4^- and vanadate actions can be explained by the activation of Ca^{2+}-activated K^+ channels because both agents increase intracellular Ca^{2+} concentration and serosal K^+ conductance(Fig. 5, Table 1) and there are clear evidences for the activation of serosal K conductance by increase in intracellular Ca^{2+} concentration (Wong et al., 1990 ; Anderson and Welsh, 1991). In our experiment, AlF_4^--induced Cl^- secretion was inhibited by pertussis toxin(Fig. 6), but the vanadate action was not. This result indicates that increase in $[Ca^{2+}]_i$ by AlF_4^- results from the increase of phosphoinositide metabolites level through the activation of pertussis toxin sensitive G protein, whereas change in $[Ca^{2+}]_i$ by vanadate is mediated by a different mechanism, probably, inhibition of Ca^{2+}-ATPase.

In the present study, Ca^{2+}-independent increase of I_{sc} by AlF_4^- and vanadate showed characteristics similar to that by cAMP-mediated secretagogue : time dependency of their actions which is similar to that of forskolin-induced I_{sc}, synergistic actioin of carbachol in the presence of AlF_4^- or vanadate, activation of apical Cl^- conduct-

ance by AlF_4^- or vanadate when serosal side was permeabilized(Fig. 1, Fig. 3 & Fig. 5).

In the case of vanadate this action can be explained by increase in cellular cAMP content(Table 1). However, AlF_4^- did not affect on cytosolic cAMP or cGMP concentration. Furthermore, AlF_4^--induced increase in apical conductance cannot be explained by activation of apical Ca^{2+}-activated Cl^- channel, because in this experiment carbachol which increases cellular Ca^{2+} level does not change in apical conductance as reported by Anderson and Welsh(1991) (Fig. 4, Fig. 5). The underlying mechanisms of the stimulation of Cl^- conductance by AlF_4^- are not clear. Recent studies have indicated that adenosine and neutrophil-derived secretagogue(NDS) induce Cl^- secretion by activation of pathways distal to cAMP in T84 cells without alterations in concentrations of any known 2nd messengers(Barrett et al., 1990 ; Madara et al., 1992 ; Madara et al., 1993). Therefore, activation of apical Cl^- conductance by AlF_4^- seems to be resulted from mechanism similar to that of adenosine or/and NDS. The other possibility is that G protein stimulation by AlF_4^- is involved in increase of apical conductance, because Tilly and coworkers reported the presence of G protein-regulated Cl^- channel in intestinal cells, which is independent of ATP, cAMP and Ca^{2+}(Tilly et al., 1991). In our experiment, this possibility is supported by the almost complete inhibition of AlF_4^--induced Cl^- secretion by the pretreatment of pertussis toxin(Fig. 6).

In this experiment, vanadate is the most potent secretagogue to Cl^- secretion in T84 cells inspite of the minimal increase of intracellular cAMP and Ca^{2+} concentration. The discrepancy of the vanadate action between Isc and secondary messenger levels can be explained by inhibition of phosphatase activity because vanadate can stimulate Cl^- secretion by the inhibition of phosphatase activity in human intestine 407 cell(Tilly et al., 1993).

Vanadate and AlF_4^- have similar chemical structure and they have been used for G protein study, but the results in this study clearly show that they have different action mechanism on Cl^- secretion in T84 cells. Recently, we also reported that vanadate and AlF_4^- have differential actions on contraction of canine trachealis muscle(Lee et al., 1994). Differences of action mechanism between vanadate and AlF_4^- may be related from the fact that AlF_4^- has a stronger action on heterotrimeric G-proteins than vanadate, which also inhibits 'P'-ATPase and phosphatase activity.

The synergism by carbachol in the presence of cyclic nucleotide has been reported in T84 cells(Cartwright et al., 1985 ; Lenvine et al., 1991 ; Warhurst et al., 1991). However, the mechanism has not yet been clarified. There are two possible mechanisms. One possibility is the simultaneous activation of apical Cl^- channels by cAMP and serosal K^+ channels by Ca^{2+} result in an enhanced secretory response(Cartwright et al., 1985). The other possible mechanism is crosstalk between Ca and cAMP messenger systems(Lenvine et al., 1991 ; Barret et al., 1993). Our data demonstrate that carbachol can induce a synergism in the presence of not only forskolin or vanadate but also AlF_4^- which does not increase cAMP or cGMP concentrations but increases apical conductance(Fig. 3, Table 1). Therefore, the synergism is considered to be due to simultaneous increase of mucosal Cl^- and serosal K^+ conductance.

In conclusion, our data represent the first evidence that AlF_4^- and vanadate can stimulate Cl^- secretion in intact intestinal cells. AlF_4^- and vanadate can increase Cl^- secretion via simultaneous stimulation of mucosal Cl^- channel and serosal Ca^{2+} activated K^+ channel. However, the AlF_4^- action is mostly attributed to stimulation of pertussis toxin-sensitive G-proteins, whereas the vanadate action mostly results from G protein-independent mechanisms.

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