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Cromakalim and lemakalim activate Ca^{2+}-dependent K^+ channels in canine colon

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Abstract. The effects of cromakalim (BRL 34915) and its (−) optical isomer, lemakalim (BRL 38227) on the activity of 265-pS Ca^{2+}-activated K^+ channels (BK channels) were examined in cell-attached and inside-out patches from canine colonic myocytes. In cell-attached patches lemakalim increased the open probability (P_o) of BK channels. Mean NP_o, where N is the number of channels per patch, at + 50 mV increased from 0.08 to 0.26 (20 μM lemakalim). In inside-out patches, cromakalim and lemakalim increased channel NP_o rapidly and reversibly. This increase in NP_o was due to a shift in half-maximal activation. Glyburide (20 μM) prevented the increase in NP_o caused by lemakalim in cell-attached patches and reversed the increase in NP_o in inside-out patches. Under conditions where Ca^{2+}-activated K^+ channels were maximally activated, lemakalim failed to increase current or induce a second type of K^+ channel activity. When tetraethylammonium (200 μM) was added to the pipette solution to block the BK channel half maximally, lemakalim also failed to induce a second type of channel. Adenosine triphosphate (1 or 2 mM) applied to the inner surface of inside-out patches had no effect on P_o of BK channels. Finally, the effects of lemakalim on ensemble average currents, constructed from multiple openings of BK channels in cell-attached patches was found to successfully mimic the effects of the drug on whole-cell membrane currents. We conclude that cromakalim and lemakalim activate BK channels in canine colonic cells. Whether this action participates in the membrane hyperpolarization and the decrease in frequency and duration of slow waves produced by these compounds in intact colonic muscles remains to be investigated.

Key words: Ionic channels – Potassium channels – Smooth muscle – Patch-clamp – Vasodilators

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Introduction

Cromakalim belongs to a class of drugs that increases Type 1 adenosine-triphosphate-activated K^+ channel (K_{ATP}) activity and leads to membrane hyperpolarization in cardiac [40], skeletal [35] and vascular muscles [18]. The Type 1 K_{ATP} channel was first described by Noma [27] in cardiac muscle. It has since been well characterized in cardiac myocytes [21], skeletal myocytes [42] and pancreatic β cells [37]. This channel is highyl selective for K^+ over Na^+, has a slope conductance of 40–80 pS, shows pronounced Mg^{2+}-dependent inward rectification, is inhibited by micromolar concentrations of ATP at the intracellular surface and is blocked by sulphonylureas (for recent reviews see [1, 29, 38]. K^+ channel openeres like cromakalim and the sulphonylureas have been reported to have effects on a variety of smooth muscle tissues such as vascular, gastrointestinal, tracheal and bladder [48]. However, direct evidence for the presence of Type 1 K_{ATP} channels has not been found in these preparations. In vascular smooth muscle, many of these compounds are potent vasodilators [36] and pharmacological studies have implicated K_{ATP} channels as the primary site of action [34]. Yet electrophysiological studies have provided evidence that these compounds may modulate the activity of large conductance K_{ATP} channels [30, 43], large conductance Ca^{2+}-activated K^+ channels [13, 23], delayed rectifier K^+ channels [2] and small conductance Ca^{2+}-activated K^+ channels [28] in vascular smooth muscle (for recent review, see [20]).

Little is known about the mechanism of action of these drugs in the gastrointestinal system. Post et al. [32] and Farraway and Huizinga [12] have reported that in the circular layer of canine proximal colon, cromakalim (BRL 34915) and its (−) optical isomer, lemakalim (BRL 38227), causes hyperpolarization of resting membrane potential, shortening of the duration of slow waves by abolition of the plateau phase and a decrease in slow wave frequency. These phenomena are consistent with an increase in K^+ permeability. In whole-cell voltage-clamp experiments it was found that cromakalim increased a

"time-independent" (quasi-instantaneous) component of outward current [32]. Cromakalim, but not lemakalim, was also found to inhibit L-type Ca^{2+} currents in this preparation.

The predominant K^+ channels found in patches from colonic myocytes are 265-pS Ca^{2+}-activated K^+ channels (BK channels) [5]. The physiological significance of these channels depends on their ability to conduct current under physiological conditions. The voltage and Ca^{2+} dependence of open probability (P_o) of this channel suggests, that it may play a role in the termination of slow waves [5]. Photolysis of the caged Ca^{2+} compound nitr-5 resulted in shortening of slow waves in the canine antrum [7] and canine colon (N. Publicover, unpublished observation). In canine colon BK channels are regulated by cyclic-adenosine-monophosphate (cAMP) dependent phosphorylation [8] and are modulated by muscarinic stimulation [9]. Whether these channels also contribute to the resting membrane potential (RMP) of colonic myocytes remains an open question.

The purpose of the present study was to determine, whether: (i) BK channels in colonic myocytes are activated by cromakalim and lemakalim, (ii) these effects could occur under resting conditions (holding potential, HP -70 mV, resting $[Ca^{2+}]_i$), (iii) in canine colon, K^+ channels other than the BK channel are activated by lemakalim and, (iv) the effects of these drugs on BK channels can account for the drug-induced changes in whole-cell currents.

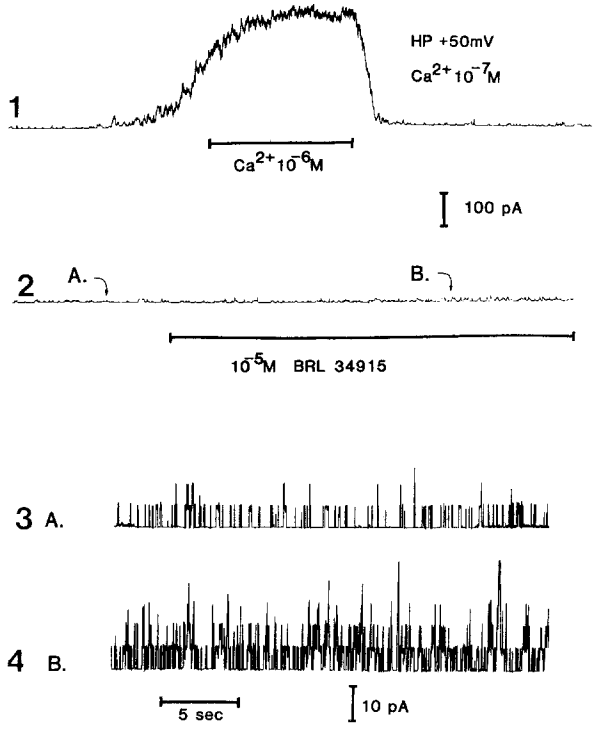

Fig. 1. Effect of cromakalim on Ca^{2+}-activated K^+ channels (inside-out patch). *Trace 1* shows that at $+50$ mV and 10^{-7} M Ca^{2+}, channel activity is very low. A change in bath Ca^{2+} to 10^{-6} M resulted in enormous activation of these channels. *Trace 2* shows that application of 10^{-5} M cromakalim to the bath solution resulted in activation of channels to a lesser extent than what is obtainable with a change in $[Ca^{2+}]$. Magnification of parts *A* and *B* of *trace 2* are shown in *traces 3* and *4* respectively

Materials and methods

Myocytes from the circular layer of the canine proximal colon were prepared as previously described [24]. Recordings of single-channel currents were made from cell-attached and excised inside-out patches using standard patch-clamp techniques [17]. High resistance seals (>5 GΩ) were formed using borosilicate electrodes (5–10 MΩ). After excision of patches, the pipette tip was inserted in a small perfusion chamber with a volume of approximately 2 µl to allow rapid change of the bath solution.

Patch-clamp studies were performed with a standard amplifier (AXOPATCH 1D, Axon Instruments, Foster City, CA, USA or a EPC-7, List Electronics, Darmstadt/Eberstadt, FRG), and data were stored on video-tape with a PCM-1 digital VCR-instrumentation recorder adaptor (Medical Systems, Greenvale, NY, USA). The data were replayed through an 8-pole Bessel filter (Ithako, Ithaka, NY, USA) at 4 kHz. Recordings were digitized with pClamp Software (Version 5.5, Axon Instruments) at a sampling rate of 10 kHz. Unitary amplitudes were measured with an amplitude-window detector (IPROC software, Axon Instruments). The current relative to baseline current (leakage) was averaged over time using IPROC and divided by the single-channel amplitude to determine NP_o (where N is the number of channels per patch). In some experiments, amplitude histograms were constructed and fit using a single-channel analysis program kindly provided by Dr. Mark Nelson. Voltage ramp protocols were applied using an IBM compatible AT clone, pClamp software and a digital analog interface (Scientific Solutions, Solon, OH, USA). The patch potential was changed over 4 s in decrements of 0.24 mV starting at positive potentials. During each ramp 2000 data points were sampled at a frequency of 500 Hz. Each ramp was repeated 5–15 times.

Solutions and drugs

Cell-attached configuration. Pipette and bath solutions (in mM): KCl, 145; $MgCl_2$, 2.3; dextrose, 5.5; ethylenebis(oxonitrilo)tetraacetate (EGTA), 0.1; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 7.2 (pH 7.4). In some of the experiments KCl was partially substituted by K^+ glutamate in the pipette solution.

Inside-out configuration. Pipette filling solution (in mM): KCl, 140; $CaCl_2$, 0.941; dextrose, 10; EGTA, 1; HEPES, 10 (pH 7.4). Bath solution (in mM): KCl, 140; $CaCl_2$, 0.61 to 1.0; dextrose, 10; EGTA, 1; HEPES, 10 (pH 7.4). The concentration of Ca^{2+} in bath and pipette solutions was buffered by EGTA to obtain activities of 10^{-7}, 10^{-6} and 10^{-5} M respectively. Association constants for H^+, K^+, and Ca^{2+} binding to EGTA were taken from [10, 11] and calculated with a program developed by Chi-Ming Hai (University of Virginia, Charlottesville, Va., USA). Ca^{2+} concentrations of 0.61, 0.941 and 1.00 mM were used in solutions containing 1 mM EGTA to obtain Ca^{2+} activities of 10^{-7}, 10^{-6} and 10^{-5} M.

Whole-cell configuration. Bath: NaCl, 130; $NaHCO_3$, 10; KCl, 4.2; $K_2H_2PO_4$, 1.2; $MgCl_2$, 0.5; $CaCl_2$, 1.5; HEPES, 10; dextrose, 5.5 (pH 7.4). Pipette: K^+ gluconate, 120; KCl, 20; $MgCl_2$, 0.5; EGTA, 5; HEPES, 5 (pH 7.2).

Chemicals

Cromakalim (BRL 34915) and lemakalim (BRL 38227) were gifts from SmithKline Beecham (Surrey, UK), glyburide was obtained from Sigma (St. Louis, MO, USA). These drugs were stored at 4° C as a 20 mM stock solution in dimethylsulfoxide (DMSO). A solution of 0.2% DMSO in 140 mM KCl solution had no effect on channel activity in inside-out patches ($n=5$). The final concentration of DMSO in the bath solution was 0.2% or less.

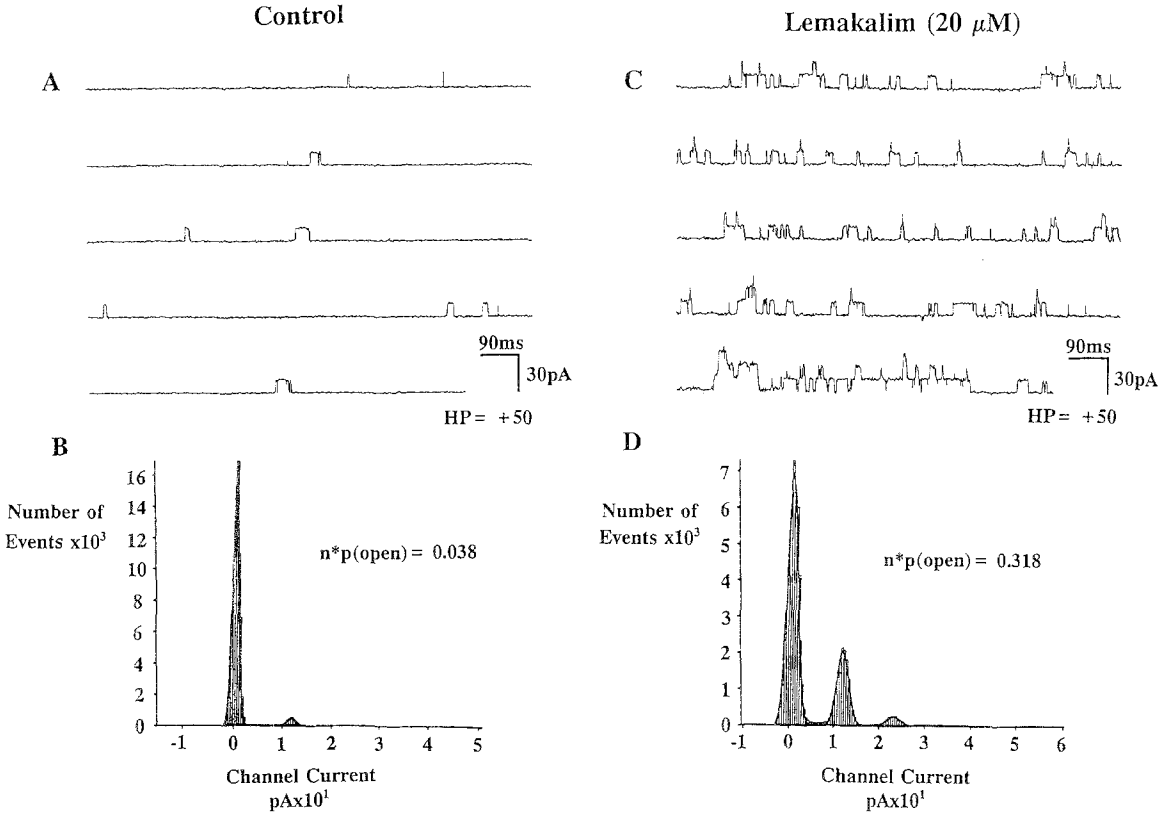

Fig. 2A–D. Effect of lemakalim on Ca^{2+}-activated K^+ channels (cell-attached patch). Membrane potential was zeroed with isotonic KCl in the bath solution. **A** Channel activity at a patch potential of + 50 mV. Single-channel openings are upward deflections of current. **B** Single-channel amplitude histogram of the data in **A**. **C** Channel activity at a membrane potential of + 50 mV in the presence of 20 μM lemakalim. The amplitude histogram in **D** shows the change in channel activity. NP_o increased from 0.038 to 0.318 in the presence of lemakalim, where N is the number of channels per patch and P_o is open probability

Results

Cromakalim and lemakalim

Lemakalim (BRL 38227) increases whole-cell outward current in a very similar manner as the racemic mixture cromakalim (BRL 34915) [32]. We therefore compared the effect of cromakalim and lemakalim on P_o of Ca^{2+}-activated K^+ channels in inside-out patches. Activity of BK channels from inside-out patches was recorded at + 50 mV HP and 10^{-7} M Ca^{2+}. In five patches, cromakalim (10^{-5} M) caused a significant increase in NP_o from 0.082 to 0.162 (1.7 ± 0.5-fold, $P < 0.05$). Illustrated in Fig. 1 is the effect of Ca^{2+} and cromakalim in one of these experiments. P_o at + 50 mV and 10^{-7} M Ca^{2+} was very low. When the Ca^{2+} in the bath was increased to 10^{-6} M, channel activity increased dramatically revealing the presence of many channels in this patch. Channel activity is returned to control levels upon perfusion of 10^{-7} M Ca^{2+} (Fig. 1, trace 1). Trace 2 is a continued recording from the same patch (10^{-7} M Ca^{2+}). Application of 10^{-5} M cromakalim to the bath solution (intracellular surface of the patch) resulted in an increase in channel activity. This increase was significant but small compared to the effect of Ca^{2+}. Traces 3 and 4 (Fig. 1) are magnifications of trace 2 before (A) and after (B) application of cromakalim. Single-channel amplitude was not affected by cromakalim.

When 10^{-5} lemakalim was added to the bath solution P_o of BK channels in six patches increased significantly from an NP_o of 0.052 to 0.19 (4.3 ± 3.2 fold, $P < 0.05$). The difference in the effectiveness of cromakalim or lemakalim to increase activity of BK channels at this concentration was not significant ($P > 0.05$). In most patches the increase in NP_o caused by cromakalim or lemakalim had a rapid onset (less than 1 min after start of bath perfusion), reached a new steady value and was reversible.

We also tested, whether lemakalim would increase channel activity in cell-attached patches. Cells were depolarized with 145 mM KCl and channel activity recorded with a pipette potential of − 50 mV (patch potential + 50 mV). Under these conditions NP_o averaged 0.080 ± 0.016. Lemakalim (20 μM) caused a substantial increase in P_o to 0.26 ± 0.063 ($n = 6$, $P < 0.05$) (Fig. 2). The time course of this effect, however, was very different from inside-out patches. It usually took several minutes for the onset to occur and activity increased gradually reaching its maximum after 40–60 min. The average increase in channel activity was 3.2 ± 1.2 fold ($n = 9$) after 40–60 min of exposure. Table 1 summarizes the effect of lemakalim at two membrane potentials. Since BK channels are notorious for spontaneous short term and long term changes in P_o, control experiments were performed to test the time course of NP_o in cell-attached

Table 1. Effect of lemakalim and glyburide on NP_o (cell-attached patch)

Potential	Conditions	
	Control	20 μM lemakalim
+ 30 mV (n = 3)	0.009 ± 0.002	0.025 ± 0.007
+ 50 mV (n = 6)	0.080 ± 0.016	0.260 ± 0.063*
		20 μM lemakalim + 20 μM glyburide
+ 30 mV (n = 3)	0.030 ± 0.008	0.021 ± 0.010
+ 50 mV (n = 3)	0.067 ± 0.021	0.017 ± 0.032

*, $P < 0.05$

NP_o values were calculated from minute long recordings of channel activity at given potentials by dividing the time averaged current by the single-channel amplitude according to $I_{average} = NP_o i_{channel}$

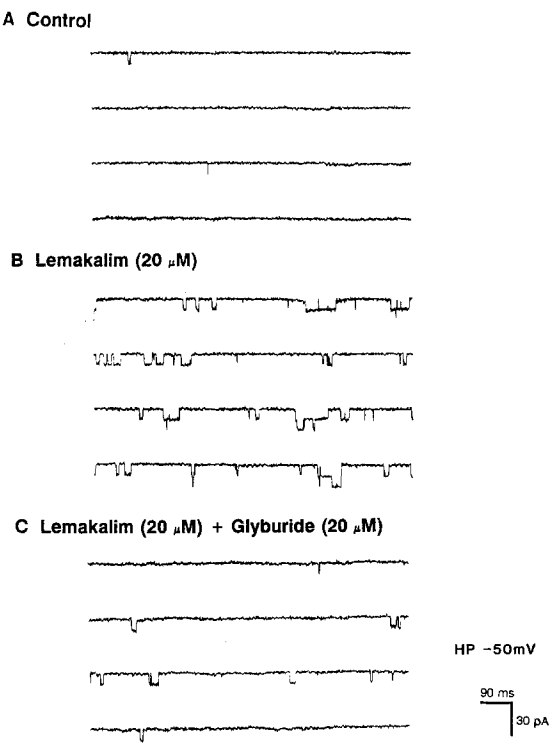

Fig. 3A–C. Effect of lemakalim and glyburide on Ca^{2+}-activated K^+ channel activity (cell-attached patch). **A** Control recording at −50 mV with a large diameter pipette tip, near the cell's resting membrane potential in vivo. **B** The increase in NP_o produced by 20 μM lemakalim. **C** In this experiment, the increase in NP_o was partially reversible when 20 μM glyburide was added to the bath solution

patches over 60 min. In standard bath solution (145 mM KCl) channel activity remained fairly stable over 60 min and, if at all, slightly declined ($n = 2$). In an additional control experiment, channel activity was monitored in 145 KCl with 0.2% DMSO added to the bath solution. In this experiment NP_o also slightly decreased over 60 min ($n = 3$).

The P_o of BK channels in canine colonic myocytes is extremely low under resting conditions and channel openings in cell-attached patches are rarely observed with membrane potentials below + 50 mV [5]. For example in one experiment channel activity was monitored at −75 mV (patch potential) in a cell-attached patch. Two openings of about 10 ms duration each were observed over a 20-min period. The patch was excised and exposed to 10^{-6} M Ca^{2+}. This revealed that the patch contained about 25 channels. Thus at −75 mV and at intracellular levels of Ca^{2+} the single-channel P_o was calculated to be less than 10^{-6}. Since lemakalim not only shortens colonic slow waves, but also hyperpolarizes the tissue at the myenteric border, we were interested to determine whether the increase in BK channel activity by lemakalim could also be demonstrated in cell-attached patches at a potential close to the RMP of circular muscle in situ. For these experiments large diameter pipettes with a tip resistance of less than 1 MΩ were used to increase the number of channels per patch. BK channel activity was monitored at −50 mV patch potential. Under control conditions, NP_o was 0.001 but increased dramatically to 0.204 after addition of 20 μM lemakalim to the bath solution (Fig. 3).

BK channels are sensitive to intracellular Ca^{2+} and membrane potential. At a given Ca^{2+} concentration P_o increases *e*-fold for about 17 mV change in membrane potential [5]. Experiments were performed to determine the effect of lemakalim on the voltage dependence of activation with voltage ramp protocols and compare these with current from whole-cell voltage-clamp. Voltage ramps were applied in the hyperpolarizing direction (e.g. + 100 mV to −100 mV) to take advantage of the rapid deactivation time constant of BK channels in this preparation [5, 6]. Currents from up to 15 voltage ramps were averaged. When lemakalim was added to inside-out patches with 10^{-7} M Ca^{2+} at the intracellular side, outward current increased at potentials above + 50 mV (Fig. 4A). With 10^{-6} M Ca^{2+} at the intracellular side, channel activation occurred at much less positive potentials. When lemakalim was applied to a patch in 10^{-6} M Ca^{2+} this resulted in a further shift in the activation of these channels to negative potentials (Fig. 4B). With cell-attached patches lemakalim (20 μM) caused an increase in current similar to the one seen with inside-out patches at low Ca^{2+} concentrations (Fig. 4C). Current responses to voltage ramps were also studied in the whole-cell configuration, where the contribution of currents other than through BK channels was minimized by 5 mM 4-aminopyridine (4-AP) (to block delayed rectifier channels [45]) and 10^{-6} M nisoldipine (to block L-type Ca^{2+}-channels [25] to avoid further activation of Ca^{2+}-dependent outward current as a result of Ca^{2+} influx through voltage sensitive Ca^{2+} channels). Lemakalim increased the current in the positive potential range in a similar fashion as in isolated patches in four out of four experiments (Fig. 4D). The steeper onset of current at potentials above + 60 mV may be due to contamination of the whole-cell current with a 4-AP-insensitive component of the delayed rectifier current [45]. The difference current is indeed very similar to the current induced by lemakalim in cell-attached patches.

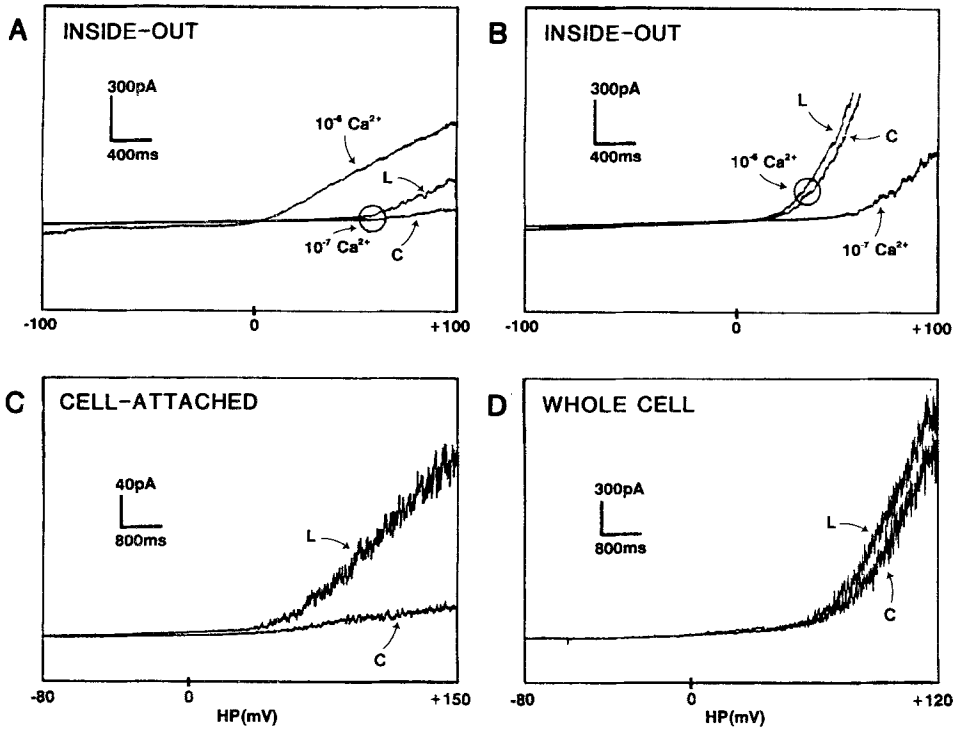

Fig. 4 A – D. The effect of lemakalim on K^+ currents was studied with voltage ramp protocols under several patch-clamp configurations. **A, B** Current responses averaged from 15 sweeps of depolarizing ramps for an inside-out patch containing many 265-pS Ca^{2+}-activated K^+ channels (BK channels) with a bath [Ca^{2+}] of 10^{-7} M (**A**) and 10^{-6} M (**B**). **C** Current responses averaged from 7 sweeps of depolarizing ramps for a cell-attached patch. Resting membrane potential (RMP) was adjusted to 0 mV with isotonic KCl in the bath solution. **D** The whole-cell current response to a depolarizing ramp. Bath solution was standard Hank's and contained 5 mM 4-aminopyridine and 10^{-6} M nisoldipine to minimize the contribution of currents other than the Ca^{2+}-dependent K^+ current. *C*, control; *L*, 20 μM lemakalim added to bath solution

Glyburide

Glyburide is an effective inhibitor of cromakalim-induced Rb^+ efflux in vascular smooth muscle [33] and appears to be a specific blocker of type 1 K_{ATP} channels in β cells and cardiac muscle [15, 44, 47]. We tested the effect of 20 μM glyburide on lemakalim-induced activation of BK channels in cell-attached and inside-out patches. In most cell-attached patches glyburide was not, or only partially (Fig. 3C) able to reverse the activation of BK channels induced by lemakalim. However when lemakalim and glyburide were added simultaneously to the bath solution, glyburide effectively prevented activation of BK channels by lemakalim in all cells tested. These data are summarized in Table 1. When glyburide (20 μM) was added to the bath solution in the absence of lemakalim, channel activity increased only slightly over 60 min (from 0.13 ± 0.1 to 0.20 ± 0.08, $n = 3$).

Application of 10 μM glyburide to the bath solution of inside-out patches readily reversed the activation of BK channels by lemakalim. The time course of NP_o for one such experiment is shown in Fig. 5. 10 μM Glyburide by itself had no effect on BK channel activity when applied to the intracellular side of excised patches ($n = 3$).

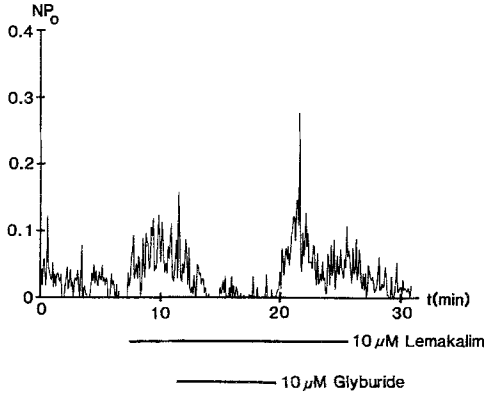

Fig. 5. Glyburide reverses increase in NP_o induced by lemakalim (inside-out patch). Channel activity from a patch held at + 50 mV in 10^{-7} M Ca^{2+} containing many BK channels was monitored and NP_o determined from the analysis of an events list. When 10 μM lemakalim was perfused into the bath channel activity increased ($NP_o = 0.17$ before and 0.45 after lemakalim). This increase was completely reversed upon addition of 10 μM glyburide. The increase in NP_o was reversible after washout of lemakalim

Are other K^+ channels activated by lemakalim?

Standen et al. [43] have described a Ca^{2+}- and tetraethylammonium-(TEA) insensitive large conductance channel in vascular smooth muscle which is inhibited by millimolar concentrations of ATP and activated by cromakalim. The slope conductance and current/voltage relationship of this channel type is so similar to the large conductance Ca^{2+}-activated K^+ channel, that it would be very different to distinguish this channel type in patches containing a large number of BK channels. We undertook several experiments to test the hypothesis, that the increase in activity of large conductance channels induced by lemakalim in our patches was due to a second type of large conductance channels similar to the channel found by Standen et al. [43] rather than an increase in P_o of BK channels.

Inside-out patches, in symmetrical 140/140 mM KCl, were exposed to high (10^{-5} M) Ca^{2+}, which resulted in full activation of BK channels. If application of lemakalim induced a second type of channel, this

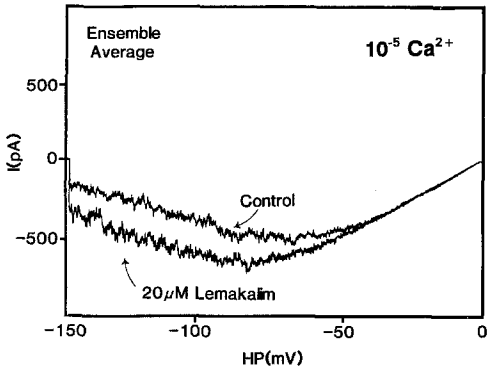

Fig. 6. Lemakalim shifts the activation curve of BK channel but does not activate additional types of K^+ channels. Current responses to a hyperpolarizing ramp from 0 to −150 mV applied to an inside-out patch containing many BK channels were averaged. The patch was exposed to symmetrical 140 mM KCl with 10^{-5} M Ca^{2+} at the intracellular surface. With these conditions the reversal potential is 0 mV and the channels are fully active at a membrane potential positive to −50 mV. At potentials negative to −50 mV, BK channels start to deactivate. Lemakalim did not increase the ensemble averaged current in the potential range where BK channels were maximally activated (−50 to 0 mV), but only negative to −50 mV, where the BK channels were partially deactivated

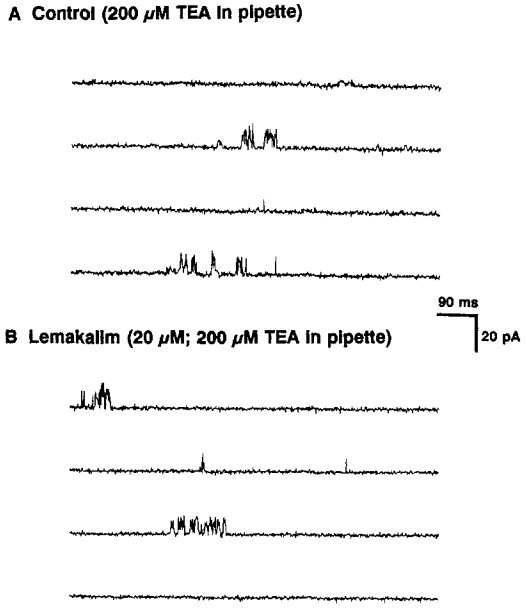

Fig. 7A, B. Activity of BK channels monitored in a cell-attached patch with 200 μM tetraethylammonium (TEA) in the pipette solution. **A** This concentration led to a partial flicker type block of BK channels. **B** When 20 μM lemakalim was added to the bath solution, channel activity did not increase. TEA-insensitive large conductance channels were not induced by lemakalim either

lemakalim-induced current should result in a further increase of current in the voltage range where BK channels are maximally active. Shown in Fig. 6 are the results of one such experiment. Current response to voltage ramps from 0 to −150 mV were averaged and plotted. The reversal potential for K^+ was 0 mV under these conditions, and no current was obtained at 0 mV. From 0 to −50 mV current increased linearly with driving force through maximally activated Ca^{2+}-dependent K^+ channels. At potentials negative to −50 mV BK channels deactivated and the corresponding inward current decreased. At −150 mV channel P_o was below 10% and the resulting inward current was very small despite a large driving force. Application of lemakalim (20 μM) did not affect inward K^+ current in the range from 0 to −50 mV, where BK channels were maximally activated. At potentials negative to −50 mV, where BK channels were partially deactivated, lemakalim caused an increase of inward K^+ current. The magnitude of this increase was similar to the increase observed with patches held at 10^{-6} M Ca^{2+}, suggesting that the increase in K^+ current was due to a shift in the activation curve of BK channels rather than an activation of a second type of large conductance K^+ channels.

The cromakalim sensitive large conductance K^+ channels described by Standen et al. [43] were not blocked by 1 mM TEA. In contrast, BK channels from gastrointestinal tract are blocked by application of TEA to the outer surface of the membrane with a K_D of about 100–300 μM [7]. We performed experiments in which TEA (200 μM) was included in the pipette solution. This caused a "flicker type" block of BK channels and reduced the apparent single-channel current by about 50%. When lemakalim was added to the bath solution, a TEA-insensitive, large conductance K^+ channel was not observed in either inside-out ($n = 3$) or cell-attached ($n = 6$) patches. The increase in P_o of the half blocked BK channels caused by lemakalim was reduced under these conditions (Fig. 7).

Occasionally 80- to 100-pS channels were observed in inside-out patches. These channels had a similar voltage and Ca^{2+} dependence as BK channels, and were never observed under conditions, where BK channels were not activated. In a few patches containing both channel types, lemakalim also increased the P_o of the smaller conductance channel. The degree to which this channel was activated by lemakalim could not be quantitated due to the large number of BK channels in these patches.

Intracellular ATP

Experiments were performed to examine, whether BK channels from canine colon are modulated by intracellular ATP. Inside-out patches were perfused with standard bath solution containing 10^{-6} M Ca^{2+}. Channel activity was recorded at a HP of −50 mV and ATP (1, 2 or 5 mM) was added to the bath solution. There was no change in NP_o or single-channel amplitude with up to 2 mM ATP ($n = 10$) (Fig. 8A). At 5 mM, ATP caused rapid membrane breakdown in three out of three patches.

In some of these experiments, the effect of ATP on voltage-dependent activation of BK channels in the range of ±100 mV was measured with voltage ramps as described previously [6]. Current responses from 15 ramps were averaged before and after application of ATP to the intracellular surface of an inside-out patch. No change or shift of the activation curve along the voltage axis was observed with concentrations up to 2 mM ATP (Fig. 8B).

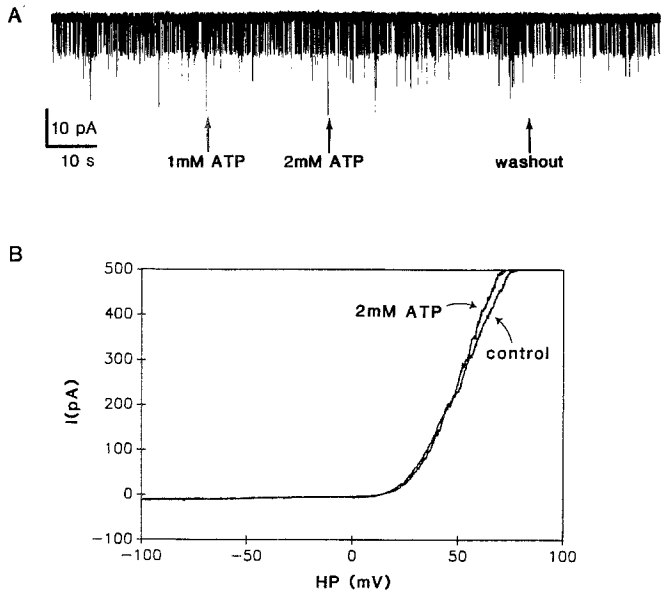

Fig. 8A, B. ATP does not block Ca^{2+}-activated K^+ channels in canine colonic myocytes. **A** A continuous recording of BK channel activity from an inside-out patch held at −50 mV membrane potential and a bath $[Ca^{2+}]$ of 10^{-7} M. Application of 1 and 2 mM ATP to the bath solution did not affect P_o. **B** Current responses to voltage ramps from an inside-out patch containing many BK channels in 10^{-6} M Ca^{2+}. Current from 15 ramps for each was averaged. Channels activated in the positive voltage range. Addition of ATP (1 mM) to the intracellular surface did not significantly change or shift the voltage dependence of BK channel activation

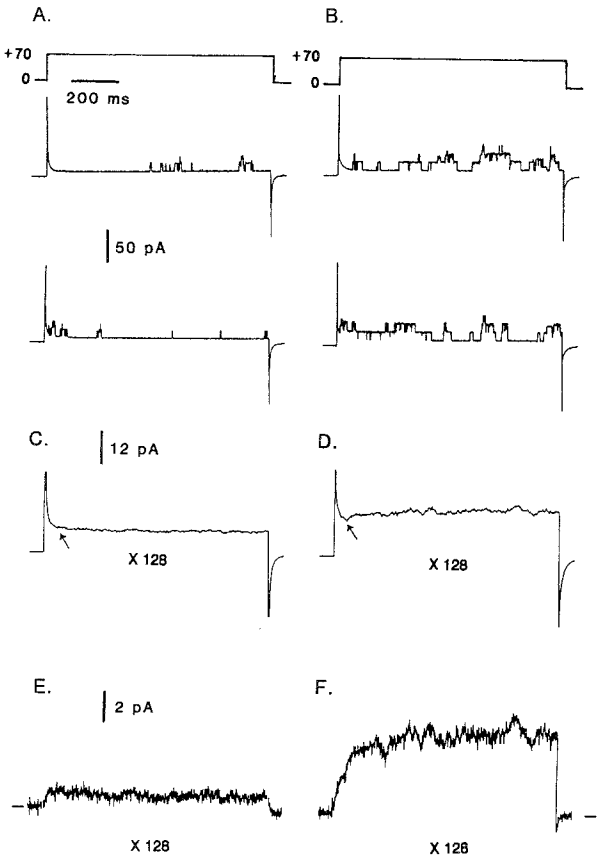

Fig. 9A–F. Ensemble average currents to depolarizing voltage steps in cell-attached patch. Channel openings in response to a step depolarization from 0 to + 70 mV (patch potential) were recorded **A** before and **B** after application of 20 μM lemakalim to the bath solution. Currents were not corrected for leak and capacitive transients. **C, D** The ensemble average current from 128 such sweeps. The fast activation of BK channels to depolarizing voltage steps makes the current appear like "time-independent"current. **E, F** The ensemble average current from the same patch after leak and capacitive current subtraction. Ensemble current activated with $\tau = 28$ ms in the control, and with $\tau = 42$ ms in the presence of lemakalim

Effects of lemakalim on ensemble averaged BK currents

With voltage step protocols, cromakalim and lemakalim affected mainly a "time-independent" or quasi-instantaneous component of whole-cell outward current in colonic [32] as well as vascular [2] smooth muscle cells. It is noteworthy that a similar "time-independent" component of whole-cell current is also affected by the vasodilator, minoxidil sulfate, in vascular smooth muscle cells [26]. Experiments were performed to examine whether the effects of lemakalim on ensemble averaged currents from patches containing numerous BK channels could account for the effect of this compound on whole-cell currents. Cell-attached patches were repetitively depolarized and membrane currents were averaged under control conditions and after exposure to lemakalim. Results from one such experiment are shown in Fig. 9. Shown in Fig. 9A, B are two examples of BK channel openings during step depolarizations from 0 to + 70 mV (patch potential) under control conditions and after exposure to lemakalim (20 μM), respectively. Lemakalim dramatically increased BK channel activity in this patch. In this experiment, membrane currents were averaged from a series of 128 identical depolarizing voltage steps in the control (Fig. 9C) and in the presence of lemakalim (Fig. 9D). For these averaged currents capacitative and leak currents were not substracted. Comparing Fig. 9C and D, it appears that lemakalim increased the magnitude of a predominantly time-independent current component following the capacitative transient (arrows). This effect is very similar to the effect of lemakalim on whole-cell currents [2, 32].

If the same data is corrected for capacitative and leak currents, then the kinetics of activation of BK channels can be observed before and after exposure to lemakalim (Fig. 9E, F). In control, the ensemble current activates with a τ of 28 ms. Exposure to lemakalim increased the amplitude of the ensemble current. The activation kinetics in the presence of drug ($\tau = 42$ ms) were similar to those observed in the control and characteristic of BK channels in colonic smooth muscle cells ($\tau = 32.1$ ms with steps to + 80 mV, [5]). This rapid activation makes it possible that under whole-cell conditions, this current might appear as a quasi-instantaneous component of membrane current unless precautions are taken to effectively eliminate the capacitative transient. It is also apparent that augmentation of BK channel activity by lemakalim might appear as an increased magnitude of a quasi-instantaneous, time-independent component of whole-cell membrane current.

Discussion

Both cromakalim and lemakalim increased P_o values of large conductance Ca^{2+}-activated K^+ channels in inside-out and cell-attached patches from canine colon. The time course of activation was much slower in cell-attached patches, suggesting that these compounds may have to diffuse through the lipid membrane bilayer in order to exert their effect. Similarly, the sulphonylurea glyburide reversed the increase in NP_o caused by lemakalim in inside-out patches, but not, or only partially, in cell-attached patches. These data demonstrate that K^+ channel openers like cromakalim and sulphonylureas are not specific for K_{ATP} channels. Our findings resemble those of Gelband et al. [13], who reported, that cromakalim increases the P_o of BK channels in smooth muscle cells from rabbit aorta, rabbit trachea and pig coronary arteries. The BK channels described in vascular smooth muscle cells by Gelband et al. [14] were also blocked by millimolar concentrations of ATP applied to the intracellular surface. Small conductance channels that are sensitive to both Ca^{2+} and ATP have been reported in vascular smooth muscle [22]. In contrast, in our study on canine colonic myocytes, ATP applied to the intracellular surface of inside-out patches had no effect on BK channel P_o. Other investigators have found no effect of cromakalim, glyburide or ATP on BK channels of smooth muscles [2, 25]. Small conductance channels that are sensitive to both Ca^{2+} and ATP have recently been reported in vascular smooth muscle [22]. These channels were activated by nicorandil [19, 20].

We examined the possibility that the increase in channel activity in our patches may have been caused by a different type of large conductance channel than BK channels. Standen et al. [43] have reported a Ca^{2+}- and TEA-insensitive K_{ATP} channel in myocytes from rabbit and rat mesenteric artery that was activated by cromakalim. This channel had a slope conductance of 135 pS at 0 mV in 60/120 mM KCl, which corresponds to a single-channel permeability of 4.5 cm s^{-1} (calculated from the constant field equation [16]). In our experiments this channel would be very difficult to distinguish from BK channels, which in colonic cells have a permeability of 5.2 cm s^{-1} (calculated from a slope conductance of 260 pS in 140/140 KCl). When the BK channel was half maximally blocked by TEA, we did not observe a large conductance, TEA-insensitive channel that was activated by lemakalim in colonic myocytes. Either this channel is not expressed in membranes of colonic myocytes, or the number of these channels is very low. Under conditions where BK channels were maximally activated with 10^{-5} M Ca^{2+} lemakalim failed to further increase ensemble average current. This strongly suggests, that the increase in large conductance channel activity observed at stationary potentials is due to an increase in the P_o of BK channels.

In some patches (5–20%) we found a 80- to 100-pS channel that was also activated by lemakalim. This channel had a similar voltage and Ca^{2+} dependence as the BK channel and was never observed under conditions where the BK channel was inactive. It is not clear whether this channel is a separate entity or a subconductance state of BK channels.

Lemakalim also increased the magnitude of ensemble average currents elicited by step depolarizations in cell-attached patches. The time constant for activation of this ensemble averaged current was in the same range as described earlier for BK channels from colonic myocytes in inside-out patches [5]. This fast activating current would appear under whole-cell conditions as a "time-independent" (quasi-instantaneous) current, similar to that observed by Post et al. [32] and Beech and Bolton [2]. Our patch-clamp experiments were performed at room temperature, while the whole-cell voltage-clamp experiments by Post et al. [32] were conducted at 37° C. Increasing bath temperature from 25° C to 37° C causes a further decrease in the time constant for activation of BK ensemble average current (A. Carl, unpublished observation).

We have previously suggested that BK channels participate in the repolarization phase of slow waves in gastrointestinal muscle cells [5, 7]. Under resting conditions, the P_o of these channels is very low and it is not clear at present, whether they contribute to the generation of RMP. In the circular layer of canine colon the RMP is graded from −80 mV at the submucosal border to −40 mV at the myenteric border [41]. The origin of this gradient is unknown at the present, but a different density and/or pump rate of electrogenic Na^+/K^+-ATPase [3] and differential expression of isoforms of the Na^+/K^+-ATPase have been implied [4]. The P_o of BK channels can be extrapolated from measurements at high Ca^{2+} and positive membrane potentials by fitting the activation curves with a Boltzman function, $P_o = 1/\{1 + \exp[-K\text{-}(V - V_{1/2})]\}$. Using $V_{1/2} = +107$ mV at 10^{-7} M and $K^{-1} = 16.9$ mV [5] gives $P_o = 1.67 \cdot 10^{-4}$ at −40 mV and $2.83 \cdot 10^{-5}$ at −70 mV membrane potential. Using a BK channel density of N of 23000/cell [5] and a slope conductance γ_{slope} of 72.4 pS at −40 mV and 43.6 pS at −70 mV (calculated from the constant field equation [16] with $[K^+]_o = 5.9$ mM, $[K^+]_i = 140$ mM and $P_K = 5.1$ cm s^{-1}) the contribution of BK channels to resting membrane conductance can be estimated from $NP_o\gamma_{slope}$. This calculation gives a total BK channel conductance, $NP_o\gamma_{slope}$ of 28 pS at −70 mV and 278 pS at −40 mV. With an input resistance of about 1 GΩ [24] (= 1000 pS membrane conductance), BK channels might contribute between 3 and 25% to resting membrane conductance. These calculations from measurements on excised patches have to be interpreted with caution however, since the activity of BK channels may be further modulated by intracellular second messengers and mechanisms like phosphorylation [8]. However, other data also supports the involvement of BK channels in maintenance of the RMP. In canine gastric antrum, UV illumination of tissue that was loaded with the light sensitive caged Ca^{2+} compound nitr-5, resulted in shortening of slow waves and a small hyperpolarization [7]. Similar effects have been observed in canine colon (N. G. Publicover, unpublished observation). Intracellular recording of membrane potential also revealed afterhyperpolarizations following long duration slow waves [39], implying a transient activation of

a K^+ current. In a recent study using whole-cell voltage-clamp it has been shown that low bath concentrations of TEA (100 μM) cause small, but significant depolarization of the RMP in isolated canine colonic smooth muscle cells [31]. Taken together, these observations suggest that while the RMP in canine colon is not exclusively generated by BK channels, BK channels may contribute significantly to the RMP. Trieschmann and Isenberg [46] have suggested, that BK channels contribute to RMP in porcine vascular smooth muscle. Although we used higher concentrations of lemakalim than are required to demonstrate a significant membrane hyperpolarization in intact tissue [32], even small increases in P_o values of BK channels induced by K^+ channel openers would result in a significant increase in membrane K^+ conductance and therefore cause membrane hyperpolarization of intact colonic tissue, consistent with earlier findings in vascular tissue [23].

The increase in P_o of BK channels induced by these compounds can readily explain the major effects of these drugs on electrical activity in situ as well as the observed increase in "time-independent" outward current recorded in whole-cell voltage-clamp experiments. However, we cannot exclude the possibility that the effect of cromakalim and lemakalim on electrical activity in situ may also involve the activation of channels that either are expressed in very low density, have a single-channel amplitude that is too small to be resolved with standard patch-clamp techniques or were inactivated under our recording conditions. Further experiments are clearly needed to provide a direct functional correlation between the effects of these compounds on BK channels and membrane hyperpolarization of colonic smooth muscle.

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References

1. Ashcroft SJH, Ashcroft FM (1990) Properties and functions of ATP-sensitive K-channels. Cell Sig 2:197–214
2. Beech DJ, Bolton TB (1989) Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. Br J Pharmacol 98:851–864
3. Burke EP, Reed JB, Sanders KM (1988) Role of sodium pump in membrane potential gradient of canine proximal colon. Am J Physiol 254:C475–C483
4. Burke EP, Sanders KM, Horowitz B (1991) Sodium pump isozymes are differentially expressed in electrically dissimilar regions of colonic circular smooth muscle. Proc Natl Acad Sci USA 88:2370–2374
5. Carl, A, Sanders KM (1989) Ca^{2+}-activated K channels of canine colonic myocytes. Am J Physiol 257:C470–C480
6. Carl A, Sanders KM (1990) Measurement of single channel open probability with voltage ramps. J Neurosci Methods 33:157–163
7. Carl A, McHale NG, Publicover NG, Sanders KM (1990) Participation of Ca^{2+}-activated K^+ channels in electrical activity of canine gastric smooth muscle. J Physiol (Lond) 429:205–221
8. Carl A, Kenyon JL, Uemura D, Fusetani N, Sanders KM (1991) Regulation of Ca^{2+}-activated K^+ channels by protein kinase A and phosphatase inhibitors. Am J Phyisol 261:C387–C392
9. Cole WC, Carl A, Sanders KM (1989) Muscarinic suppression of Ca^{2+}-dependent K current in colonic smooth muscle. Am J Physiol 257:C481–C487
10. Fabiato A (1981) Myoplasmic free calcium concentration reached during the twitch of an isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. J Gen Physiol 78:457–497
11. Fabiato A, Fabiato F (1979) Calculator programs for computing the composition of solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J Physiol (Paris) 75:463–505
12. Farraway L, Huizinga JD (1991) Potassium channel activation by cromakalim affects the slow wave type action potential of colonic smooth muscle. J Pharmacol Exp Ther 257:35–41
13. Gelband CH, Lodge NJ, van Breemen C (1989) A Ca^{2+}-activated K^+ channel from rabbit aorta: modulation by cromakalim. Eur J Pharmacol 167:201–210
14. Gelband CH, Silberberg SD, Groschner K, van Breemen C (1990) ATP inhibits smooth muscle Ca^{2+}-activated K^+ channels. Proc R Soc Lond [Biol] 242:23–28
15. Gillis KD, Gee WM, Hammoud A, McDaniel ML, Falke LC, Misler S (1989) Effects of sulfonamides on a metabolite-regulated ATP_i-sensitive K^+ channel in rat pancreatic B-cells. Am J Physiol 257:C1119–C1127
16. Goldman DE (1943) Potential, impedance, and rectification in membranes. J Gen Physiol 27:37–60
17. Hamill OP, Marty A, Neher E, Sakman B, Sigworth FJ (1981) Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
18. Hamilton TC, Weir SW, Weston AH (1986) Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. Br J Pharmacol 88:103–111
19. Kajioka S, Oike M, Kitamura K (1990) Nicorandil opens a calcium-dependent potassium channel in smooth muscle cells of the rat portal vein. J Pharmacol Exp Ther 254:905–913
20. Kajioka S, Nakashima M, Kitamura K, Kuriyama H (1991) Mechanism of vasodilation induced by potassium-channel activators. Clin Sci 81:129–139
21. Kakei M, Noma A (1984) ATP-sensitive single K^+ channel in the atrioventricular node cell of the rabbit heart. J Physiol (Lond) 352:1265–1284
22. Kitamura, K, Kuriyama H (1991) Actions of potassium channel openers on the ionic currents recorded from the vascular smooth muscle cells. J Mol Cell Cardiol 23:S98
23. Klöckner U, Trieschmann U, Isenberg G (1989) Pharmacological modulation of calcium and potassium channels in isolated vascular smooth muscle cells. Arzneimittelforschung 1:120–126
24. Langton PD, Sanders KM (1989) Participation of calcium current in colonic electrical activity. Am J Physiol 257:C451–C460
25. Langton PD, Nelson MT, Huang Y, Standen NB (1991) Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 260:H927–H934
26. Leblanc N, Wilde DW, Keef KD, Hume JR (1989) Electrophysiological mechanisms of minoxidil sulfate-induced vasodilation of rabbit portal vein. Circ Res 65:1102–1111
27. Noma A (1983) ATP-regulated K^+ channels in cardiac muscle. Nature 305:147–148
28. Okabe K, Kajioka S, Nakao K, Kitamura K, Kuriyama H, Weston AH (1990) Actions of cromakalim on ionic currents recorded from single smooth muscle cells of the rat portal vein. J Pharmacol Exp Ther 252:832–839
29. Petersen OH, Findlay I (1987) Electrophysiology of the pancreas. Physiol Rev 67:1054–1116

30. Post JM, Jones AW (1991) Stimulation of arterial ^{42}K efflux by ATP-depletion and cromakalim is antagonized by glyburide. Am J Physiol 260:A848–H854
31. Post JM, Hume JR (1992) Ionic basis for spontaneous depolarizations in smooth muscle cells. Biophy J 61:A396
32. Post JM, Stevens RJ, Sanders KM, Hume JR (1991) Effect of cromakalim and lemakalim on slow waves and membrane currents in colonic smooth muscle. Am J Physiol 260:C375–C382
33. Quast U, Cook NS (1989) In vitro and in vivo comparison of two K^+ channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. J Pharmacol Exp Ther 250:261–271
34. Quast U, Cook NS (1989) Moving together: K^+ channel openers and ATP-sensitive K^+ channels. Trends Pharmacol Sci 10:431–435
35. Quasthoff S, Spuler A, Horn-Lehman F, Grafe P (1989) Cromakalim, pinacidil and RP 49356 activate a tolbutamide-sensitive K^+ conductance in human skeletal muscle fibres. Pflügers Arch 414:S179–S180
36. Richer C, Pratz J, Mulder P, Mondot S, Giudicelli JF, Cavero I (1990) Cardiovascular and biological effects of K^+ channel openers, a class of drugs with vasorelaxant and cardioprotective effects. Life Sci 47:1693–1705
37. Rorsman P, Trube G (1985) Glucose dependent K^+-channels in pancreatic β-cells are regulated by intracellular ATP. Pflügers Arch 405:305–309
38. Rorsman P, Trube G (1990) Biophysics and physiology of ATP-regulated K^+ channels (K_{ATP}). In: Cook NS (ed) Potassium channels: structure, classification and therapeutic potential. Ellis Horwood, Chichester, pp 96–116
39. Sanders KM, Smith TK (1986) Enteric neural regulation of slow waves in circular muscle of the canine proximal colon. J Physiol (Lond) 377:297–313
40. Sanguinetti MC, Scott AL, Zingaro GJ, Siegl PKS (1988) BRL 34915 (cromakalim) activates ATP-sensitive K^+ current in cardiac muscle. Proc Natl Acad Sci USA 85:8360–8364
41. Smith TK, Reed JB, Sanders KM (1987) Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. Am J Physiol 252:C215–C224
42. Spruce AE, Standen NB, Stanfield PR (1985) Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. Nature 316:736–738
43. Standen NB, Qualye JM, Davis NW, Brayden JE, Huang Y, Nelson MT (1989) Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. Science 245:177–180
44. Sturgess NC, Kozlowski RZ, Carrington CA, Hales CN, Ashford MJL (1988) Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. Br J Pharmacol 95:83–94
45. Thornbury KD, Ward SM, Sanders KM (1991) Participation of fast-activating, voltage-dependent potassium currents in electrical slow waves of colonic circular muscle. Am J Physiol (in press)
46. Trieschmann U, Isenberg G (1989) Ca^{2+}-activated K^+ channels contribute to the resting potential of vascular myocytes. Ca^{2+}-sensitivity is increased by intracellular Mg^{2+}-ions. Pflügers Arch 414:S183–S184
47. Trube G, Rorsman P, Ohno-Shosaku T (1986) Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic beta-cells. Pflügers Arch 407:493–499
48. Weston AH (1989) Smooth muscle K^+ channel openers; their pharmacology and clinical potential. Pflügers Arch 414:S99–S105