Inhibitory effects of SKF96365 on the activities of K⁺ channels in mouse small intestinal smooth muscle cells

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ABSTRACT.   In order to investigate the effects of SKF96365 (SKF), which is a non-selective cationic channel blocker, on K⁺ channel currents, we recorded currents through ATP sensitive K⁺ (I_{KATP}), voltage-gated K⁺ (I_{Kv}) and Ca²⁺ activated K⁺ channels (I_{BK}) in the absence and presence of SKF in single small intestinal myocytes of mice with patch-clamp techniques. SKF (10 μM) reversibly abolished I_{KATP} that was induced by cromakalim (10 μM), which is a selective ATP sensitive K⁺ channel opener. These inhibitory effects were induced in a concentration-dependent and voltage-independent manner. The 50% inhibitory concentration (IC_{50}) was 0.85 μM, which was obviously lower than that reported for the muscarinic cationic current. In addition, SKF (1 μM ≈ the IC_{50} value in I_{KATP} suppression) reversibly inhibited the I_{Kv} that was induced by repetitive depolarizing pulses from −80 to 20 mV. However, the extent of the inhibitory effects was only ~30%. In contrast, SKF (1 μM) had no significant effects on spontaneous transient I_{BK} and caffeine-induced I_{BK}. These results indicated that SKF inhibited ATP sensitive K⁺ channels and voltage-gated K⁺ channels, with the ATP sensitive K⁺ channels being more sensitive than the voltage-gated K⁺ channels. These inhibitory effects on K⁺ channels should be considered when SKF is used as a cationic channel blocker.
KEY WORDS: ATP sensitive K⁺ channel, Ca²⁺ activated K⁺ channel, intestinal smooth muscle, SKF96365, voltage-gated K⁺ channel

In gastrointestinal smooth muscles, the stimulation of muscarinic receptors by cholinergic agonists, including acetylcholine, increases the intracellular concentration of Ca²⁺ ([Ca²⁺]$_i$), which results in a contractile response [45]. The increase in [Ca²⁺]$_i$ is induced by the Ca²⁺ release from internal stores and Ca²⁺ entry into the cell through L-type voltage-gated Ca²⁺ channels (Ca$_V$1.2). The activation of Ca$_V$1.2 can be achieved by depolarization due to muscarinic stimulation of non-selective cationic channels [18, 23, 50]. Cationic channels are thought to consist of transient potential canonical channels 4 and 6 (TRPC4 and 6) [43], which are also permeable to Ca²⁺ and involved in Ca²⁺ entry [47]. In addition, muscarinic receptors directly or indirectly regulate the activities of K⁺ channels that are expressed in gastrointestinal smooth muscle cells, including ATP sensitive K⁺ (K_{ATP}), voltage-gated K⁺ (K_V) and large conductance Ca²⁺ activated K⁺ (BK) channels, through which the electrical activities of the muscles are modulated [5, 6, 12, 14, 20].

SKF96365 (SKF) is an imidazole compound that inhibits receptor-mediated Ca²⁺ entry [27]. SKF has been reported to inhibit the opening of TRPC channels in guinea-pig [52] and mouse ileal cells [9]. Although other inhibitors of TRPC channels, such as quinine, quinidine, La³⁺ and flufenamic acid, are well known [9, 15, 24], these chemicals are not specific for the TRPC channels. Therefore, in investigations of the physiological and pharmacological properties of muscarinic receptor-gated TRPC channels, SKF has been widely used as a channel inhibitor in gastrointestinal smooth muscle cells [9, 26, 52]. However, it has been reported that application of SKF evoked membrane depolarization in gastrointestinal smooth muscles [15, 51], indicating additional effects of SKF on ion channels other than TRPC channels. The depolarizing action can make it more difficult to analyze the physiological roles of TRPC channels. Schwarz *et al.* [36] have suggested that SKF blocks inwardly and outwardly rectifying K⁺ currents in endothelial cells (IC_{50}; ≥40 μM). Thus, it is possible that SKF can block K⁺ channels that are expressed in gastrointestinal smooth muscles together with TRPC channels, thus resulting in membrane depolarization. However, the effects of SKF on the activities of the K⁺ channels that are expressed in gastrointestinal smooth muscle cells remain to be elucidated. In the present study, we investigated the effects of SKF on the currents through the K_{ATP} (I_{KATP}), K_V (I_{Kv}) and BK (I_{BK}) channels in longitudinal smooth muscle cells that were isolated from mouse small intestine, which have often been used to investigate the activation mechanisms of TRPC channels.

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MATERIALS AND METHODS

The nomenclature of the ion channels was in accordance with Alexander *et al.* [2]. All of the animal care and experimental procedures described below complied with the guidelines of the local animal ethics committee of the Faculty of Life Sciences, Kyoto Sangyo University.

Cell preparation: Mice (hybrid of 129S4 or 129S6 and CF1) of either sex who were above 2-month-old and who weighed (mean ± standard deviation) 25.1 ± 3.0 g were sacrificed by cervical dislocation. A 15-cm-long gut segment was then removed from the region over the jejunum and ileum, except for the 2.0-cm terminal portion, and placed in physiological salt solution (PSS; for composition, see below). The gut segment was cut into 1.0- to 1.5-cm pieces, and the longitudinal muscle layer was carefully peeled off from each of the pieces. Single smooth muscle cells were isolated enzymatically from the longitudinal muscle layers as described previously [40]. The cells were suspended in PSS containing 0.5 mM $CaCl_2$. A small aliquot was placed on coverglass and stored at 4°C for 2–7 hr until its use on the same day.

Whole-cell current recording: Whole-cell membrane current recordings were performed at room temperature (21–25°C) with standard patch-clamp techniques [11] with 4–7 MΩ patch pipettes. The current signals were amplified with a current amplifier (CEZ-2300 or 5100, Nihon Kohden Corp., Tokyo, Japan) filtered at 1 KHz and captured at a sampling rate of 4 KHz with an analog-digital converter (Digidata 1440A, Molecular Devices, LLC, Sunnyvale, CA, U.S.A.) that was interfaced with a computer (Think Centre A58 Small, Lenovo, Morrisville, NC, U.S.A.) that was running the pCLAMP program (version 10, Molecular Devices, LLC).

For the I_{KATP} recordings, the cells were bathed in a 60-mM K^+ solution and intracellularly dialyzed with a K^+-rich high BAPTA pipette solution (for compositions, see below) at a holding potential of −80 mV in order to increase the driving force of K^+ and minimize the activities of the K_V and BK channels [12, 20, 31]. Under these conditions, the K^+ equilibrium potential (E_K) was estimated at −21 mV, and the K^+ currents were inwardly activated. I_{Kv} was recorded in cells that was bathed in Ca^{2+}-free PSS (for composition, see below) and dialyzed with the K^+-rich high-BAPTA pipette solution in order to minimize the currents through the BK and $Ca_V1.2$ channels. The whole-cell voltage clamp mode was held at −80 mV, and 2 sec step pulses to 20 mV that were generated by the pCLAMP program were repeatedly applied to the cell every 20 sec to elicit I_{Kv}. When currents were recorded through I_{BK}, a K^+-rich low EGTA pipette solution (for composition, see below) was used. The cells were bathed in PSS and held at a holding potential of 0 mV. Recordings of the muscarinic cationic channel currents (mI_{TRPC}) were made from cells that were bathed in Cs^+-rich external solution and intracellularly dialyzed with Cs^+-rich pipette solution (for compositions, see below) at a holding potential of −60 mV in order to eliminate any K^+ currents.

Solutions: The PSS that was used in the experiments had the following composition (mM): NaCl, 126; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1.2; glucose, 14; and HEPES, 10.5; and the pH was adjusted to 7.2 with NaOH. The 60-mM K^+ solution was made by changing the concentrations of NaCl and KCl in the PSS to 72 mM and 60 mM, respectively. If necessary, the KCl in the 60-mM K^+ solution was replaced with CsCl. The Ca^{2+}-free PSS was prepared by omitting $CaCl_2$ from the PSS and adding 0.5 mM EGTA. The Cs^+-rich external solution had the following composition (mM): CsCl, 120; glucose, 12; and HEPES, 10; and the pH was adjusted to pH 7.4 with CsOH. The compositions of each patch-pipette solution (mM) were as follows. The K^+-rich low-EGTA pipette solution contained KCl, 134; Na_2GTP, 1.0; $MgCl_2$, 1.2; MgATP, 1.0; glucose, 14; EGTA, 0.05; and HEPES, 10.5; and the pH was adjusted to 7.2 with KOH. For the K^+-rich high-BAPTA pipette solution, $CaCl_2$ was removed, and 20 mM BAPTA was added. In this case, in order to adjust the total concentration of K^+ to 134 mM and the pH to 7.2, the concentration of KCl was reduced to ~67 mM, and KOH was added. The Cs^+-rich pipette solution contained CsCl, 80; Na_2GTP, 1.0; creatine, 5; MgATP, 1.0; glucose, 20; HEPES, 10; BAPTA, 10; and $CaCl_2$, 4.6 (calculated free calcium=100 nM), with the pH adjusted to 7.4 with CsOH.

Drugs: The following drugs were used: 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy] ethyl-1H-imidazole hydrochloride (SKF), cromakalim (Tocris Bioscience, Bristol, U.K.), carbamylcholine chloride (CCh), glibenclamide (Sigma-Aldrich Co. LLC, St. Louis, MO, U.S.A.), tetraethylammonium Chloride (TEA; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and caffeine (Wako Pure Chemical Industries Ltd., Osaka, Japan). Cromakalim and glibenclamide were dissolved in dimethyl sulfoxide to form a stock solution and was stored at −20°C. The drug concentrations herein are expressed as the final concentrations that were applied to the cells.

Data analysis: The amplitude of I_{KATP} that was induced by cromakalim was measured, and the difference in the current level after the application of glibenclamide (10 μM) was calculated. In order to determine the current-voltage (I-V) relationships of I_{KATP}, a 350-msec ramp pulse that ranged from −100 mV to 60 mV was applied to the cells before and after SKF application, and the I-V curves were constructed by subtracting the current after the application of glibenclamide from the current in the presence of cromakalim alone or with SKF [31]. The amplitude of I_{Kv} was measured by subtracting the leakage component from the outward current that was induced by the depolarizing pulses [44]. The leakage component was estimated in each experiment as follows. A 10-mV hyperpolarizing pulse was applied to the cells at the holding potential (−40 mV), and the amplitude of the current was considered the leakage current for the 10-mV step. The leakage current that corresponded to the 100-mV depolarizing step pulse was subtracted from each of the outward currents that were evoked by the depolarizing pulse. The amplitudes of the spontaneous transient outward I_{BK} (STOC) and caffeine-induced I_{BK} were calculated as the difference from the holding current. The amplitude of mI_{TRPC} was calculated as the difference from the holding current before the applica-

tion of CCh. The extent of the SKF-induced inhibition of each current was expressed as the percentage reduction from the control current level immediately before its application.

The values are presented herein as mean ± S.E.M. The statistical significance of the difference between two groups was tested with a Student's unpaired or paired t-test. When multiple groups were compared, a one-way analysis of variance (ANOVA) that was followed by a posthoc Bonferroni-type multiple comparison test was used. The differences were considered significant when the P values were less than 0.05. The averaged curves that represented the relationship between the concentration of SKF and its inhibitory effects were constructed by curve fitting with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, U.S.A.), and these provided the SKF concentration that was required to produce half maximal inhibition (IC$_{50}$). The cells that were obtained from male and female mice showed similar amplitudes for I$_{KATP}$, I$_{Kv}$, STOC and mI$_{TRPC}$.

RESULTS

Current through the K$_{ATP}$ channels in mouse small intestinal smooth muscles: The cells were dialyzed with the K$^+$-rich high-BAPTA pipette solution and bathed in the 60-mM K$^+$ external solution at a holding potential of −80 mV. Under these conditions, application of the K$_{ATP}$ channel opener cromakalim (10 μM) induced an inward current, as has been reported by Nuttle & Farley [31]. The current developed progressively with time and reached a plateau about 1–3 min later (Fig. 1A). Further application of the K$_{ATP}$ channel inhibitor, glibenclamide (10 μM), almost completely abolished the current. The mean amplitude of the cromakalim-induced current was −895.2 ± 66.5 pA (n=39). When the extracellular K$^+$ in the bath solution was completely replaced with Cs$^+$, the cromakalim-induced inward current was immediately abolished at −80 mV (n=5; data not shown). The I-V relationships of the cromakalim-induced current were determined by applying a voltage ramp pulse that ranged from −100 mV up to +60 mV over 350 msec (Fig. 1Ba). As shown in Fig 1Bb, the I-V curve was linear across the voltages tested. The polarity of the current was reversed at −18.8 ± 2.0 mV (n=7), which corresponded to the estimated E$_K$ of −21 mV, thus indicating that this current was carried through K$^+$ channels. These results indicated that the cromakalim-induced current was carried through K$_{ATP}$ channels.

SKF reversibly suppressed the K$_{ATP}$ channel current (I$_{KATP}$): After the cromakalim-induced I$_{KATP}$ reached a steady level, SKF (10 μM) was applied in the presence of cromakalim. As shown in Fig. 2A, SKF rapidly decreased the amplitude of I$_{KATP}$ and abolished the current within about 1 min. The time constant (τ) of the suppression (7.3 ± 1.6 sec, n=7) was significantly shorter than the value (35.6 ± 5.8 sec, n=10) in the glibenclamide-induced abolition of I$_{KATP}$. The removal of SKF from the bath solution allowed the recovery of I$_{KATP}$ with a slower time course. Figure 2Ba shows a typical example of I$_{KATP}$ suppression that was induced by cumulative applications of SKF with a series of ascending concentrations (0.03–10 μM). The inhibitory ef-
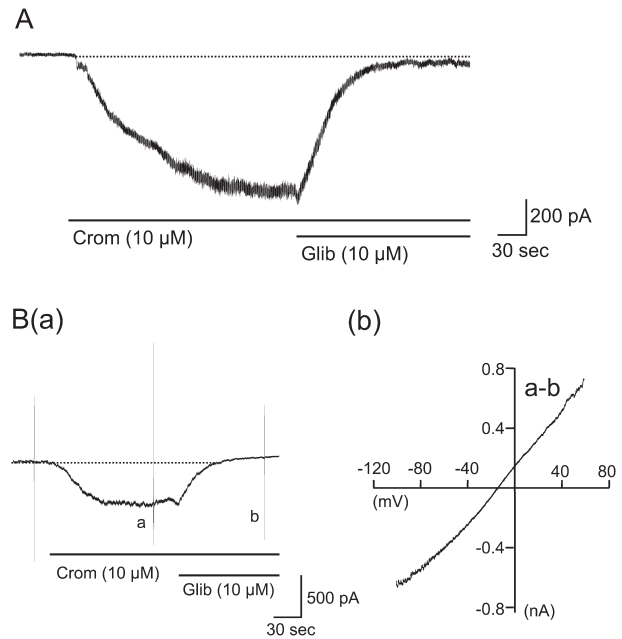

Fig. 1. Characterization of cromakalim-induced K$_{ATP}$ channel currents (I$_{KATP}$) in single longitudinal smooth muscle cells isolated from mouse small intestine. Cells were dialyzed with the K$^+$-rich high-BAPTA pipette solution and bathed in the 60-mM K$^+$ external solution at a holding potential of −80 mV. Cromakalim (10 μM) was extracellularly applied to the cells. A: a typical example of the cromakalim-induced sustained inward I$_{KATP}$. B shows the current-voltage (I-V) relationship of cromakalim-induced I$_{KATP}$. (a): a typical current trace, where a voltage ramp pulse from −100 mV up to +60 mV over 350 msec was applied in the presence of cromakalim alone (current a) and in combination with glibenclamide (10 μM) (current b). (b): the I-V curve constructed by subtraction of the current b from the current a.

fects increased with increasing SKF concentrations, and the maximal inhibition was attained at 10 μM. The mean IC$_{50}$ value that was estimated by the curve fitting of the data from each myocyte was 0.85 ± 0.12 μM (n=6) (Fig. 2Bb).

In order to investigate the voltage dependency of the SKF-induced I$_{KATP}$ suppression, the ramp pulses were applied in the absence and presence of SKF (1 μM), and the I-V curves were obtained, as shown in Fig. 2Ca. The I-V curves of I$_{KATP}$ remained linear, and the reversal potential was almost unchanged after the application of SKF (cromakalim alone, −18.5 ± 0.82 mV and cromakalim with SKF, −16.4 ± 0.97 mV; n=6) (Fig. 2Cb). I$_{KATP}$ suppression at −80, −40 and 40 mV was comparable (Fig. 2Cc). These results indicated that the SKF-induced I$_{KATP}$ suppression was voltage-independent.

Comparison of the SKF-induced mI$_{TRPC}$ suppression with the I$_{KATP}$ suppression: The cells that were bathed in the Cs$^+$-rich external solution were clamped at a voltage of −60 mV with patch pipettes that were filled with the Cs$^+$-rich pipette solution in order to block any K$^+$ currents ([Ca^{2+}]$_i$ buffered to 100 nM). Under these conditions, the application of CCh (100 μM) induced an inward mI$_{TRPC}$, as previously reported
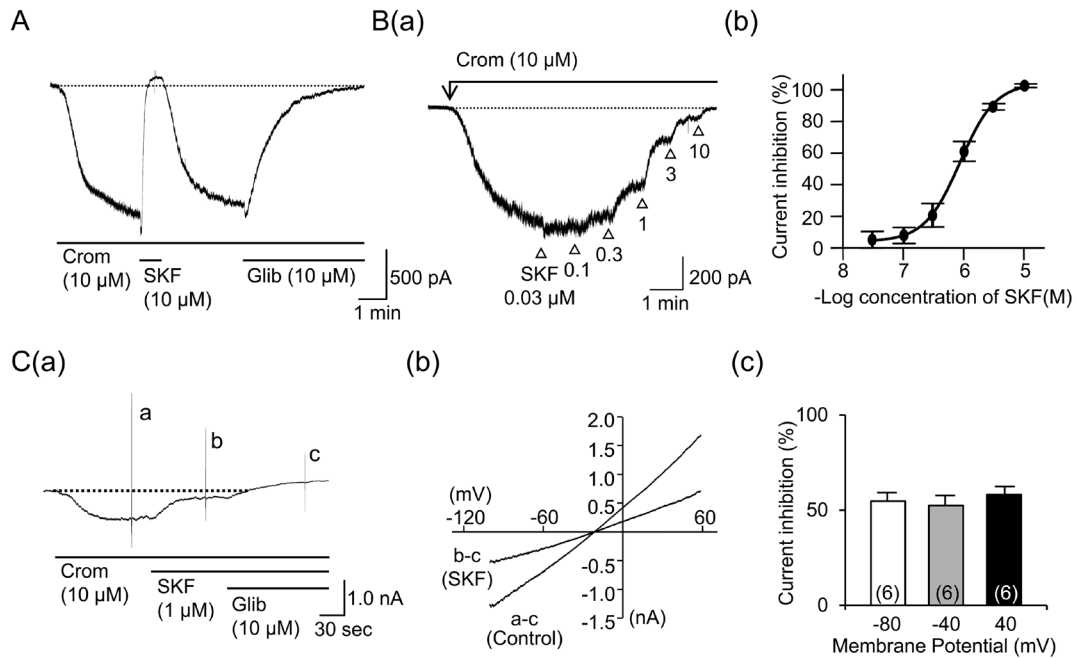
Fig. 2. Effects of SKF96365 (SKF) on cromakalim-induced I_{KATP}. I_{KATP} was induced by cromakalim (10 μM) in the cells under the same condition as those mentioned in Fig. 1. A shows a typical current trace when SKF (10 μM) was extracellularly applied to the cell in the presence of cromakalim (10 μM). B shows a relationship between concentration of SKF and its inhibitory effect on I_{KATP}. (a): a typical example of the I_{KATP} suppression induced by cumulative application of SKF (0.03-10 μM). (b): the averaged concentration-inhibition curve of I_{KATP}. Each point indicates the mean ± S.E.M. of measurements in 6 cells. C shows a voltage sensitivity of the SKF-induced I_{KATP} suppression. (a): a typical example of I_{KATP} suppression induced by SKF (1 μM), where a voltage ramp pulse from −100 mV up to +60 mV over 350 msec was applied in the presence of cromakalim (10 μM) alone (current a), cromakalim + SKF (1 μM) (current b) and those agents together with glibenclamide (10 μM) (current c). (b): the I-V curves constructed by subtraction of the current c from the current a or b. (c): the mean percentage of I_{KATP} suppression at each holding potential. Each column indicates mean + 1 S.E.M. The numbers of cells used are presented in parenthesis.

by Sakamoto *et al.* [34]. SKF (10 μM), which completely abolished I_{KATP}, was applied to the bath solution in the presence of CCh when the mI_{TRPC} reached a steady level. The amplitude of the mI_{TRPC} gradually decreased after the application of SKF and reached a plateau about 1 min later (Fig. 3Aa). However, the mean suppression of mI_{TRPC} was only 55.5 ± 5.6% (n=8) (Fig. 3B). The time constant (τ) of the inhibitory effect was 38.7 ± 8.3 sec (n=7), which was significantly longer than the value in the I_{KATP} suppression. When a higher concentration of SKF (30 μM) was applied, it almost completely abolished the mI_{TRPC} (n=4, Fig. 3Ab and 3B). The abolition had a longer time constant (τ), 12.6 ± 5.3 sec (n=3), than the value in I_{KATP} suppression.

SKF reversibly suppressed I_{Kv}: The cells that were bathed in the Ca^{2+}-free PSS were clamped at a voltage of −80 mV with patch pipettes that were filled with the K^+-rich high-BAPTA pipette solution. Under these conditions, a 2 sec-step pulse to 20 mV was repeatedly applied every 20 sec in order to elicit I_{Kv}. Applications of the depolarizing pulse evoked an initial transient outward current (IK_{Vpeak}) with an additional sustained component ($IK_{Vsustained}$), as was previously described in mouse intestinal smooth muscles [25]. As shown in Fig. 4Aa, the amplitudes of both IK_{Vpeak} and $IK_{Vsustained}$

reduced after the application of SKF (1 μM). The I_{Kv} suppressions developed progressively with time and reached a plateau about 1–2 min later (Fig. 4Ab). The suppression of the IK_{Vpeak} had a longer time constant (τ) (66.8 ± 23.1 sec, n=8) than the $IK_{Vsustained}$ suppression did (21.1 ± 5.4 sec, n=8), although the difference was not statistically significant. The mean suppressions of IK_{Vpeak} and $IK_{Vsustained}$ by SKF (1 μM) were significantly smaller than I_{KATP} suppression (Fig. 4Ac). The removal of SKF allowed for a partial recovery of IK_{Vpeak} and $IK_{Vsustained}$ with a slow time course (Fig. 4Ab). The restored I_{Kv} was strongly inhibited by the application of the K^+ channel blocker TEA (30 mM).

The cumulative application of SKF (0.03–30 μM) at increasing concentrations inhibited both IK_{Vpeak} and $IK_{Vsustained}$ in a concentration-dependent manner. The inhibitory effects reached a maximum at 30 μM (Fig. 4Ba). However, the $IK_{Vsustained}$ suppressions were significantly larger than the IK_{Vpeak} suppressions at 3, 10 and 30 μM (Fig. 4Bb). The mean IC_{50} value that was estimated by the curve fitting of the data from each myocyte was 3.3 ± 0.24 μM in the IK_{Vpeak} (n=5), which was significantly higher than the value in the $IK_{Vsustained}$ (2.0 ± 0.35 μM, n=5). These IC_{50} values were significantly higher than the value in the I_{KATP} suppression.

A(a)

CCh
(100 μM)   SKF (10 μM) |50 pA

              20 sec

(b)

CCh
(100 μM)   SKF (30 μM) |100 pA
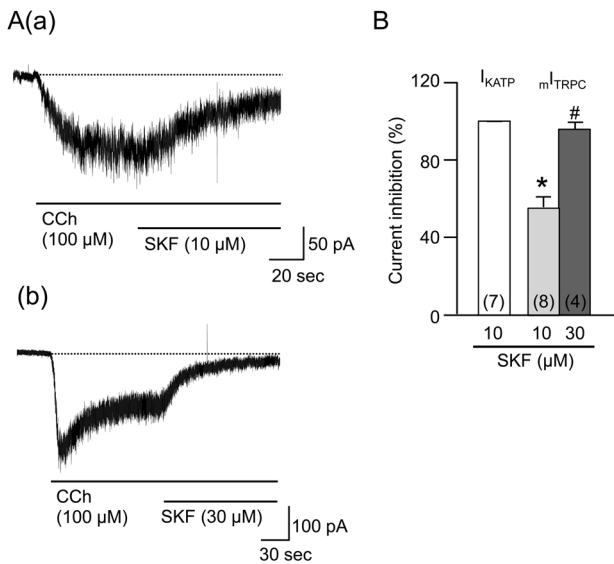
              30 sec

B

Fig. 3.   Effects of SKF96365 (SKF) on carbachol (CCh)-induced mI_{TRPC}. Cells bathed in the Cs^+-rich external solution were held under voltage clamp at −60 mV using patch pipettes filled with the Cs^+-rich pipette solution to block any K^+ currents. Aa and Ab show a typical current trace when SKF (10 and 30 μM) was extracellularly applied to the cell in the presence of CCh (100 μM), respectively. B shows the mean percentages of I_{KATP} and mI_{TRPC} suppressions. Each column indicates mean + 1 S.E.M. The numbers of cells used are presented in parenthesis. * in (B) represents significantly smaller inhibition of mI_{TRPC} induced by SKF (10 μM) relative to the I_{KATP} one ($P<0.05$). # in (B) represents significantly greater inhibition of mI_{TRPC} induced by SKF (30 μM) relative to the corresponding one induced by SKF (10 μM) ($P<0.05$).

I_{BK} was less sensitive to SKF: It is well known that Ca^{2+} releases from Ca^{2+} stores can induce I_{BK} responses [34, 40]. Thus, under the appropriate conditions (see methods), I_{BK} that was evoked by the potent Ca^{2+} releaser caffeine was recorded in the presence of SKF. As shown in Fig. 5A, the STOCs that were evoked by Ca^{2+} sparks [7] occurred with various frequencies and amplitudes, as previously reported by Sakamoto *et al.* [33]. When $[Ca^{2+}]_i$ was strongly chelated, the STOCs were not observed at all (see Figs. 1 and 2). The K^+ channel blocker TEA (5 mM) almost abolished the STOCs. In the presence of TEA, the application of CCh and the subsequent application of caffeine failed to evoke the I_{BK} (data not shown, n=3). These findings strongly indicated that the STOCs and caffeine-induced I_{BK} were conducted through BK channels. SKF (1 μM) had no obvious effects on the STOCs (Fig. 5A and 5B). The change rate of the STOC amplitude was similar to the time-dependent rundown that was observed in the cells without SKF (Fig. 5C). Caffeine (10 mM) consistently evoked a brief I_{BK}, even in the presence of SKF (1 μM) (Fig. 5B). The mean amplitude of the I_{BK} that was induced by caffeine did not differ significantly from the value without SKF (Fig. 5D). The higher concentration of SKF (10 μM) reduced the STOCs, but not the caffeine-induced I_{BK} (Fig. 5C and 5D).

DISCUSSION

Activation of the muscarinic receptors induces the opening of cationic channels comprising TRPC4 and 6 [43]. SKF has been used as an inhibitor of the TRPC channels [9, 26, 52]. However, it has been reported to inhibit other types of ion channels, including Ca_V1, Ca_V2, Ca_V3 [27, 37], Na_V1 [13] and inwardly or outwardly rectifying K^+ channels [36]. In this study, we investigated the effects of SKF on the activities of the K_{ATP}, K_V and BK channels that are expressed in gastrointestinal smooth muscle cells.

K_{ATP} channels have been suggested to regulate electrical excitability in gastrointestinal smooth muscles [19–21]. Cromakalim induced a sustained inward current that was abolished by glibenclamide in small intestinal smooth muscles of the mouse, as has been reported by Nuttle & Farley [31]. The I-V curve of the cromakalim-induced current was almost linear, which was consistent with the results obtained for rabbit esophageal [12] and swine tracheal [31] smooth muscle cells. The reversal potential of the current was similar to the estimated E_K value, and the replacement of extracellular K^+ with Cs^+ abolished the current, which indicated that the charge carrier of this current was K^+. These results indicated that the cromakalim-induced current was carried through the K_{ATP} channels. The extracellular application of SKF reversibly inhibited the cromakalim-induced I_{KATP} in a concentration-dependent manner. The I_{KATP} was abolished at a SKF concentration of 10 μM. Although SKF (10 μM) also inhibited the CCh-induced mI_{TRPC}, the mean percentage of suppression was only about 50% in the present study, which was consistent with the IC_{50} (6.5–14.5 μM) that has been reported for SKF-induced mI_{TRPC} suppression [52]. The mean IC_{50} value (0.85 μM) for the SKF-induced I_{KATP} suppression in the present study was obviously lower than the reported IC_{50} value for the mI_{TRPC} suppression, which suggested that SKF suppressed the K_{ATP} channels with a higher affinity than that required for inhibition of TRPC channels.

The K_{ATP} channels are composed of Kir6 and SUR subunits. Two Kir6 genes (Kir6.1 and Kir6.2) and two SUR genes (SUR1 and SUR2) have been identified [1]. The pharmacological properties of the K_{ATP} channels, including the sensitivity to its channel opener and blocker, depend on the channel subunits [16, 17, 49]. K_{ATP} channels, which have been reported in pancreatic β cells, cardiac muscles and vascular smooth muscles, consist of the Kir6.2/SUR1 [10, 28], Kir6.2/SUR2A [4] and Kir6.1/SUR2B [29, 39, 49] subunits, respectively. Nakayama *et al.* [30] have reported the expression of Kir6.1 and SUR2B in mouse ileal smooth muscles. Quayle *et al.* [32] have reported that the IC_{50} values of glibenclamide, tolbutamide, which is a selective K_{ATP} channel inhibitor, and TEA, which is a nonselective K^+ channel blocker, were 101 nM, 351 μM and 6.2 mM, respectively, in the smooth muscles from the rabbit mesenteric artery. Taken together with our results, the order of affinity to the K_{ATP} channels appears to be glibenclamide >SKF >> tolbutamide >> TEA. Future investigations are needed on the sensitivity of SKF to other types of K_{ATP} channels

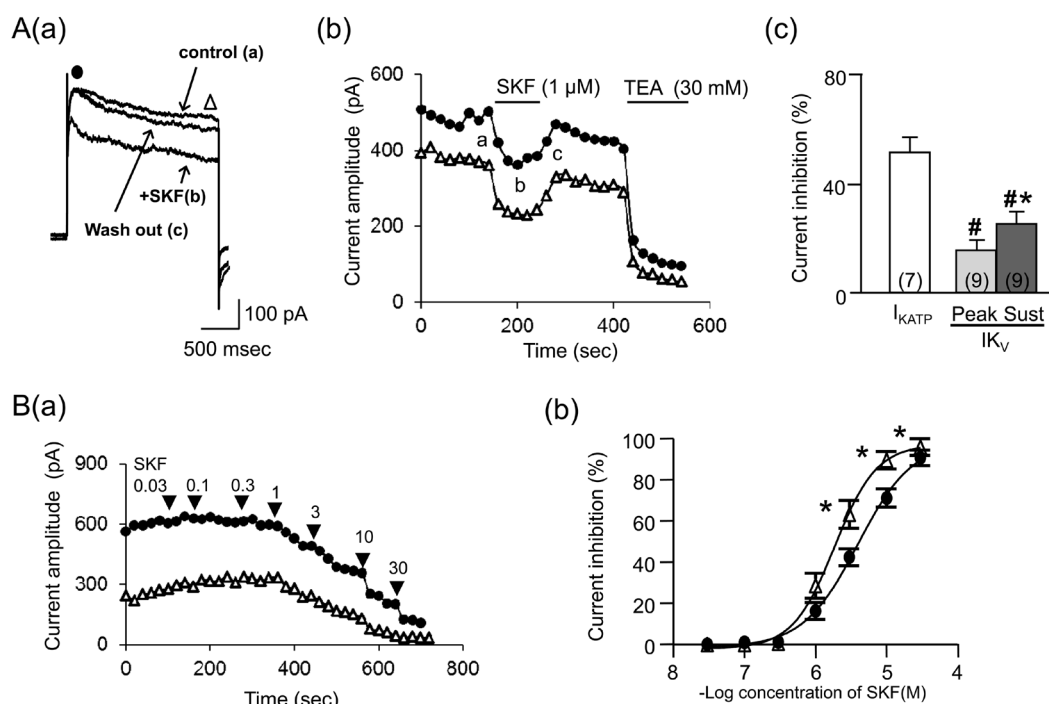Because the I-V curve of the I_{KATP} that was obtained from

Fig. 4.   Effects of SKF on voltage-gated K⁺ channel currents (I_{Kv}). Cells bathed in the Ca^{2+}-free PSS were held under voltage clamp at −80 mV using patch pipettes filled with the K⁺-rich high-BAPTA pipette solution. A 2 sec step pulse to 20 mV was repeatedly applied every 20 sec in order to elicit I_{Kv}. Aa: IK_{Vpeak} (●) and $IK_{Vsustained}$ (Δ) recorded in a cell when SKF (1 μM) was extracellularly applied. Ab: time courses of the change in IK_{Vpeak} (●) and $IK_{Vsustained}$ (Δ) in the cell in a plotted against time (the beginning of depolarizing pulse applications was taken as zero) when SKF (1 μM) was extracellularly applied. Points a–c in the graph correspond with actual I_{Kv} records (a–c) in Aa. Ac shows the mean percentages of I_{KATP} and I_{Kv} suppressions induced by SKF (1 μM). Each column indicates mean + 1 S.E.M. The numbers of cells used are presented in parenthesis. B (a): time courses of the change in IK_{Vpeak} (●) and $IK_{Vsustained}$ (Δ) when SKF (0.03–30 μM) was cumulatively applied. Bb: the averaged concentration-inhibition curves of IK_{Vpeak} (●) and $IK_{Vsustained}$ (Δ). Each point in Bb indicates the mean ± S.E.M. of measurements in 3–5 cells. # in (Ac) represents significantly smaller inhibition of I_{Kv} induced by SKF (1 μM) relative to the I_{KATP} one (*P*<0.05). Asterisks in (Ac) and (Bb) represent significantly greater inhibition of the $IK_{Vsustained}$ relative to the IK_{Vpeak} (*P*<0.05).

the myocytes in the presence of SKF remained linear, the inhibitory effects of SKF on I_{KATP} seemed to be voltage-independent. Glibenclamide inhibits the K_{ATP} by occupying sulfonylurea receptors [8]. The I_{KATP} abolition that was induced by glibenclamide developed more slowly than that induced by SKF, as shown by the time constants of the abolitions. Thus, SKF may inhibit the K_{ATP} in a different manner from that of glibenclamide. The short time course of the SKF-induced I_{KATP} suppression implies the involvement of a simple mechanism, such as direct blockade of the channel pore, which is located in Kir6, as was suggested in caffeine-induced I_{KATP} suppression [41]. If so, the binding site might not be a voltage sensor of the channels, and its binding would be reversible. Future investigations with the single channel patch clamp technique are needed to elucidate the blockade mechanisms of SKF.

Zholos *et al*. [52] have reported that the inhibition of mI_{TRPC} by SKF was greater at positive membrane potentials than that at negative ones, which indicated the voltage-dependency of its inhibitory effects. Thus, the mechanisms of SKF-induced suppression of the mI_{TRPC} may be somewhat

different from those for the I_{KATP}. This idea is supported by the fact that the time constant of the SKF-induced mI_{TRPC} suppression was much longer than the corresponding value of I_{KATP} suppression. The different characteristics of these suppressions can provide useful information when SKF is used as a blocker of TRPC or K_{ATP}.

K_V channels, which are widely expressed in gastrointestinal smooth muscles, are involved in the setting of the resting membrane potential and the determination of amplitude and rhythmicity of action potentials [22, 48]. Application of the depolarizing pulse evoked a biphasic outward current that consisted of IK_{Vpeak} and $IK_{Vsustained}$ and that was strongly inhibited by TEA. SKF (1 μM), which was a concentration that corresponded to the IC_{50} in the I_{KATP} suppression, reversibly inhibited the I_{Kv}. However, the mean percentages of the SKF-induced I_{Kv} suppressions were only ~30%, and the IC_{50} values (>2 μM), which were significantly higher than the value in the I_{KATP} suppression, were similar to the value reported for mI_{TRPC} suppression. A great contribution of the K_V4 and K_V2 family to the IK_{Vpeak} and $IK_{Vsustained}$, respectively, has been suggested [3, 25, 35]. The heterogeneity of
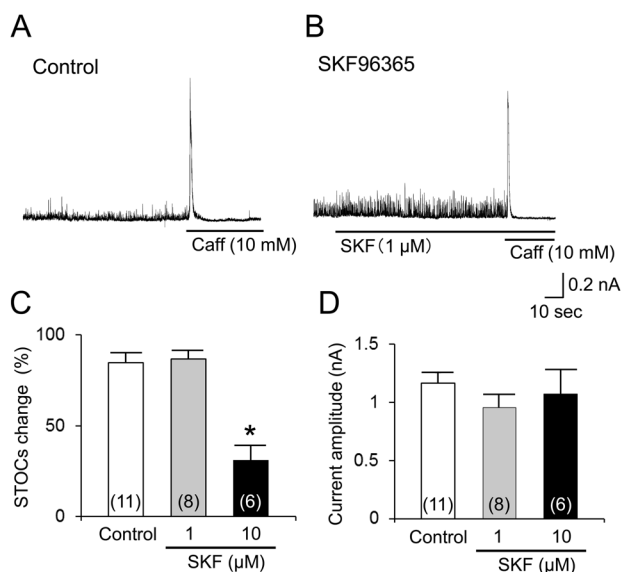
Fig. 5.   Less sensitivity of SKF to Ca^{2+} activated K⁺ channel current (I_{BK}). Cells were bathed in PSS and held at a holding potential of 0 mV using the K⁺-rich low EGTA pipette solution. Spontaneous transient or caffeine (10 mM)-induced Ca^{2+} release events were detected as I_{BK}. A and B: typical current traces in the absence (A) and presence (B) of SKF (1 μM), respectively. C shows the mean change rates of spontaneous transient outward I_{BK} (STOCs) in the presence or absence of SKF. D shows summary of caffeine-induced I_{BK} in the presence or absence of SKF. Each column indicates mean + 1 S.E.M. The numbers of cells used are presented in parentheses. * in (C) represents significantly larger reduction in STOCs in the presence of SKF (10 μM) relative to the time-dependent rundown of STOCs in the absence of SKF ($P<0.05$).

I_{Kv} can make it difficult to determine the sensitivity of the K_V family to SKF due to the lack of commercial blockers with high selectivity. However, the $IK_{Vsustained}$ suppression was greater than the IK_{Vpeak} suppression with a lower IC_{50} value ($IK_{Vsustained}$: 2.2 μM vs. IK_{Vpeak}: 3.3 μM). Furthermore, the time constant of the $IK_{Vsustained}$ suppression was shorter than the value for the IK_{Vpeak} suppression. These findings may reflect decreased sensitivity of the K_V4 family for SKF compared with K_V2. The short time course of the SKF-induced $IK_{Vsustained}$ suppression may imply a direct bind of SKF to the channel, as indicated in TEA or 4-Aminopyridine-induced K_V channel suppressions [38, 42] as well as the I_{KATP} suppression.

In gastrointestinal smooth muscles, BK channels are expressed and supposed to involve in a negative feedback mechanism to prevent cytosolic Ca^{2+} overload induced by the muscarinic Ca^{2+} mobilization [46]. SKF (1 μM) had no significant effects on STOCs and caffeine-induced I_{BK} which reflected the Ca^{2+} release from the internal stores in the myocytes. These results indicated that BK channel is less sensitive to SKF. Taken together, SKF can inhibit K_{ATP}, K_V and BK channels in addition to TRPC channel, and the order of affinity of SKF to those channels appears to be $K_{ATP} > K_V \approx TRPCs > BK$. SKF (3–50 μM) has been reported to evoke

membrane depolarization in gastrointestinal muscles (15, 51). The depolarization can be explained by the inhibitory effects of SKF on those K⁺ channels. The depolarization can cause to underestimate a contribution of TRPC channel to mechanical and electrical responses. Thus, the inhibitory effects of those K⁺ channels should be considered when SKF is used as a TRPC channel blocker in gastrointestinal smooth muscles. Characterizing the SKF-induced suppression, such as IC_{50} and voltage-dependency, and additional tests with other TRPC blockers may be necessary to assess an involvement of TRPC channel in a response.

In conclusion, SKF can suppress K_{ATP}, K_V and BK channels. The order of affinity of SKF to those channels is $K_{ATP} > K_V > BK$.

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