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Cross talk of cAMP and flavone in regulation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and $Na^+/K^+/2Cl^-$ cotransporter in renal epithelial A6 cells

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Abstract

We have reported that in renal epithelial A6 cells flavones stimulate the transepithelial Cl^- secretion by activating the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and/or the $Na^+/K^+/2Cl^-$ cotransporter. On the other hand, it has been established that cAMP activates the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter. However, no information is available on the interaction between cAMP and flavones on stimulation of the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter. To clarify the interaction between cAMP and flavones, we studied the regulatory mechanism of the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter by flavones (apigenin, luteolin, kaempherol, and quercetin) under the basal and cAMP-stimulated conditions in renal epithelial A6 cells. Under the basal (cAMP-unstimulated) condition, these flavones stimulated the Cl^- secretion by activating the $Na^+/K^+/2Cl^-$ cotransporter without any significant effects on the CFTR Cl^- channel activity. On the other hand, these flavones diminished the activity of the cAMP-stimulated $Na^+/K^+/2Cl^-$ cotransporter without any significant effects on the CFTR Cl^- channel activity. Interestingly, the level of the flavone-induced Cl^- secretion under the basal condition was identical to that under the cAMP-stimulated condition. Based on these results, it is suggested that although both cAMP and flavones activate the $Na^+/K^+/2Cl^-$ cotransporter, these flavones have more powerful effects than cAMP on the $Na^+/K^+/2Cl^-$ cotransporter.

Keywords: CFTR; Cotransport; cAMP; Forskolin; Epithelium; Kidney

1. Introduction

Epithelia play physiologically crucial roles in defending our body from environmental changes by controlling salts/ions and water transport across epithelia. Therefore, understanding the regulatory mechanism of ion transport in epithelia is very important as it prevents our body from disorder of homeostasis. cAMP is known to regulate the Cl^- transport across epithelia, such as the airway [1], the intestine [2], and the kidney [1–3]. Recent studies in renal epithelial A6 cells reveal that cAMP stimulates the Cl^- transport by activating the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter [3,4].

Flavones are multifunctional compounds extracted from soybeans and other various plants. Recent studies report the action of flavones on cell cycle, apoptosis, ion transport, and gene expression [5,6]. Our previous study indicated that some flavones (genistein, apigenin, and daidzein) stimulated the Cl^- secretion in A6 cells by activating the CFTR Cl^- channel and/or the $Na^+/K^+/2Cl^-$ cotransporter independent of the inhibitory action on protein tyrosine kinase [7]. On the other hand, it has been already established that cAMP stimulates the Cl^- secretion by activating the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter similar to flavones. However, we

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na^+ channel; G_t, transepithelial conductance; I_{sc}, short-circuit current; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; PD, transepithelial potential difference; PKA, protein kinase A; PKI, myristoylated protein kinase A inhibitor (14–22) amide.

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have no information on the interaction between flavones and cAMP on the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter. In the present study, to clarify the interaction between flavones and cAMP on the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter, we investigated the regulation of the Cl^- secretion by flavones under the basal and cAMP-stimulated conditions.

2. Materials and methods

2.1. Chemicals

Apigenin, KT5720, and myristoylated protein kinase A inhibitor (14–22) amide (PKI) were purchased from Calbiochem. All other chemicals were obtained from Sigma.

2.2. Solution

The bathing solution contained 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES with pH 7.4. DMSO was used as a solvent for flavones, forskolin, benzamil, and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB). DMSO alone at the concentration used in the preset study had no effects on the short-circuit current (I_{sc}) and the transepithelial conductance (G_t).

2.3. Cell culture

We purchased A6 cells from American Type Culture Collection in the 68th passage, and cultured A6 cells (passages 75–84) on plastic flasks in a culture medium of NCTC-109 modified for amphibian cells containing 10% fetal bovine serum and streptomycin and penicillin. The flasks were kept in a humidified incubator at 27° with 2.0% CO_2 in air. For I_{sc} and G_t measurements, we seeded cells onto polycarbonate porous membranes attached to the bottom of plastic cups (6.5 mm transwell filter; Tissue culture-treated Transwell; Costar Corporation) at density of 5×10^4 cells/well for 14–16 days.

2.4. Measurements of I_{sc} and G_t

Monolayers grown on polycarbonate porous membranes were rinsed with the same solution as the experimental solution, and were transferred to a modified Ussing chamber (Jim's Instrument). I_{sc} and G_t were measured with an amplifier VCC-600 (Physiologic Instrument) as previously reported [8,9]. Briefly, the transepithelial potential difference (PD) was measured continuously under an open-circuit condition. For measurement of G_t, we applied a pulse of 1 μA constant current for 0.5 s to the monolayer under the open-circuit condition every 10 s, calculating the G_t from the change in the PD (ΔPD) caused by the constant current pulse (1 μA) using Ohm's law ($G_t = 1\ \mu A/\Delta PD\ (mV)$) [8,10,11]. The NPPB-sensitive G_t was used as an indicator of the Cl^- conductance (channel activity) [3,4]. The I_{sc} was calculated using Ohm's law with the G_t and PD ($I_{sc} = G_t \cdot PD$) [12]. The linear current–voltage relationship and the direct measurement of I_{sc} under the voltage-clamp condition indicate that the calculated I_{sc} under the open-circuit condition was identical to the directly measured I_{sc} under the voltage-clamp condition. A positive current represents a net flow of cation from the apical to basolateral solutions or a net flow of anion from the basolateral to apical solutions. Therefore, the Cl^- secretion is represented as a positive current (I_{sc}) in the present study. The NPPB-sensitive I_{sc} was used as an indicator of the Cl^- secretion [3,4]. The bathing solution was stirred with 21% O_2/79% N_2. The PD was measured with a pair of calomel electrodes that were immersed in a saturated KCl solution and bridged to the Ussing chamber by a pair of polyethylene tubes filled with a 2 M KCl solution containing 2% agarose.

2.5. Temperature

All experiments were performed at 22–23°.

2.6. Data presentation and statistics

Results are expressed as the mean ± standard error (SE). Statistical significance was determined by Student's t test or one-way ANOVA, and $P < 0.05$ was considered significant.

3. Results and discussion

As previously reported, in renal epithelial A6 cells the Cl^- secretion is mediated through two steps: (1) the step of Cl^- uptake through the $Na^+/K^+/2Cl^-$ cotransporter at the basolateral membrane, and (2) the step of Cl^- release through the CFTR Cl^- channel at the apical membrane [13]. In the present study, we measured the NPPB-sensitive I_{sc} as an indicator of the transcellular Cl^- secretion and the NPPB-sensitive conductance (G_t) as an indicator of the CFTR Cl^- channel activity [4,13,14]. We have recently reported that apigenin, a flavone, activates the $Na^+/K^+/2Cl^-$ cotransporter without any significant effects on the CFTR Cl^- channel activity under the cAMP-unstimulated condition [7]. However, we have no information on the interaction between flavones and cAMP on the CFTR Cl^- channel and the $Na^+/K^+/2Cl^-$ cotransporter. To study the interaction between flavones and cAMP, we used apigenin, luteolin, kaempherol, and quercetin (Fig. 1) under the cAMP-stimulated and -unstimulated conditions in renal epithelial A6 cells. In all of the experiments, to isolate the Cl^- secretion from the Na^+ absorption, 10 μM benzamil, which is a specific inhibitor of epithelial Na^+ channels (ENaC) and completely blocks the ENaC at the concentration (10 μM) [15], was added to the apical side 10 min

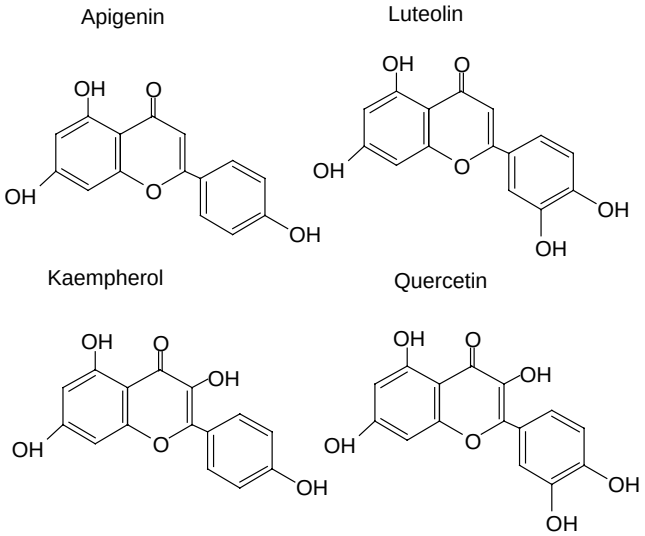

Fig. 1. Structures of flavones; apigenin, luteolin, kaempherol and quercetin.

before application of flavones or forskolin. Bilateral addition of 100 μM quercetin caused an increase in the NPPB-sensitive I_{sc} (Fig. 2A), suggesting that quercetin stimulates the transcellular Cl^- secretion. On the other hand, quercetin did not significantly increase the G_t (Fig. 2B). All of these flavones stimulated the NPPB-sensitive I_{sc} (Fig. 3A) without any significant effects on the NPPB-sensitive G_t (Fig. 3B). These results suggest that these flavones stimulate the Cl^- secretion by activating the $Na^+/K^+/2Cl^-$ cotransporter without any significant effects on the CFTR Cl^- channel activity. Notably, it is very interesting that four flavones selected on the basis of their structures had similar function to activate the $Na^+/K^+/2Cl^-$ cotransporter, suggesting that the function of these flavones is related to their structures.

We next examined the effects of these flavones on the cAMP-stimulated Cl^- secretion. We applied 100 μM quercetin bilaterally 30 min after addition of 10 μM forskolin (an activator of adenylate cyclase) to increase the cytosolic cAMP content. Quercetin caused a transient increase in the forskolin-stimulated I_{sc} within 1 min after its application (closed circles in Fig. 4A). After this transient phase, quercetin diminished the forskolin-stimulated I_{sc} (closed circles in Fig. 4A). Although quercetin showed a tendency to transiently elevate the G_t, quercetin had no significant effects on G_t at the steady state (Fig. 4B). Apigenin, luteolin, kaempherol, and quercetin diminished the NPPB-sensitive I_{sc} (Fig. 5A) without any significant effects on the NPPB-sensitive G_t (Fig. 5B). The flavone-induced level of the NPPB-sensitive I_{sc} under the forskolin-stimulated condition (Fig. 5A) was identical to that caused by flavone alone without forskolin (Fig. 3A). These observations suggest that the flavone diminished the cAMP-stimulated Cl^- secretion by decreasing the activity of the cAMP-stimulated $Na^+/K^+/2Cl^-$ cotransporter at the steady state.

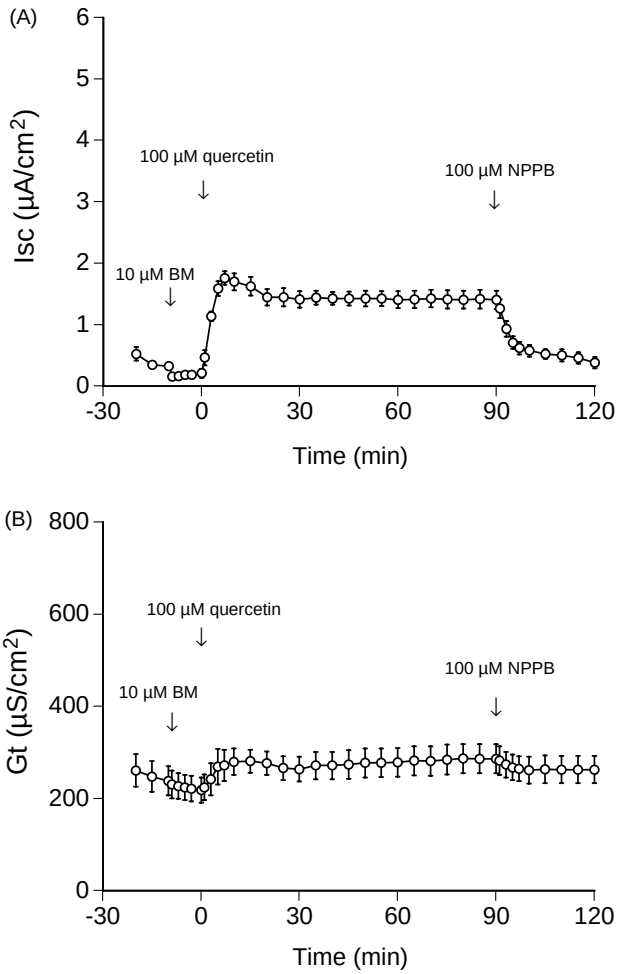

Fig. 2. Time course of the action of 100 μM quercetin on I_{sc} (A) and G_t (B) under the basal (unstimulated) condition in monolayered renal epithelial A6 cells. Benzamil (BM), 10 μM, was added to the apical side 10 min before application of quercetin. Quercetin, 100 μM, was applied to the bilateral sides at 0 min. Apical application of 100 μM NPPB was performed 90 min after addition of quercetin. (A) Quercetin increased the I_{sc}, most of which was sensitive to 100 μM NPPB. (B) Quercetin had no significant effects on G_t. Data are presented as the mean ± SE (N = 5).

Taken together, these observations shown in the present study indicate that the flavone has a more powerful effect than cAMP on the $Na^+/K^+/2Cl^-$ cotransporter. To confirm this point, we applied forskolin to A6 cells that had been treated with quercetin. Forskolin (10 μM) had no significant effects on the I_{sc} in A6 cells treated with quercetin (closed circles in Fig. 6A). On the other hand, the G_t was elevated by application of forskolin (10 μM) even in A6 cells treated with quercetin (closed circles in Fig. 6B). Similar to the case of quercetin, in A6 cells treated with luteolin, kaempherol, or apigenin, forskolin had no significant effects on the NPPB-sensitive I_{sc} (Fig. 7A), but the NPPB-sensitive G_t was elevated by forskolin (Fig. 7B). These observations strongly indicate that the flavone has a more powerful effect than cAMP on the $Na^+/K^+/2Cl^-$ cotransporter.

It is well known that cAMP activates the $Na^+/K^+/2Cl^-$ cotransporter *via* phosphorylation of the cotransporter

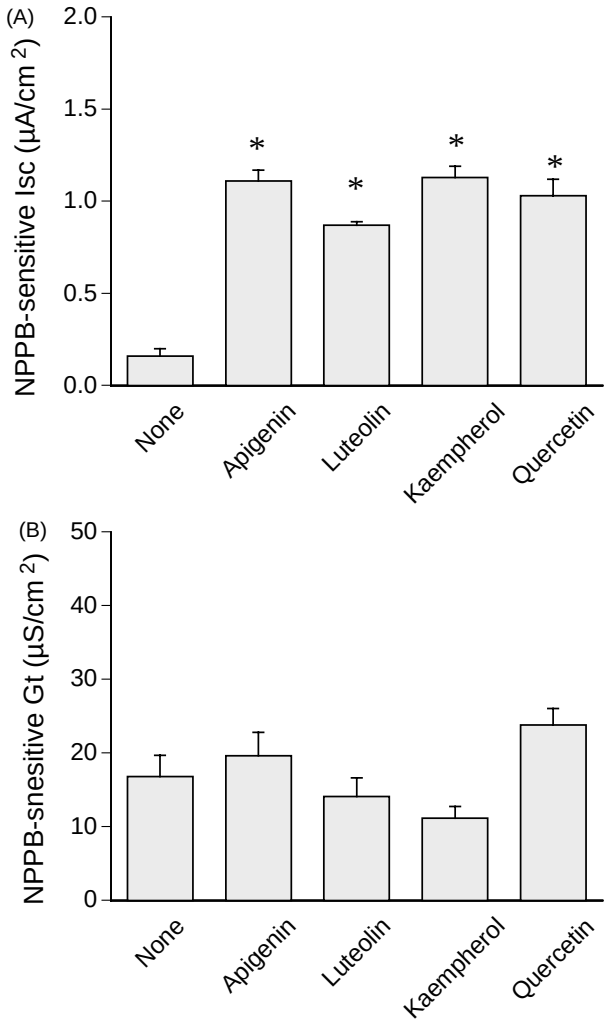

Fig. 3. Effects of 100 μM apigenin, luteolin, kaempherol, and quercetin on I_{sc} (A) and G_t (B) under the basal (unstimulated) condition in monolayered renal epithelial A6 cells. Benzamil, 10 μM, was added to the apical side 10 min before application of flavones. Flavones, 100 μM, were applied to the bilateral sides; in the control case shown as "None" (absence of flavones), DMSO alone was applied as a solvent for flavones. Apical application of 100 μM NPPB was performed 90 min after addition of flavones. Data are presented as the mean ± SE (N = 5–7). *A significant increase compared with "None" (control) ($P < 0.05$).

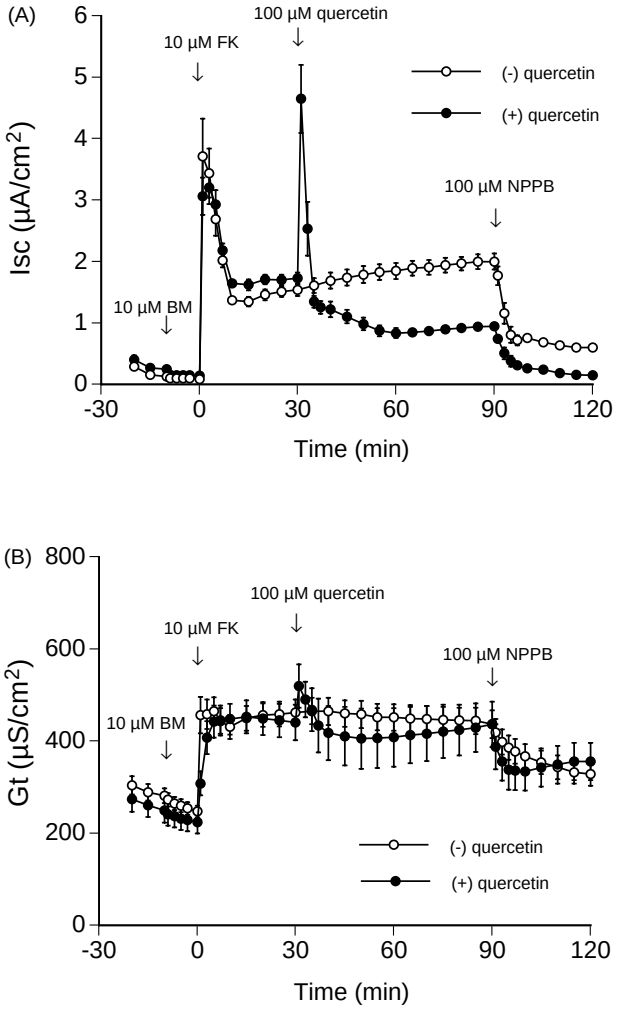

Fig. 4. Time course of the action of 100 μM quercetin on I_{sc} (A) and G_t (B) under the forskolin-stimulated condition in monolayered renal epithelial A6 cells. Benzamil (BM), 10 μM, was added to the apical side 10 min before application of quercetin (open and closed circles). Forskolin, 10 μM, was applied to the basolateral side at 0 min (open and closed circles). Quercetin, 100 μM, was added to the bilateral sides 30 min after application of forskolin (closed circles); in the control case (open circles; absence of quercetin), DMSO alone was applied as a solvent for quercetin. Apical application of 100 μM NPPB was performed 90 min after addition of forskolin (open and closed circles). (A) Quercetin caused an increase in I_{sc} followed by a decrease in I_{sc} (closed circles). NPPB of 100 μM diminished the I_{sc} (open and closed circles). (B) Quercetin had no significant effects on G_t, although quercetin showed a tendency to cause a transient increase in G_t (closed circles). The G_t was decreased by application of 100 μM NPPB. Data are presented as the mean ± SE (N = 5).

protein [16,17]. On the other hand, quercetin and apigenin are known as nonspecific kinase inhibitors and could inhibit the protein kinase A (PKA) at the concentration used in the present study. Therefore, we studied whether the flavone diminishes the cAMP-stimulated Cl^- secretion by inhibiting PKA. We applied KT5720 and PKI, PKA inhibitors, to A6 cells. PKI (2 μM; IC_{50} = 36 nM) did not significantly diminish the forskolin-stimulated I_{sc} (closed circles in Fig. 8A) or the NPPB-sensitive I_{sc} (Fig. 8B). KT5720 (2 μM; IC_{50} = 56 nM) also had no significant effects on the forskolin-stimulated NPPB-sensitive I_{sc} (Fig. 8B). On the other hand, pretreatment with 2 μM KT5720 or 2 μM PKI for 2 hr partially decreased the forskolin-stimulated I_{sc} likely to 5 μM H89 [7], suggesting that KT5720 and PKI at the concentration inhibit PKA in A6 cells. These observations indicate that the decrease in the forskolin-stimulated Cl^- secretion by the flavone would not be caused by inhibition of PKA, although some more experimental results are required to completely confirm the conclusion. We demonstrate that the flavone used in the present study keeps the activity of the $Na^+/K^+/2Cl^-$ cotransporter at the identical level irrespective of the cAMP stimulation. This means that; (1) the flavone could act on the $Na^+/K^+/2Cl^-$ cotransporter irrespective of its phosphorylation, and (2) the activity of the flavone-regulated

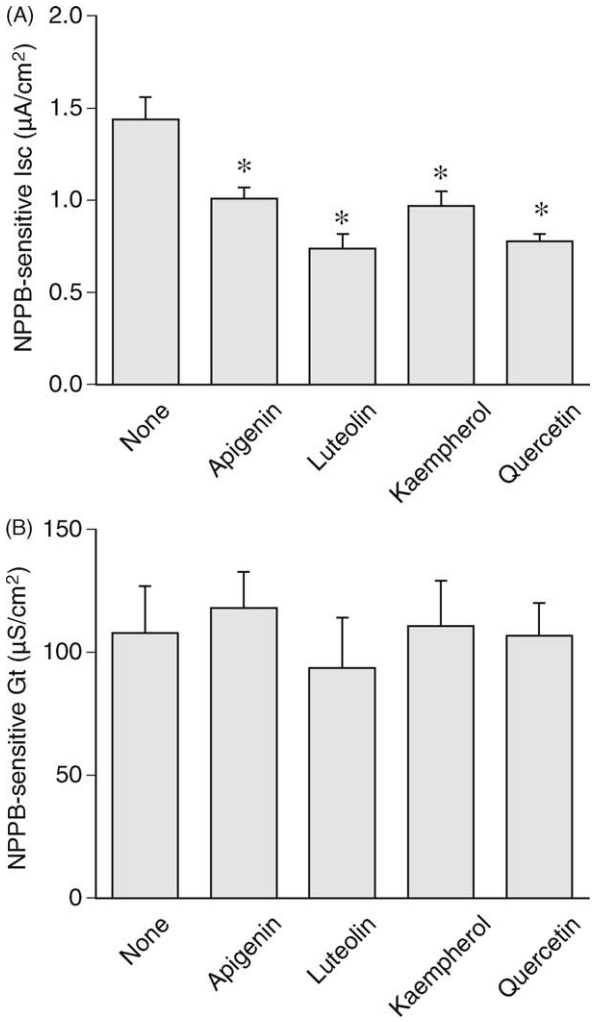

Fig. 5. Effects of 100 μM apigenin, luteolin, kaempherol, and quercetin on I_{sc} (A) and G_t (B) under the forskolin-stimulated condition in monolayered renal epithelial A6 cells. Benzamil, 10 μM, was added to the apical side 10 min before application of flavones. Forskolin, 10 μM, was applied to the basolateral side. Flavones, 100 μM, were added to the bilateral sides 30 min after application of forskolin; in the case shown as "None" (absence of flavones; forskolin alone), DMSO alone was applied as a solvent for flavones. Apical application of 100 μM NPPB was performed 90 min after addition of forskolin. (A) Unlike the basal (unstimulated) condition, flavones significantly diminished the NPPB-sensitive I_{sc}. (B) Flavones had no significant effects on the NPPB-sensitive G_t. Data are presented as the mean ± SE (N = 5–7). *A significant decrease compared with "None" (control) ($P < 0.05$).

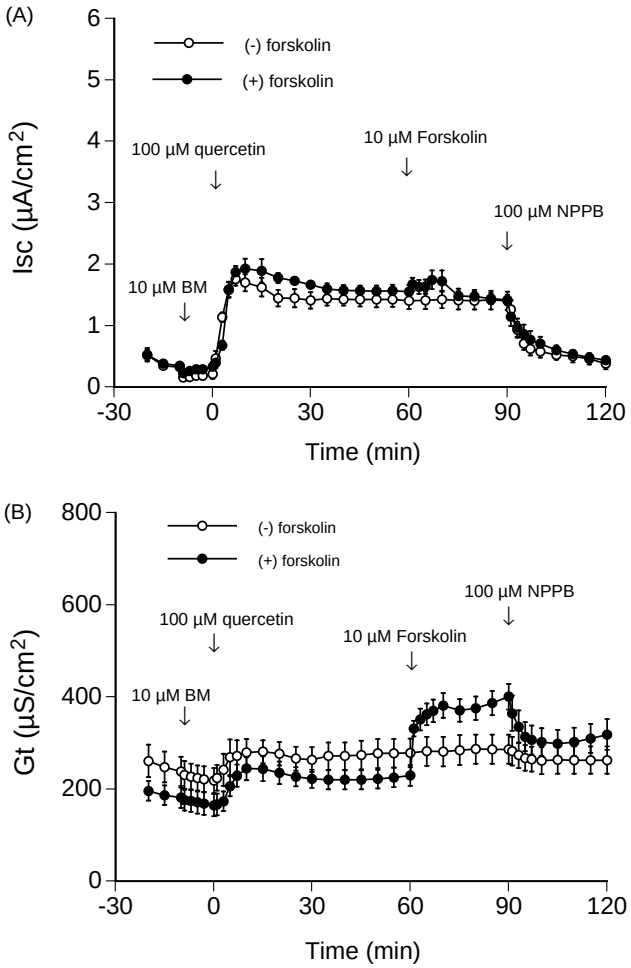

Fig. 6. Time course of the action of 10 μM forskolin on I_{sc} (A) and G_t (B) under the quercetin-stimulated condition in monolayered renal epithelial A6 cells. Benzamil (BM), 10 μM, was added to the apical side 10 min before application of quercetin (open and closed circles). Quercetin, 100 μM, was applied to the bilateral sides at 0 min (open and closed circles). Forskolin, 10 μM, was added to the basolateral side 60 min after application of quercetin (closed circles); in the control case (open circles; absence of forskolin), DMSO alone was added as a solvent for forskolin. Apical application of 100 μM NPPB was performed 90 min after addition of quercetin (open and closed circles). (A) Quercetin increased the I_{sc} (open and closed circles). Forskolin had no significant effects on I_{sc} (closed circles). NPPB diminished the I_{sc} irrespective of forskolin application (open and closed circles). (B) Quercetin had no significant effects on G_t, although quercetin showed a tendency to increase the G_t (open and closed circles). Forskolin significantly elevated the G_t (closed circles). NPPB diminished the G_t (open and closed circles). Data are presented as the mean ± SE (N = 5).

$Na^+/K^+/2Cl^-$ cotransporter is independent of phosphorylation of the cotransporter.

Quercetin caused transient increases in I_{sc} and G_t under the cAMP-stimulated condition, although quercetin had no significant effects on G_t at the steady state. As previously reported [14], cAMP stimulates translocation of CFTR Cl^- channels to the apical membrane in A6 cells. Therefore, flavones might modify the trafficking rate of the channel between the intracellular store site and the apical membrane, increasing transiently the number of the channel staying at the apical membrane. This might cause the transient increase in I_{sc} and G_t.

As it is well known, cAMP activates the $Na^+/K^+/2Cl^-$ cotransporter and also does activate the CFTR Cl^- channel, increasing the Cl^- transport. The present study indicates that, unlike cAMP, apigenin, luteolin, kaempherol, and quercetin activated only the $Na^+/K^+/2Cl^-$ cotransporter without any significant effects on the CFTR Cl^- channel activity. As previously reported [7], we can select the type of flavone which activates the $Na^+/K^+/2Cl^-$ cotransporter and/or the CFTR Cl^- channel. Further, we can control the amount of the Cl^- secretion to the favorite level irrespective

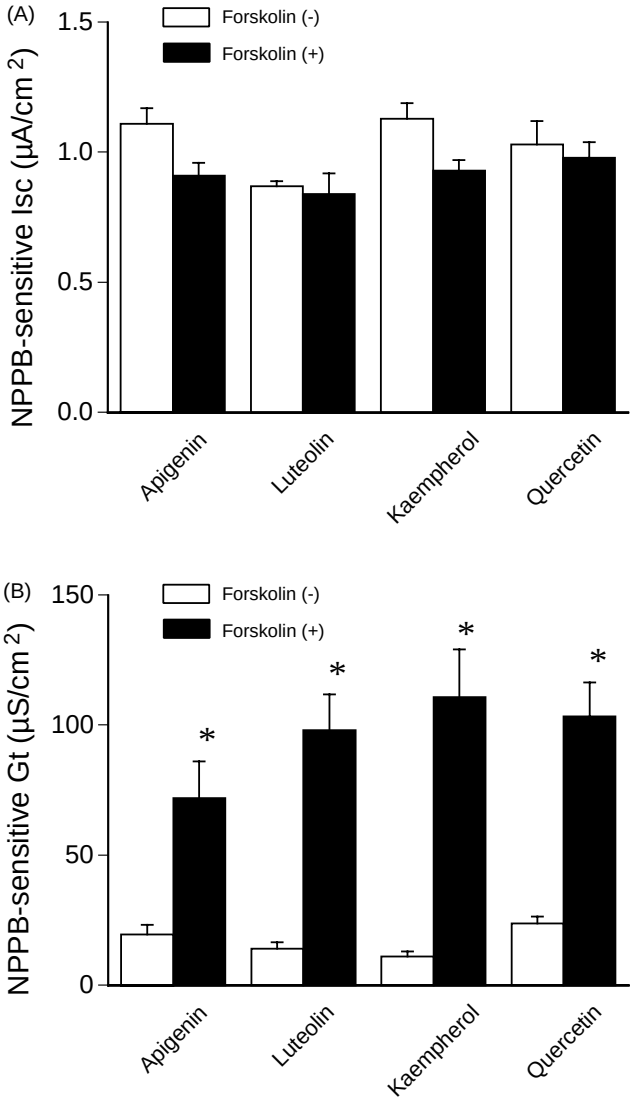

Fig. 7. Effects of 10 μM forskolin on the NPPB-sensitive I_{sc} (A) and G_t (B) under the flavone-stimulated condition in monolayered renal epithelial A6 cells. Benzamil, 10 μM, was added to the apical side 10 min before application of flavones. Flavones, 100 μM, were applied to the bilateral sides. Forskolin, 10 μM, was added to the basolateral side 60 min after application of flavones; in the control case (absence of forskolin), DMSO alone was added as a solvent for forskolin. The apical application of 100 μM NPPB was performed 90 min after addition of flavones. (A) Forskolin had no significant effects on the NPPB-sensitive I_{sc}. (B) Forskolin significantly elevated the NPPB-sensitive G_t. Data are presented as the mean ± SE (N = 5–7). *A significant increase compared with control (absence of forskolin) ($P < 0.05$). Open column, forskolin (−); closed column, forskolin (+).

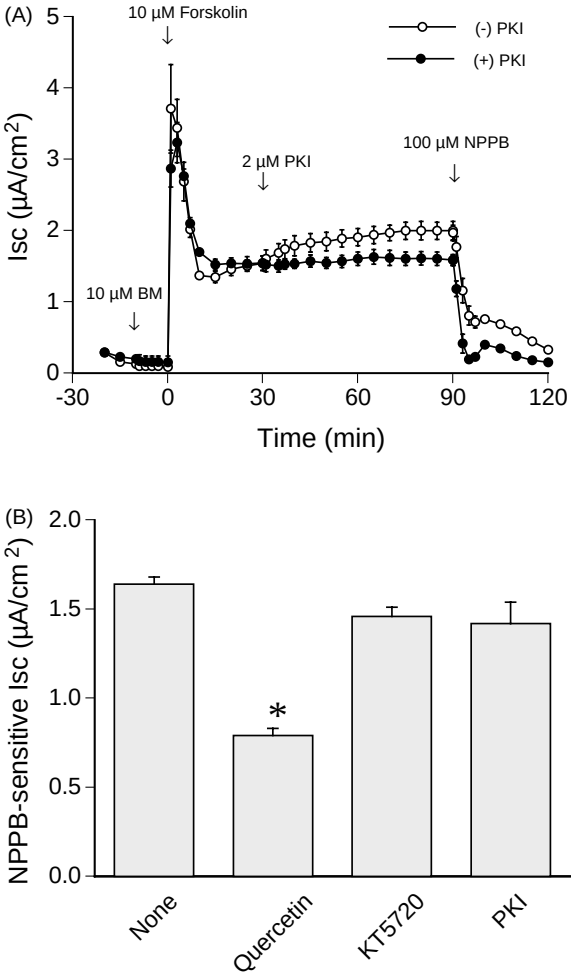

Fig. 8. Effects of PKA inhibitors (KT5720 and PKI) on the forskolin-stimulated I_{sc} in monolayered renal epithelial A6 cells. Benzamil (BM), 10 μM, was added to the apical side 10 min before application of forskolin. Forskolin, 10 μM, was applied to the basolateral side at 0 min. PKI or KT5720, 2 μM, was added to the bilateral sides 30 min after application of forskolin. In the control case (no addition of PKI or KT5720), distilled water alone was applied as a solvent for PKI and KT5720. Apical application of 100 μM NPPB was performed 90 min after addition of forskolin. (A) The time course of PKI effects on the I_{sc} (PKI, closed circles; control (no addition of PKI), open circles). (B) Effects of PKA inhibitors (KT5720 and PKI) and quercetin on the forskolin-stimulated NPPB-sensitive I_{sc}. Quercetin, 100 μM, was applied to the bilateral sides 30 min after addition of forskolin. Data are presented as the mean ± SE (N = 5–7). *A significant decrease compared with "None" (control, absence of quercetin, KT5720 or PKI) ($P < 0.05$).

of the activity of the CFTR Cl^- channel using flavones. For example, in a case that some compounds, such as cholera toxin [18], induce a huge amount of Cl^- secretion [19], we have to regulate the Cl^- secretion to the normal level for keeping homeostasis. However, it is difficult to control the level of Cl^- secretion to the moderate level using Cl^- channel blockers and/or inhibitors of the $Na^+/K^+/2Cl^-$ cotransporter, since the blockers and inhibitors may abolish the Cl^- secretion. On the other hand, even in a case that epithelia could not secrete Cl^- due to lack of cAMP-dependent signals or adenylate cyclase-coupled receptors, the flavone could stimulate Cl^- secretion. In both cases, we may control the Cl^- secretion to the moderate level using the flavone used in the present study. Thus, the flavone would be a useful tool for therapeutics of disorder of Cl^- secretion.

Acknowledgments

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