We found recently that thromboxane A$_2$ is a novel secretagogue for Cl$^-$ secretion in isolated rat colon [1] and that 9,11-epithio-11,12-methano-thromboxane A$_2$ (STA$_2$), a stable thromboxane A$_2$ analogue [2], can mimic the effect of endogenous thromboxane A$_2$ [1]. STA$_2$ was suggested to open an apical cAMP-dependent Cl$^-$ channel in rat colonic crypt cells [1]. On the other hand, we found that STA$_2$ increases intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in colonic crypt cells [3].

Carbachol has been reported to increase [Ca$^{2+}$], via a M$_3$ muscarinic receptor in rat colonic crypt cells [4]. This increase leads to an opening of basolateral Ca$^{2+}$-activated K$^+$ channels and hyperpolarizes the cell, and then maintains an electrochemical driving force for Cl$^-$ secretion at the apical membrane [5]. Carbachol-stimulated, Ca$^{2+}$-dependent K$^+$ channels were reported in rat [6] and human [5, 7] colonic crypt cells.

So far, the effects of thromboxane A$_2$ on the basolateral Ca$^{2+}$-activated K$^+$ channels have not yet been examined. In the present study, using isolated rat colonic mucosa and colonic crypts, we investigated the relationship between STA$_2$-induced increase in [Ca$^{2+}$], and an opening of the Ca$^{2+}$-activated K$^+$ channels.

**Methods**

The following procedures were performed in accordance with the guidelines presented by the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University. The mucosa-submucosa preparation (hereafter, simply described as the mucosa) was obtained from female Wistar rats (Japan SLC, Shizuoka, Japan) weighing 140–200 g. The animals had free access to water and food until the day of the experiment. Animals were sacrificed rapidly by
stunning and cervical dislocation. The serosa and muscularis propria were stripped away by hand to obtain mucosa of the distal part of the colon descendens. Colonic crypts were isolated from the mucosa as previously described [8].

The intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of indo-1-loaded cells located at the middle of isolated colonic crypts was measured with a ACAS 570 interactive confocal laser cytometer (Meridian, Oke-mos, MI, U.S.A.) as described elsewhere [3]. The bathing solution for the measurement of [Ca\(^{2+}\)]\(_i\) contained (in mM): 140 NaCl, 5.4 KCl, 1.25 Ca\(_{2+}\), 1 MgCl\(_2\) and 10 HEPES; the pH was adjusted to 7.4 with NaOH.

Whole-cell current-clamp experiments of the crypt cells were performed using an EPC-9 patch-clamp system (HEKA Elektronik, Schulze GmbH., Lambrecht, Germany) as previously described [1]. The extracellular bathing solution contained (in mM): 140 sodium gluconate, 5.4 potassium gluconate, 1.25 CaSO\(_4\), 1 MgSO\(_4\) and 10 HEPES; the pH was adjusted to 7.4 with NaOH. The intracellular pipette solution contained (in mM): 140 potassium gluconate, 0.041 CaSO\(_4\), 2 MgSO\(_4\), 0.1 EGTA, 10 HEPES and 2 ATP; the pH was adjusted to 7.2 with KOH.

In the Ussing chamber experiments, the isolated mucosa was fixed in a modified chamber, and short-circuit current (I\(_{sc}\)), transepithelial potential difference (P\(_d\)), and tissue conductance (G\(_t\)) were measured with an amplifier (CEZ-9100, Nihon Kohden Co., Tokyo, Japan) as previously described [1]. The direction of I\(_{sc}\) from the mucosal to serosal side was expressed as positive, and the reference of P\(_d\) was taken on the serosal side. The 4.5K\(^+\)-7Cl\(^-\) solution contained (in mM): 107 sodium gluconate, 4.5 KCl, 25 NaHCO\(_3\), 1.8 Na\(_2\)HPO\(_4\), 0.2 NaH\(_2\)PO\(_4\), 1.25 CaCl\(_2\), 4.5 CaSO\(_4\), 1 MgSO\(_4\) and 12 glucose. The 20K\(^+\)-7Cl\(^-\) solution contained (in mM): 91.5 sodium gluconate, 15.5 potassium gluconate, 4.5 KCl, 25 NaHCO\(_3\), 1.8 Na\(_2\)HPO\(_4\), 0.2 NaH\(_2\)PO\(_4\), 1.25 CaCl\(_2\), 4.5 CaSO\(_4\), 1 MgSO\(_4\) and 12 glucose. These solutions were gassed with carbonogen (5% CO\(_2\)-95% O\(_2\)) at a pH of 7.4. The mucosa was pre-treated with ouabain (5 mM at the serosal side; Sigma Chemical Co., St. Louis, MO, U.S.A.) to block the current generated by Na\(^+\),K\(^+\)-ATPase [9]. Nystatin (200 μg/ml at the mucosal side; Wako Pure Chemical Industries, Osaka, Japan) was used to permeabilize the mucosal side of membranes selectively.

To further confirm the lack of effect of STA\(_2\) on basolateral K\(^+\) channels, we performed Ussing chamber experiments. Mucosal administration of nystatin (200 μg/ml), a monovalent ionophore, increased I\(_{sc}\) with a transient peak phase and a subsequent plateau phase in the presence of a serosally directed K\(^+\) gradient (4.5K\(^+\)-7Cl\(^-\) solution at the serosal side and 20K\(^+\)-7Cl\(^-\) solution at the mucosal side). This increase shows that nystatin selectively permeabilized the mucosal membrane as previously described [9]. Nystatin increased I\(_{sc}\) from 18.6±1.4 to 61.3±6.4 μA/cm\(^2\) at the peak phase (n=8, p<0.01; measured at 8.2±1.7 min after the addition of nystatin), and to 35.3±2.4 μA/cm\(^2\) at the plateau phase (n=8, p<0.01; measured at 19.8±2.2 min after the addition of nystatin). Under the present experimental conditions, the contribution of Cl\(^-\) current to I\(_{sc}\) was negligibly small because the solutions contained only 7 mM Cl\(^-\) [1]. When the plateau phase was observed, STA\(_2\) (0.1 μM at the serosal side) or carbachol (10 μM at the serosal side) was pre-treated with ouabain (5 mM at the serosal side) or carbachol (10 μM at the serosal side; Sigma Chemical Co., St. Louis, MO, U.S.A.) to block the current generated by Na\(^+\),K\(^+\)-ATPase [9].

Results and Discussion

First, we tested the effects of STA\(_2\), a stable thromboxane A\(_2\) analogue, and carbachol on [Ca\(^{2+}\)]\(_i\) in the cells located at the middle of isolated colonic crypts. Under the same experimental conditions as here, we found previously that STA\(_2\)- and carbachol-induced increases in [Ca\(^{2+}\)]\(_i\) showed a transient peak phase at 5–10 s after addition of the stimulant [3]. This phase was due to the release of Ca\(^{2+}\) from intracellular stores [3,4]. In the present study, therefore, the [Ca\(^{2+}\)]\(_i\) of the crypt cells was measured 7 s after the addition of STA\(_2\) (0.1 μM) or carbachol (10 μM) in order to estimate the amplitude of the transient phase (Fig. 1A, B). We selected these concentrations of STA\(_2\) and carbachol because the amplitude of the transient phase elicited by STA\(_2\) was not significantly different from that elicited by carbachol (Fig. 1C). We used 0.1 μM STA\(_2\) and 10 μM carbachol in the following experiments.

Next, we examined whether STA\(_2\) hyperpolarizes the cells located at the middle of crypts under whole-cell current-clamp recording with Cl\(^-\)-free extracellular and intracellular solutions. STA\(_2\) (0.1 μM) did not affect the cell membrane potential (Fig. 2). On the other hand, carbachol (10 μM) hyperpolarized the cells as previously described [10]. These results suggest that STA\(_2\) does not open the basolateral Ca\(^{2+}\)-activated K\(^+\) channels in rat colonic crypt cells but carbachol does.


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side) was added. STA2 did not significantly change $I_{sc}$ (Fig. 3A, B), $I_d$ (Fig. 3C) or $G_i$ (Fig. 3D), while carbachol significantly increased $I_{sc}$, $I_d$ and $G_i$ (Fig. 3). When the $K^+$ gradient across the serosal membrane was absent ($4.5K^+-7Cl^-$ solution at both serosal and mucosal sides), the effect of carbachol on $I_{sc}$ was very small (Fig. 4). These results suggest that carbachol, but not STA2, increases the $K^+$ current via the opening of $Ca^{2+}$-activated $K^+$ channels in the basolateral membrane.

We found previously that STA2 causes $Cl^-$ secretion in isolated rat colonic mucosa in the presence of a normal concentration of $Cl^-$ in the solutions, and that STA2 depolarized the cell in isolated rat colonic crypts when normal $Cl^-$-containing solutions were used [1]. These results indicate that STA2 opens the apical $cAMP$-dependent $Cl^-$ channels that are also activated by forskolin and vasoactive intestinal peptide [11, 12]. On the other hand, we found that STA2 increases $[Ca^{2+}]_{i}$ in rat colonic crypt cells [3], and confirmed this effect again in the present study (Fig. 1). In fact, the cloned TXA2 receptor expressed in cultured cells was found to link with both the $cAMP$- and $Ca^{2+}$-signaling pathways [13]. In the present experiment, however, STA2 did not open the basolateral $Ca^{2+}$-activated $K^+$ channels, while carbachol did (Figs. 2–4). In contrast to STA2, Strabel and Diener [14] reported that carbachol does not directly activate apical $Cl^-$ channels, but it opens basolateral $Ca^{2+}$-activated $K^+$ channels. Opening of the $K^+$ channels by carbachol leads to hyperpolarization of the crypt cells, and this stimulates $Cl^-$ secretion via elevating a favorable electrochemical driving force for $Cl^-$ exit at the apical membrane [5,14].

Why STA2 does not activate the $Ca^{2+}$-activated $K^+$ channels in spite of its ability to increase $[Ca^{2+}]_{i}$? Two possible reasons are considered. First, a group(s) of

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**Fig. 1. Effects of STA2 and carbachol on the $[Ca^{2+}]_{i}$ of single cells in isolated rat colonic crypts.**

A, B: $[Ca^{2+}]_{i}$ of the cells located at the middle of crypts was measured just before (0 s) and 7 and 60 s after the addition of 0.1 $\mu$M STA2 (A) or 10 $\mu$M carbachol (B). $n=10$. C: The net increase in $[Ca^{2+}]_{i}$ at the transient peak phase was measured 7 s after the addition of 0.1 $\mu$M STA2 (open column) or 10 $\mu$M carbachol (closed column). $n=10$. NS, not significantly different ($p>0.05$).

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**Fig. 2. Effects of STA2 and carbachol on the membrane potential of single cells in isolated rat colonic crypts.**

A: A typical trace of the membrane potential ($V_m$) of the cells located at the middle of crypts. The $V_m$ was recorded at the zero-current clamp mode. The extracellular bathing solution containing STA2 (0.1 $\mu$M) or carbachol (10 $\mu$M) was perfused as indicated. B: Maximal net changes of the $V_m$ ($\Delta V_m$) obtained 60–90 s after the addition of STA2 (open column) or carbachol (closed column) were measured. $n=3$. $* p<0.05$. 

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intracellular Ca$^{2+}$ stores sensitive to the STA2-elicited pathway may be different from those sensitive to carbachol-elicited pathway. The location of STA2-sensitive Ca$^{2+}$ stores may not be sufficiently close to the sites essential for activation of the K$^+$ channels. Second, an opening of the Ca$^{2+}$-activated K$^+$ channels may be blocked by elevation of the intracellular cAMP level induced by STA2. Bleich et al. [6] reported that forskolin inhibits the Ca$^{2+}$-dependent K$^+$ channels activated by carbachol in rat colonic crypt cells. Apparently, further studies based on these points are necessary to clarify the physiological property of the STA2-induced increase in [Ca$^{2+}$].

We conclude that opening of the basolateral Ca$^{2+}$-activated K$^+$ channels is not involved in the mechanism of STA2-induced Cl$^-$ secretion in the rat colonic mucosa.

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