Multiple components of delayed rectifier K^+ current in canine colonic smooth muscle

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1. We investigated the pharmacology and voltage-dependent activation and inactivation kinetics of the 'delayed rectifier' K^+ current, I_{dK}, in canine colonic myocytes and developed protocols which separate this current into three distinct components that differ in their kinetics and pharmacology.

2. Block of I_{dK} by TEA or 4-aminopyridine (4-AP) alone was incomplete. Maximal concentrations of TEA or 4-AP blocked 76% (EC_{50} = 2·6 mM) and 51% (EC_{50} = 69 mM) of current, respectively. In the presence of 10 mM 4-AP, I_{dK} could be blocked completely by TEA.

3. TEA and 4-AP had distinct effects on current activation: time constants for activation of I_{dK} at +10 mV were 25·6 ± 4·4 ms under control conditions, 40·3 ± 7·6 ms in the presence of 10 mM 4-AP and 16·7 ± 2·3 ms with 10 mM TEA in the bath solution. 4-AP block and removal of block were use dependent, but no frequency dependence or voltage dependence of steady-state block could be detected. These data are consistent with the presence of a rapidly activating 4-AP-sensitive current, $I_{dK(f)}$, and a more slowly activating TEA-sensitive current component, $I_{dK(s)}$.

4. A third component of the delayed rectifier current, $I_{dK(n)}$, was revealed when 10 mM TEA was included in the pipette solution. $I_{dK(n)}$ was rapidly activating, had a membrane potential at half-maximal inactivation ($V_{\frac{1}{2}}$) for steady-state inactivation 13 mV negative of that for the mixed I_{dK}, was completely insensitive to 4-AP (10 mM) and was blocked by external TEA with an EC_{50} of 7·7 mM.

5. These data demonstrate that the delayed rectifier current in canine colonic smooth muscle is composed of three currents, $I_{dK(f)}$, $I_{dK(s)}$ and $I_{dK(n)}$. All three currents are insensitive to charybdotoxin (100 nM).

Two main classes of macroscopic K^+ current have been described in gastrointestinal smooth muscle cells: a Ca^{2+}-activated K^+ current mediated by BK channels (Walsh & Singer, 1981; Benham, Bolton, Lang & Takewaki, 1986) and a Ca^{2+}-insensitive, voltage-activated K^+ current mediated by delayed rectifier channels (Ohya, Terada, Kitamura & Kuriyama, 1986; Noack, Deitmer & Lammel, 1986; Bielefeld, Hume & Krier, 1990). Ca^{2+}-activated K^+ current has been characterized extensively in many preparations (for review see Latorre, 1994) including the canine colon (Carl & Sanders, 1989). However, much less is known about delayed rectifier currents and their role in the electrical activity of smooth muscles.

The expression 'delayed rectification' was originally coined for a K^+-selective membrane conductance in the squid axon that activates with a delay after a depolarizing voltage step (Hodgkin & Huxley, 1952). This term is currently used to denote axon-like voltage-dependent and Ca^{2+}-independent K^+ currents. Delayed rectifier current appears to play a critical role in the electrical activity of the canine colon. In cells from the circular muscle layer this current activates rapidly upon depolarization and tends to limit the amplitude of upstroke depolarization of electrical slow waves (Thornbury, Ward & Sanders, 1992*a*). Since it does not fully inactivate during 5–10 s depolarizing test pulses, it may provide a balance for the Ca^{2+} current during the plateau phase of slow waves and therefore may be critical for shaping the electrical event that ultimately determines the amount of Ca^{2+} entering the cell and cellular contraction.

While delayed rectifier current (I_{dK}) has been found in every kind of smooth muscle investigated, the properties of these currents in different tissues and species are diverse. For example, the sensitivity of I_{dK} to

4-aminopyridine (4-AP) in smooth muscles varies over a large range, depending on tissue source and species. Voltage-dependent K^+ outward current that was very sensitive to 4-AP (half-maximal block below 0·5 mM) was described in rabbit pulmonary artery (Okabe, Kitamura & Kuriyama, 1987), rabbit portal vein (Beech & Bolton, 1989) and canine trachea (Muraki, Imaizumi, Kojima, Kawai & Watanabe, 1990). Boyle, Tomasic & Kotlikoff (1992) recently found a 13 pS K^+ channel that was strongly inhibited by 1 mM 4-AP in inside-out patches from porcine airway smooth muscle. In contrast, voltage-sensitive K^+ currents that were less sensitive to 4-AP (half-maximal block above 0·5 mM) have been described in rabbit ileum (Ohya *et al.* 1986), guinea-pig gastric antrum (Noack *et al.* 1986), rat anococcygeus smooth muscle (McFadzean & England, 1992), guinea-pig portal vein (Pfrunder & Kreye, 1992), rabbit coronary artery (Volk, Matsuda & Shibata, 1991), canine coronary artery (Buljubasic, Rusch, Marijic, Kampine & Bosnjak, 1992), canine renal artery (Gelband & Hume, 1992) and feline cerebral artery (Bonnet, Rusch & Harder, 1991). It appears that the term 'delayed rectifier' has been used for a number of different currents. However, it is not clear whether these currents represent activation of single or multiple components of K^+ conductances. It was suggested by Thornbury *et al.* (1992*a*) that, in colonic smooth muscle cells, a 4-AP-insensitive current with a more negative inactivation range may contribute to 'delayed rectifier' current.

4-AP-sensitive delayed rectifier channels appear to play an important role in the electrical slow waves of the canine colonic circular muscle layer and may be a target for neuronal control of slow wave activity. 4-AP (10 mM) caused a significant increase in slow wave duration (Thornbury *et al.* 1992*a*). Du, Carl, Smith, Sanders & Keef (1994) found that cAMP-mediated hyperpolarization in the canine colon was abolished by pretreatment with 4-AP but not TEA. While 'delayed rectifier' current in the colon is sensitive to both 4-AP and TEA, we will show in this study, that 'delayed rectifier' current can be separated into a 4-AP-sensitive, TEA-insensitive current and two distinct TEA-sensitive, 4-AP-insensitive current components.

METHODS

Mongrel dogs of either sex were killed with an overdose of sodium pentobarbitone (45 mg kg^{-1}). The abdomen was opened and a segment of proximal colon 6–14 cm from the ileocolonic sphincter was removed. The colon was opened along the mesenteric border, cleared of remaining faecal material and a 3×3 cm piece of tissue was placed in a dissecting dish containing oxygenated Ca^{2+}–Mg^{2+}-free Hanks' solution of the following composition (mM): 141·2 Na^+, 5·8 K^+, 130·4 Cl^-, 15·5 HCO_3^-, 0·34 HPO_4^{2-}, 0·44 $H_2PO_4^-$, 10 dextrose, 2·9 sucrose. This solution had a pH of 7·4 at 37 °C when bubbled to equilibrium with 97% O_2 – 3% CO_2.

Patch clamp recordings

Myocytes from the circular layer of the canine proximal colon were prepared as previously described (Carl & Sanders, 1989). Current was recorded using the cell-attached or whole-cell mode of the patch clamp technique. High resistance seals (> 5 GΩ) were formed using borosilicate electrodes (1·5–3 MΩ). A standard amplifier (Axopatch-1D, Axon Instruments) was used for current recordings. Data were filtered at 5 kHz and analysed using pCLAMP software (version 5.1.1, Axon Instruments). All data were digitized at a 10 kHz sampling rate except the steady-state inactivation experiments (Fig. 7) which were sampled at 200 Hz. Capacitance was compensated for and residual capacitance current was digitally removed. Current from X mV hyperpolarizing pulses was multiplied by (test potential − holding potential)/X and subtracted from currents recorded during test pulses. Series resistance was not compensated for. Series resistance was between 4 and 8 MΩ. Amplitude of currents was always below 1000 pA resulting in a voltage error of less than 8 mV. Data were corrected for a −10 mV liquid junction potential.

Solutions

Standard bath solution contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1·2 $MgCl_2$, 10 dextrose, 10 Hepes, 5 Tris (pH 7·4). In most experiments $CaCl_2$ was replaced by equimolar $MnCl_2$ to remove Ca^{2+} inward currents. In some experiments TEA (up to 30 mM) was added to the bath solution. When 100 mM TEA was applied, an equimolar amount of NaCl was removed to maintain osmolarity.

The standard pipette solution contained (mM): 20 KCl, 110 potassium gluconate, 5 $MgCl_2$, 2·5 K_2ATP, 0·1 Na_2GTP, 2·5 disodium creatine phosphate, 5 Hepes, 1 BAPTA (pH 7·0). All patch clamp experiments were carried out at room temperature (24 °C).

Analysis of data

Significant differences between means were calculated by Student's t test and values were considered significantly different at $P < 0·05$. n values represent the number of cells. Data are expressed as means ± standard error of the mean (S.E.M.).

Drugs used

4-Aminopyridine (4-AP) and tetraethylammonium (TEA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Charybdotoxin was obtained from Peninsula Inc. (Belmont, CA, USA).

RESULTS

A delayed rectifier current, I_{dK}, in canine colon measured during voltage ramps is sensitive to TEA and 4-AP but block of this current is incomplete, even when high concentrations are used (Du *et al.* 1994). In order to determine whether I_{dK} can be separated into distinct components we measured dose–response relations for TEA and 4-AP block. Since the dynamics of block may be distorted during voltage ramps, we investigated current block using depolarizing test steps: Fig. 1 shows whole-cell current recorded from a holding potential of −60 mV to test potentials ranging from 0 to +30 mV. On average, I_{dK} was blocked 53 ± 4% ($n = 13$) by 10 mM TEA

(Fig. 1*B*) and 34 ± 9% ($n = 9$) by 10 mM 4-AP (Fig. 1*C*) at a test potential of 0 mV.

Figure 2 shows dose–response curves for block of I_{dK} at +20 mV measured at the end of 100 ms test pulses. TEA dose–response curves were obtained from seven cells under control conditions (*A*; ○) and from four cells with 10 mM 4-AP added to the pipette solution (*A*; ●). Data were averaged and fitted by a sigmoidal function of the form:

$$I_{drug}/I_{control} = (1 - C)/(1 + ([drug]/EC_{50})^{n_H}) + C, \quad (1)$$

where EC_{50} is the concentration for half-maximal effect, n_H is the Hill coefficient, and C is the residual current at super-maximal concentrations. Concentrations for half-maximal block of current by TEA were 2·6 ($n = 7$, control) and 2·2 mM ($n = 4$, with 10 mM 4-AP). Similarly, 4-AP dose–response curves were obtained under control conditions (*B*; □, no drugs added to bath solution except increasing concentrations of 4-AP) and in the presence of 100 mM TEA (■). Data from ten cells (control) and six cells (100 mM TEA) were averaged and fitted by a sigmoidal function of the form of eqn (1). Concentrations for half-maximal block of current by 4-AP were 69 ($n = 10$, control) and 23 μM ($n = 6$, in the presence of 100 mM TEA). Both drugs, when applied alone, showed incomplete block at maximal concentrations (76% block for TEA, 51% block for 4-AP) but abolished 98–100% of control current when applied together. This finding supports the notion that 'delayed rectifier' current in this preparation may be composed of both 4-AP-sensitive and TEA-sensitive components. In order to test this idea we investigated the block of current by TEA and 4-AP in more detail and developed protocols to separate individual current components.

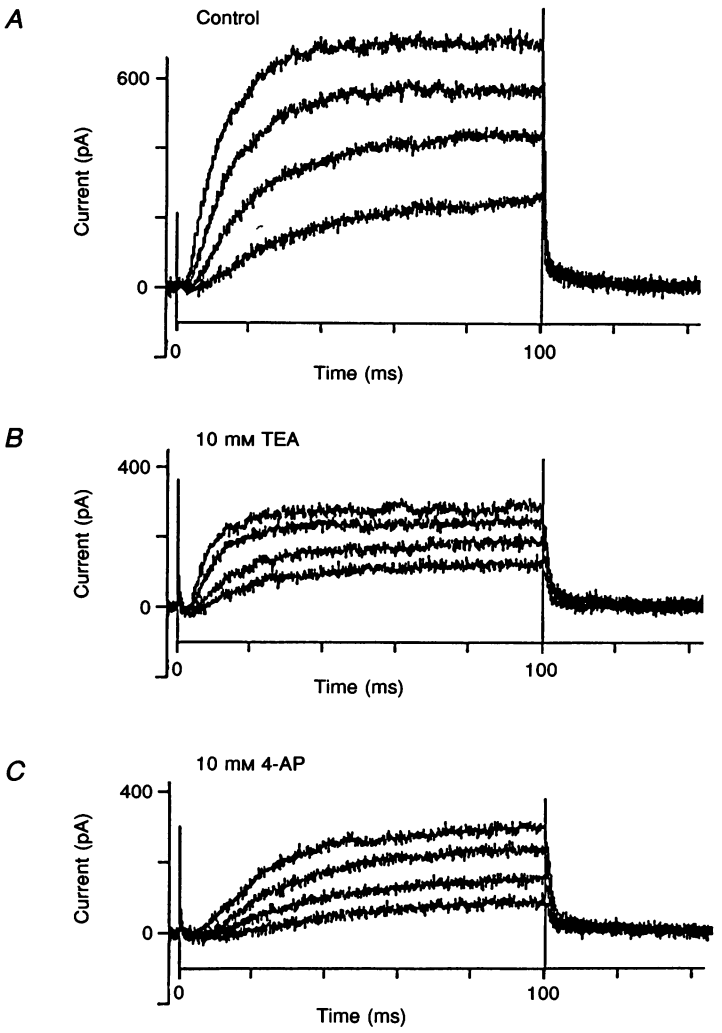

Figure 1. Block of delayed rectifier current by TEA and 4-AP

Delayed rectifier current was elicited with step depolarizations from a holding potential of −60 mV to test potentials ranging from 0 to +30 mV. Leak and capacitative currents were digitally subtracted using properly scaled small hyperpolarizing test pulses (see Methods). The early (within 10 ms) current was very sensitive to 4-AP but not to TEA.

Delayed rectifier current as shown in Fig. 1 activated in a time- and voltage-dependent manner and reached a plateau within 100 ms. Time constants of activation, τ_{act}, were obtained by fitting single exponential functions to the current. After bath application of 10 mM TEA, a rapidly activating current remained (Fig. 1*B*). In contrast, residual current after application of 10 mM 4-AP to the bath solution was slowly activating (Fig. 1*C*). On average, times for half-maximal activation were 25·6 ± 4·4 (control, $n = 17$), 16·7 ± 2·3 (10 mM TEA, $n = 11$) and 40·3 ± 7·6 ms (10 mM 4-AP, $n = 8$) at +10 mV test potential. Figure 3 shows time constants of activation as a function of test potentials. τ_{act} decreased linearly with membrane depolarization, but activation of 'delayed rectifier' current was slower in the presence of 10 mM 4-AP and faster in the presence of 10 mM TEA compared with control conditions at all potentials between 0 and +30 mV (Fig. 3*A*).

If the 'delayed rectifier' current in this preparation were simply the sum of a 4-AP- and a TEA-sensitive component, then the current in the presence of 4-AP should have properties similar to the TEA difference current (control current − current in presence of TEA). Indeed, the TEA difference current had a τ_{act} of 36·4 ± 9·7 ms ($n = 13$, +10 mV) a value similar to the current in the presence of 4-AP ($P > 0{\cdot}05$), while the 4-AP difference current had a τ_{act} of 14·2 ± 5·1 ms ($n = 8$, +10 mV) a value similar to the current in the presence of TEA ($P > 0{\cdot}05$), as shown in Fig. 3*B*.

Accordingly, reduction of current by TEA and 4-AP was significantly time dependent: 10 mM TEA blocked only 34 ± 6% of control current after 10 ms but 53 ± 4% after 100 ms, at +20 mV ($n = 13$). Reduction of outward current by 4-AP was time dependent in the opposite direction: at +20 mV, 10 mM 4-AP blocked 64 ± 4% of the control current after 10 ms but only 34 ± 9% after

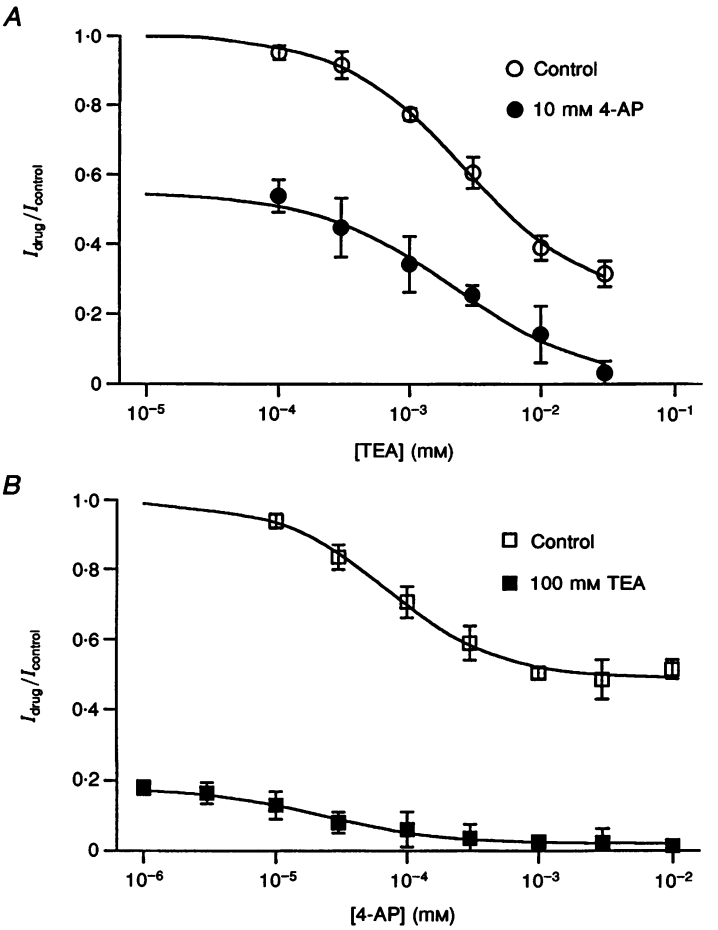

Figure 2. Dose–response relations for block of delayed rectifier current by TEA and 4-AP

Current was measured at the end of a 100 ms depolarizing pulse to +20 mV from a holding potential of −60 mV. *A* shows block of current by TEA using standard pipette solution (○) and in cells where 10 mM 4-AP had been added to the pipette solution (●). *B* shows block of current by 4-AP using standard solutions (□) and in cells where 100 mM TEA had been added to the bath solution (substituted for NaCl) (■).

100 ms ($n = 9$). These data can be interpreted in two ways. (1) The time-dependent current reduction by 4-AP may be due to block of two distinct K^+ channels with different activation kinetics and different sensitivity to 4-AP, i.e. two distinct K^+ currents underlie I_{dK}: one current might be rapidly activating, very sensitive to 4-AP and less sensitive to 10 mM TEA ($I_{dK(f)}$) and the other current slowly activating, more sensitive to TEA and less sensitive to 4-AP ($I_{dK(s)}$). (2) Another interpretation would be that the time dependence of 4-AP block may be due to a complicated mechanism of 4-AP block. In order to determine whether state or voltage dependence of 4-AP block could explain these results, we investigated the mechanism of I_{dK} block by 4-AP.

Use dependence of 4-AP block

In order to examine whether the observed time dependence of 4-AP block might be due to a slow unbinding of 4-AP from channel molecules opened during depolarizing pulses, we investigated the use dependence of 4-AP block. Figure 4 shows current activated during 100 ms duration test pulses to +20 mV from a holding potential of −90 mV. Test pulses were elicited every 10 s and several control responses were measured. Current activated and reached a peak within 100 ms. Next, 1 mM 4-AP was perfused into the bath solution and the cell held at −90 mV for 2 min more after complete bath exchange. When depolarizing voltage steps were resumed, current continuously decreased until it reached a steady level after approximately four pulses. Figure 4*A* shows current elicited from the first four test pulses (marked 1–4 in Fig. 4*C*) after application of 4-AP. These data show that 4-AP displayed a use-dependent block which can be explained if 4-AP binds preferentially to the open state of the channel (Choquet & Korn, 1992). Use dependence of 4-AP removal was also examined. During the wash-out period (5 min) the cell was held at −90 mV. Figure 4*B*

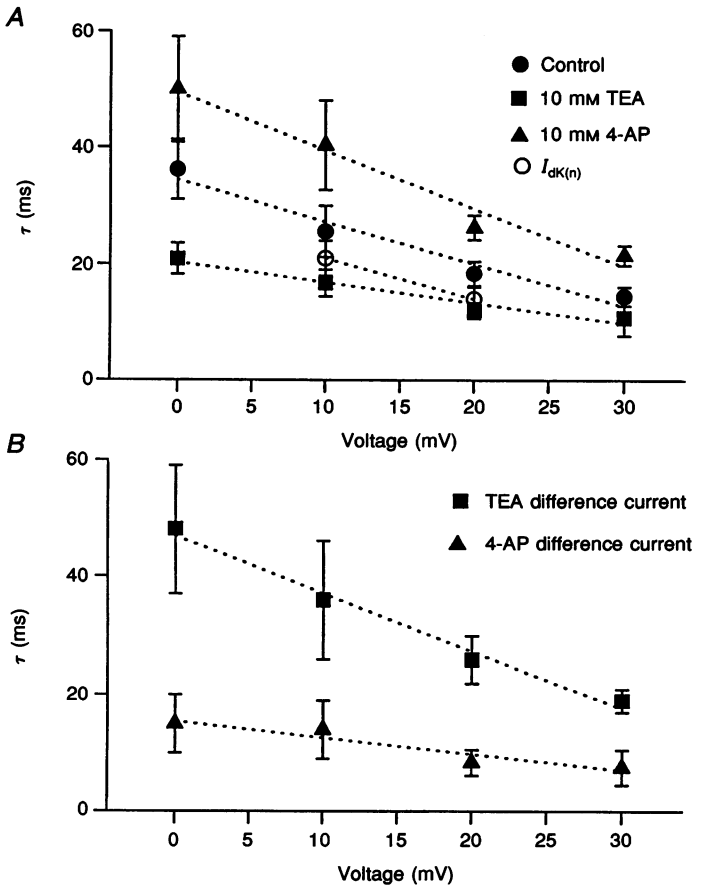

Figure 3. Time constants of activation of outward current as a function of test potential

Current activated faster at more positive potentials. Data are fitted by linear regression. *A*, TEA (10 mM, ■) decreased time constants while 4-AP (10 mM, ▲) increased time constants at all potentials. A component of delayed rectifier current isolated by including 10 mM TEA in the pipette solution ($I_{dK(n)}$, ○) was more rapidly activating than the mixed delayed rectifier current (●). *B*, time constants of activation of difference currents (control current − current in presence of 10 mM TEA, ■; control current − current in presence of 10 mM 4-AP, ▲).

shows current elicited from the first four test pulses (marked 5–8 in panel *C*) after removal of 4-AP from the bath solution. The first test pulse to +20 mV elicited only slowly activating current, while the second and all subsequent pulses elicited more rapidly activating current. Figure 4*C* shows the plot of the time course of peak current measured at the end of 100 ms test pulses during this experiment. The most straightforward explanation of these experiments is that 4-AP cannot unbind from the closed channel state, i.e. it is trapped during channel closure, and that the slow activation during the first test pulse after drug removal was due to the slow unbinding of 4-AP during channel openings (Kirsch & Drewe, 1993). Similar results were obtained in four of four cells.

Frequency dependence of 4-AP block

If the time dependence of 4-AP block were due to a slow unbinding of the 4-AP molecule from the channel during the duration of the 100 ms depolarization, then a second depolarization following the test pulse immediately after a very brief repolarization should result in a smaller current during the second test pulse compared with the first. Such frequency dependence of 4-AP block during a train of depolarizing pulses has indeed been observed in delayed rectifier current of the squid giant axon (Meves & Pichon, 1977) and nodes of Ranvier (Ulbricht & Wagner, 1976): K^+ outward current rose very slowly during the first depolarizing pulse, but faster during the second and later pulses. This phenomenon was explained by a partial removal of the 4-AP block during the first depolarization.

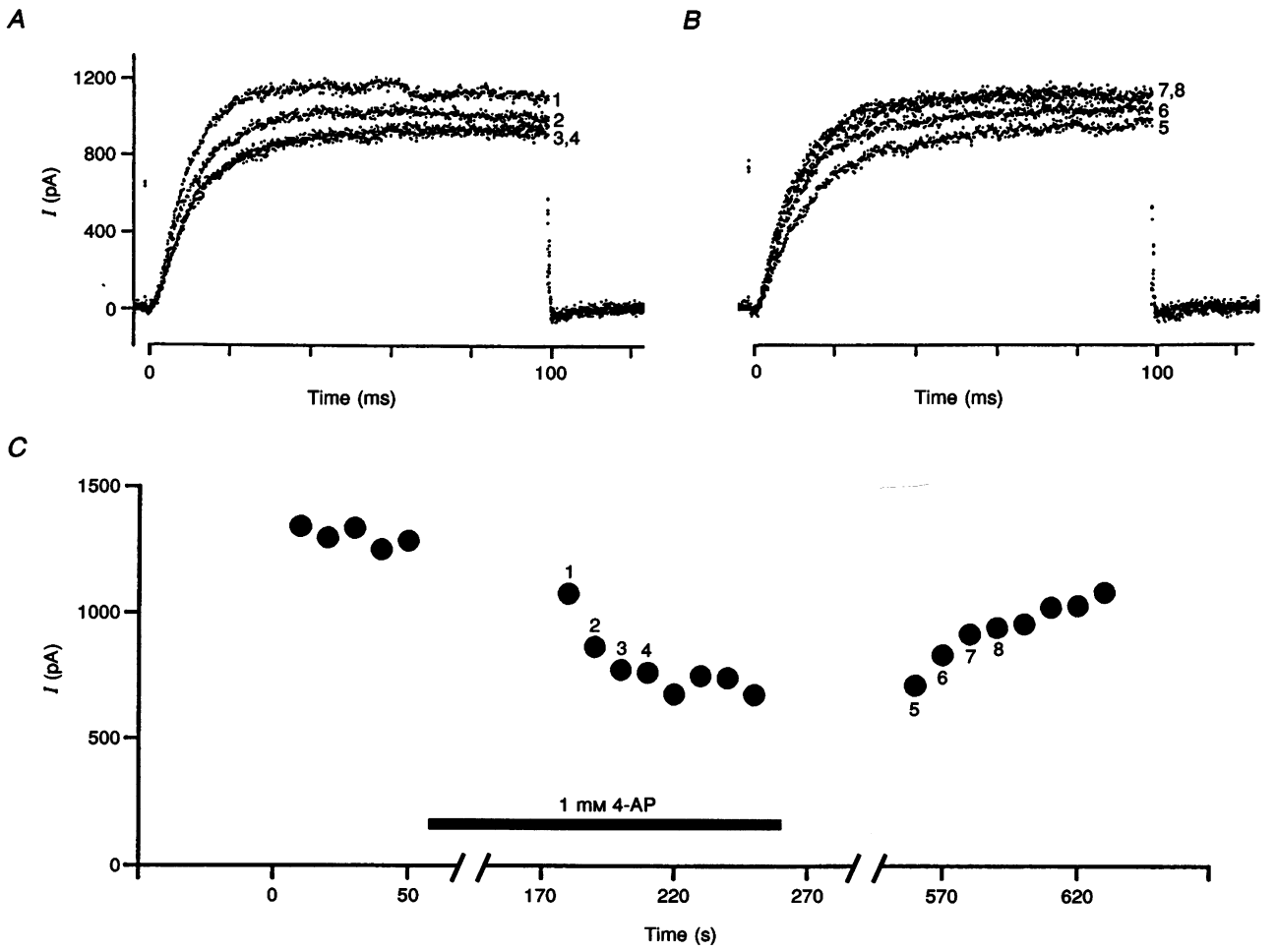

Figure 4. Use dependence of 4-AP block

4-AP (1 mM) was perfused into the bath solution and the cell was held at −90 mV during this time period. *A*, voltage steps of 100 ms duration from −90 to +20 mV resulted in successive current suppression. Currents elicited during the first 4 step depolarizations (marked 1–4) are shown. *B* shows current elicited after removal of 4-AP from the bath (5 min wash during which cell was kept at −90 mV). Current elicited during the first depolarization after drug removal was smaller than during subsequent depolarizations and recovered gradually. Currents elicited during the first 4 step depolarizations after wash-out (marked 5–8) are shown. *C*, time course of peak current measured at 100 ms.

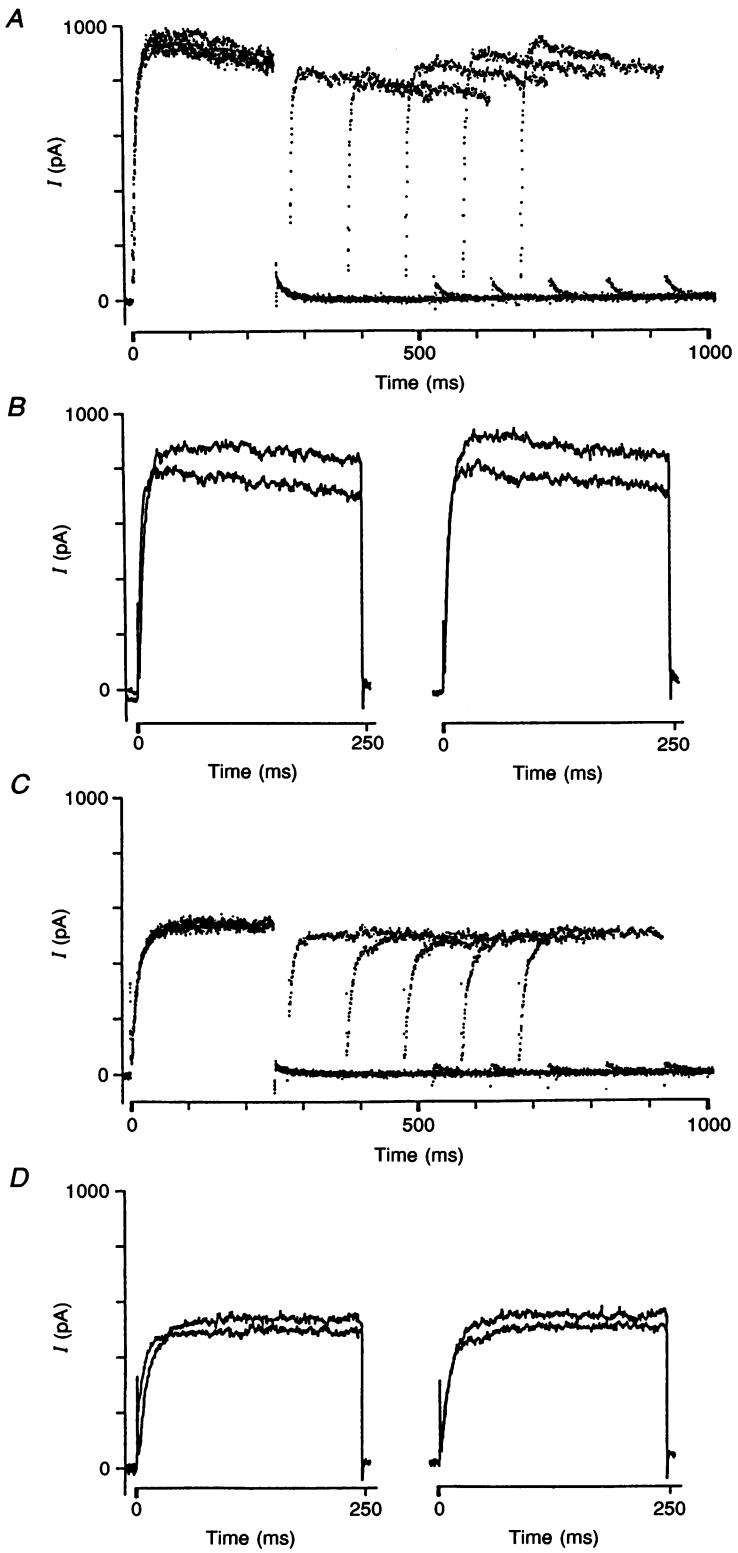

Figure 5. Frequency dependence of 4-AP block

A, current was measured during 250 ms twin voltage steps from −60 to +20 mV separated by a variable interval (50–450 ms). *B*, currents elicited by the first and second depolarization step separated by 50 (left) and 150 ms (right), respectively, are shown superimposed. Current elicited by the second pulse of the twin steps activates faster but reaches a smaller peak resulting in a cross-over of current traces. *C*, same protocol as in *A*, but in the presence of 1 mM 4-AP. *D*, currents in the presence of 1 mM 4-AP elicited by the first and second depolarization step separated by 50 and 150 ms are shown superimposed.

We investigated the frequency dependence of 4-AP block in colonic myocytes in order to determine whether the time dependence of 4-AP block (Fig. 1) might be due to such a mechanism. Current was measured during 250 ms twin voltage steps from −60 to +20 mV separated by a variable interval (50–450 ms) (Fig. 5*A*). In these experiments, current continued to inactivate despite the brief repolarizations (cumulative inactivation). Figure 5*B* shows superimposed currents elicited by the first and second depolarization step separated by 50 (left panel) and 150 ms (right panel) duration hyperpolarizations. Current elicited by the second pulse of the twin pair was smaller but activated more rapidly than current from the first pulse. This resulted in a 'cross-over' of the current traces, which was especially noticeable when the two pulses were separated by the briefest (50 ms) interval. The rapid activation of the current from the second pulse of the twin pair may have been due to incomplete deactivation of current elicited by the first pulse (note that tail currents lasted more than 50 ms in this experiment). Figure 5*C* and *D* shows results from the same cell in the presence of 1 mM 4-AP. While overall currents were about 40% smaller than under control conditions, results were qualitatively similar including the 'cross-over' phenomenon. We conclude that block of I_{dK} by 4-AP was not frequency dependent. In three similar experiments using 1 mM 4-AP we did not find any significant frequency dependence of 4-AP block, suggesting that this mechanism cannot explain the slow rate of rise of outward current we observed with 4-AP.

Voltage dependence of 4-AP block

We also examined the voltage dependence of 4-AP block. Current was activated by depolarizing pulses to potentials ranging from 0 to +30 mV from a holding potential of −60 mV, and current was measured at the end of the 100 ms duration test pulse. Figure 6 shows current block by 10 mM 4-AP as a function of test potential measured in five cells. While the degree of block from cell to cell was quite variable, there was no significant voltage dependence of block observable in these experiments.

Taken together, the use dependence of 4-AP favouring the open channel state and the absence of frequency dependence and voltage dependence suggest that the slowing of activation times in the presence of 4-AP is not due to a state-dependent block of 'delayed rectifier' current in colonic myocytes.

Block of $I_{dK(f)}$ and $I_{dK(s)}$ by internal TEA reveals a current with a low inactivation threshold

Delayed rectifier channels in squid axon are known to be sensitive to TEA applied to the cytoplasmic membrane surface (Armstrong & Binstock, 1965; Armstrong, 1975; Stanfield, 1983), while BK channels are relatively insensitive (dissociation constant, K_D, > 50 mM; Carl, Frey, Ward, Sanders & Kenyon, 1993). We undertook experiments with 10 mM TEA in the pipette solution in order to study $I_{K(Ca)}$ in isolation (Du *et al.* 1994). Indeed, in these experiments I_{dK} elicited with a voltage ramp protocol was completely suppressed, leaving a pure charybdotoxin (ChTX)-sensitive $I_{K(Ca)}$ activating at potentials positive to +30 mV (Du *et al.* 1994). Taken together with the data shown here, this suggests that both $I_{dK(f)}$ and $I_{dK(s)}$ are sensitive to block by internally applied TEA.

However, when cells were step depolarized to potentials that were below the activation range for BK channels from a very negative holding potential, a small-amplitude inactivating outward current could be observed. Figure 7 shows currents recorded from two cells, one with standard pipette solution (Fig. 7*A*) and one with 10 mM TEA added

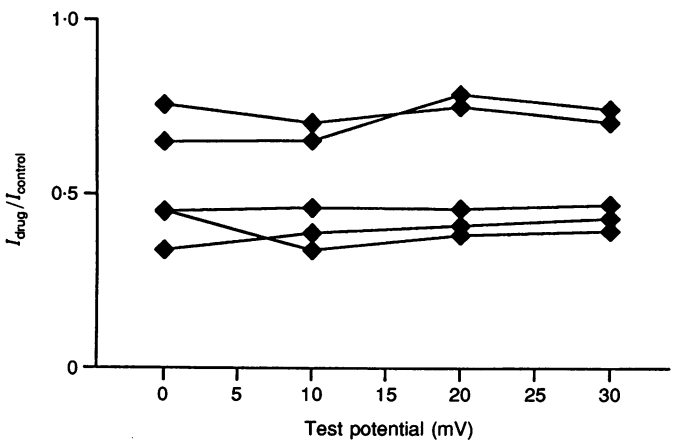

Figure 6. Voltage dependence of 4-AP block

Current was measured at the end of a 100 ms depolarizing test pulse in the presence and absence of 10 mM 4-AP. While the degree of block varied from cell to cell, there was no significant voltage dependence of block between 0 and +30 mV test potentials.

to the pipette solution (Fig. 7*B*). Current was elicited by 5 s test pulses to +10 mV following a 20 s prepulse to −90, −50 and −10 mV (note change in time scale). Current with 10 mM TEA in the pipette was small in amplitude, inactivated with a single exponential time course ($\tau = 3100 \pm 300$ ms at −10 mV, $n = 4$), and was half-inactivated at potentials 13 mV more negative than the current measured in the absence of internal TEA (Fig. 7*C*). In cells with 10 mM TEA in the pipette, current availability at −60 mV was 52·4 ± 5·9% and voltage for half-maximal inactivation, $V_{½}$, was −64 mV ($n = 9$) determined from a fit of data to a Boltzmann function:

$$I = (1 - C)/[1 + \exp(V - V_{½})/V_{s}], \quad (2)$$

where V_s is the slope factor. In contrast, current availability under control conditions at −60 mV was 65·5 ± 2·8% and $V_{½}$ was −51 mV ($n = 25$). Extracellularly applied TEA or 4-AP significantly shifted the inactivation curve to the right and left, respectively (Table 1). Current inactivation under control conditions

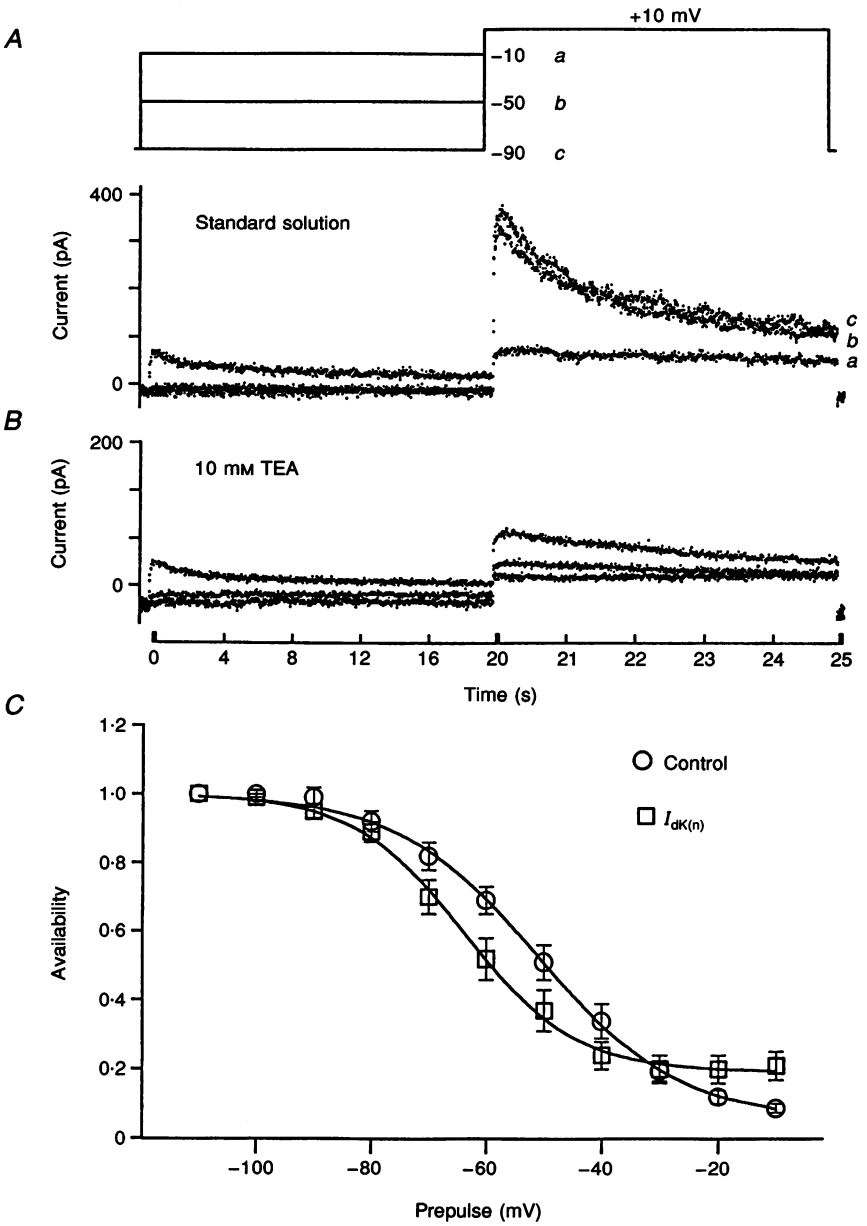

Figure 7. Isolation of low inactivation threshold current, $I_{dK(n)}$

Availability of current was measured as normalized peak current during a 5 s test depolarization to +10 mV following a 20 s conditioning pulse. *A*, under control conditions (no TEA in the pipette) delayed rectifier current was only 10% inactivated at −50 mV in this experiment. *B*, with 10 mM TEA in the pipette solution (different cell), a low inactivation threshold current was revealed. This current was 80% inactivated at −50 mV in this experiment. *C* summarizes steady-state inactivation for cells under control conditions (○) and with 10 mM TEA in the pipette solution (□).

Table 1. Properties of delayed rectifier current components

	Activation	Inactivation	Block by TEA		Block by 4-AP	
	τ_{act} at +10 mV (ms)	$V_{½}$ (mV)	EC_{50} (mM)	Max. block (%)	EC_{50} (μM)	Max. block (%)
Control	25·6 ± 4·4 (17)	−51 (25)	2·6 (7)	76 (7)	69 (10)	51 (10)
10 mM 4-AP in pipette solution	40·3 ± 7·6 (8)	−64 (9)	2·2 (4)	100 (4)	—	—
10 mM TEA in bath solution	16·7 ± 2·3 (11)	−38 (7)	—	—	23 (6)*	98 (6)*
10 mM TEA in pipette solution	21·0 ± 3·2 (5)	−64 (9)	7·7 (6)	100 (6)	No block (3)	0 (3)

τ_{act}, time constant of activation. $V_{½}$, voltage for half-maximal steady-state inactivation. Control, properties of the mixed 'delayed rectifier' current. *Values recorded in 100 mM TEA. EC_{50} values and percentage maximal block were obtained from Hill function fits to dose–response curves.

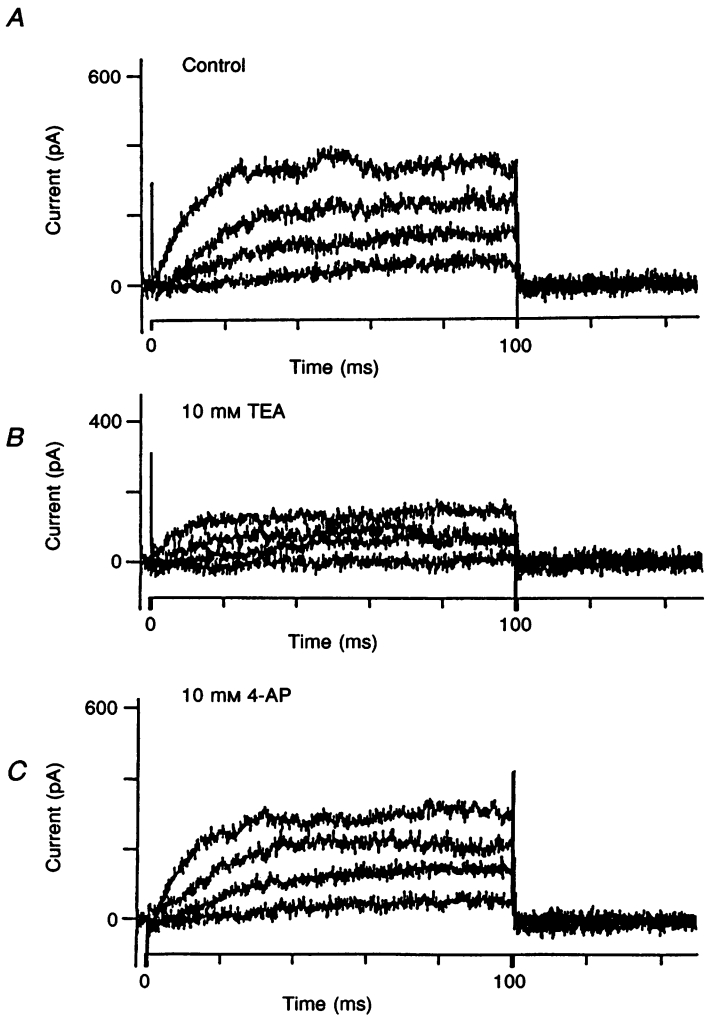

Figure 8. Pharmacology of the low inactivation threshold current, $I_{dK(n)}$

A, current was elicited by step depolarizations to test pulses ranging from −20 to +40 mV from a holding potential of −100 mV with 10 mM TEA in the pipette. *B*, 10 mM TEA applied to the bath solution blocked more than 50% of $I_{dK(n)}$. *C*, 10 mM 4-AP added to the bath solution did not affect this current.

was best fitted with two exponentials with time constants of 1000 ± 100 and 4500 ± 500 ms, respectively, at -10 mV ($n = 25$). Application of 10 mM 4-AP ($n = 8$) or 10 mM TEA ($n = 5$) to the bath solution did not significantly affect the time constants for inactivation.

We investigated the pharmacology of this low inactivation threshold current, $I_{dK(n)}$, in experiments where 10 mM TEA was included in the pipette solution. Figure 8 shows $I_{dK(n)}$ activated by test pulses ranging from -20 to $+40$ mV from a holding potential of -100 mV. This current was not affected by 10 mM 4-AP ($n = 3$, for example Fig. 8*C*) or 100 nM charybdotoxin ($n = 3$) but was sensitive to extracellular TEA application. $I_{dK(n)}$ was reduced $57 \pm 4\%$ ($n = 6$ cells) measured at +10 mV after application of 10 mM TEA to the bath solution (Fig. 8*B*). Figure 9 shows the dose–response curve for block of $I_{dK(n)}$ (●) by TEA. Half-maximal block occurred at 7·7 mM ($n = 6$). Figure 9 also shows data from the four experiments where 10 mM 4-AP was included in the pipette solution instead of TEA, for comparison (○). These data show that the low inactivation threshold current $I_{dK(n)}$ is significantly less sensitive to external TEA than the slowly activating, 4-AP-insensitive current $I_{dK(s)}$ ($P < 0{\cdot}001$).

$I_{dK(n)}$ also differed from $I_{dK(s)}$ in its activation kinetics. The time constant of activation for $I_{dK(n)}$ was $21{\cdot}0 \pm 3{\cdot}2$ ms ($n = 4$) at +10 mV compared with $40{\cdot}3 \pm 7{\cdot}6$ ms for $I_{dK(s)}$ ($P < 0{\cdot}001$). It is likely that because of the small amplitude and negative inactivation range, $I_{dK(n)}$ was missed in an earlier study using slowly depolarizing voltage ramps (Du *et al.* 1994). The properties of (i) the mixed delayed rectifier current (control current), (ii) current in the presence of 10 mM 4-AP, (iii) current in the presence of 10 mM TEA in the bath, and (iv) current with 10 mM TEA in the pipette solution are summarized in Table 1.

Current components underlying I_{dK} have similar rectifying properties

Delayed rectifier current in guinea-pig cardiac myocytes was recently shown to be composed of two components: a rapidly activating current I_{Kr}, sensitive to the class III anti-arrhythmic agent E-4031, and a more slowly activating current I_{Ks} (Sanguinetti & Jurkiewicz, 1990). Since I_{Kr} is strongly inwardly rectifying, these currents could be separated using a tail current envelope protocol. In order to determine whether the smooth muscle currents $I_{dK(f)}$ and $I_{dK(s)}$ are related to the cardiac I_{Kr} and I_{Ks}, respectively, we applied the envelope of tail test used by Sanguinetti & Jurkiewicz (1990). Outward currents were elicited by test pulses of various durations to +40 mV from a holding potential of -90 mV. Following the test pulse, cells were repolarized to -40 mV to observe tail currents. The ratio of tail currents divided by current at the end of the depolarizing test pulse was plotted as a function of test pulse duration (Fig. 10).

Tail current ratios were, on average, 0·20, in good agreement with current flow through channels that show only Goldman-type rectification (expected tail current ratio $I(-40\text{ mV})/I(+40\text{ mV})$, calculated from the Goldman–Hodgkin–Katz equation, is 0·18 for a K^+ gradient of 5·9/140 mM). No significant time dependence was observed when the test pulse duration was varied between 10 and 100 ms (Fig. 10*A*) or between 200 and 1400 ms (Fig. 10*B*). Application of 10 mM TEA resulted in

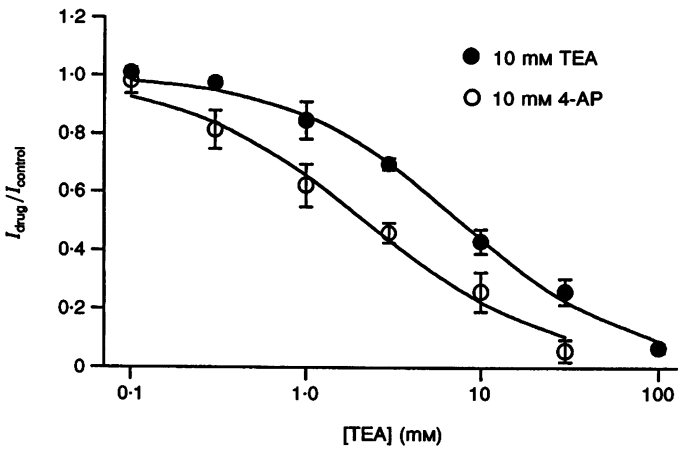

Figure 9. Dose–response relations for block of $I_{dK(n)}$ by TEA

Current was measured at the end of a 100 ms depolarizing test pulse to +10 mV from a holding potential of -100 mV. TEA (10 mM) was included in the pipette solution (●). Data are fitted by a Hill function with parameters: $EC_{50} = 7{\cdot}7$ mM, $n_H = 0{\cdot}9$, $C = 0$. ○, dose–response relation for current from cells with 10 mM 4-AP instead of TEA included in the pipette solution. Data are fitted by a Hill function with parameters: $EC_{50} = 2{\cdot}2$ mM, $n_H = 0{\cdot}8$, $C = 0$.

a 17% reduction of the tail current ratios, probably due to voltage-dependent block by the positively charged TEA ions. These results indicate that, unlike the situation in cardiac myocytes, $I_{dK(f)}$ and $I_{dK(s)}$ cannot be separated by a tail current envelope protocol, implying that the rectifying properties of these two current components are similar in colonic smooth muscle cells.

DISCUSSION

Delayed rectifier current in canine colonic smooth muscle cells appears to be composed of three distinguishable components: (1) a rapidly activating, 4-AP-sensitive, TEA-insensitive current, $I_{dK(f)}$; (2) a more slowly activating current, $I_{dK(s)}$, which is insensitive to 4-AP but blocked by external TEA; and (3) a low inactivation threshold current, $I_{dK(n)}$, which is also insensitive to 4-AP but less sensitive to TEA than $I_{dK(s)}$ and activates more rapidly than $I_{dK(s)}$ (Table 1).

The separation of $I_{dK(f)}$ and $I_{dK(s)}$ was suggested by the time dependence of 4-AP block and relies on the proposed mechanism of action of 4-AP. Block of K^+ current by 4-AP shows complicated time, voltage and use dependence in many preparations (Yeh, Oxford, Wu & Narahashi, 1976; Meves & Pichon, 1977; Thompson, 1982; Campbell, Qu, Rasmusson & Strauss, 1993). This time, voltage and use dependence is caused by the 4-AP molecule interacting selectively with particular channel states and the proportion of channels in closed, open or inactivated states at a given time and voltage. The channel state with which 4-AP preferentially interacts differs with tissue and species source, suggesting that delayed rectifier channels comprise a heterogeneous group of channels with respect to the 4-AP binding site and its interaction with other functional groups (e.g. the voltage sensor and gate of the K^+ channel molecule). For example, block of the transient outward current I_{to} by 4-AP in ventricular myocytes occurred preferentially in the closed

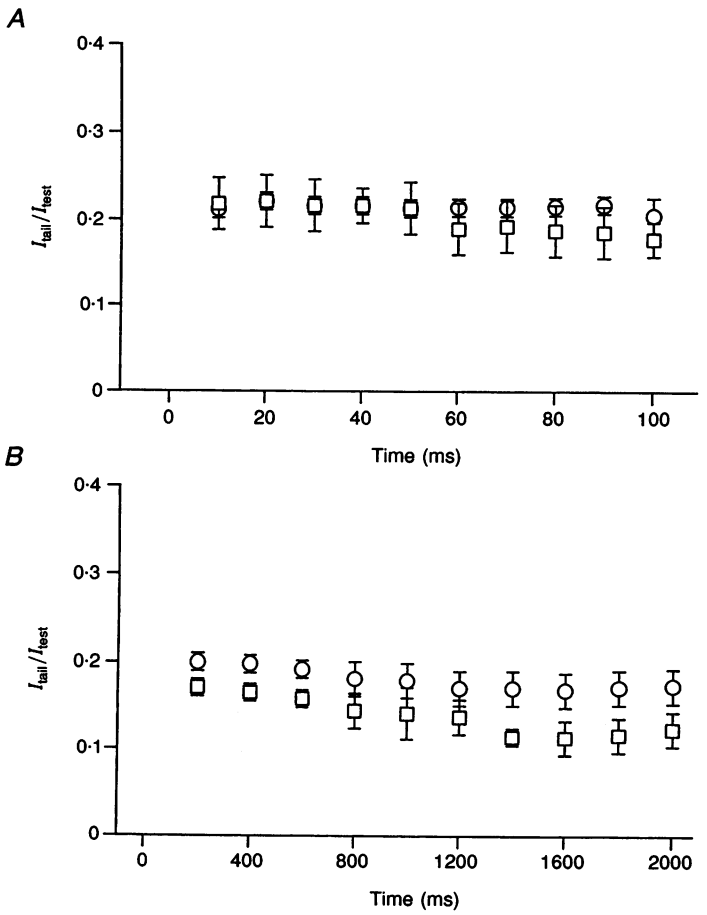

Figure 10. Tail current envelopes

Outward currents were elicited by test pulses of durations between 10 and 100 ms (*A*) or 200 and 1400 ms (*B*) to +40 mV from a holding potential of −90 mV. Following the test pulse, cells were repolarized to −40 mV to observe tail currents. The ratio of tail currents divided by current at the end of the depolarizing test pulse was plotted as a function of test pulse duration (○). After application of 10 mM TEA to the bath solution this protocol resulted in smaller tail current ratios (□).

state ('reverse use dependence'; Campbell *et al.* 1993). In contrast, channel openings were required for 4-AP to interact with K^+ channels in murine B lymphocytes (Choquet & Korn, 1992). This is similar to the proposed mechanism of block of K^+ channels in colonic smooth muscle cells (this study). Our data do not exclude the possibility that binding and unbinding of 4-AP to the channel molecule occurred independently of channel opening, but required membrane depolarization and therefore appeared to be correlated with channel gating.

In order to explain the results of Fig. 1 by 4-AP binding kinetics, one would have to assume that 4-AP binds to the channel either in the closed state or immediately after the first channel opening, and that the block becomes weakened during the on-going depolarization (Ulbricht & Wagner, 1976). However, we did not detect any noticeable frequency dependence of block. Furthermore, membrane depolarization was required for both binding and unbinding of the 4-AP molecule to occur. Finally, in the voltage range 0 to +30 mV steady-state block of delayed rectifier current by 4-AP was not voltage dependent. Therefore, the time dependence of 4-AP block cannot be explained by its voltage or use dependence and is more probably due to the presence of two distinct current components, one rapidly activating and 4-AP sensitive, the other more slowly activating and 4-AP insensitive. This conclusion is supported further by studies of the mechanism of 4-AP block of delayed rectifier channels cloned from canine colonic myocytes and expressed in *Xenopus* oocytes. cDNA from smooth muscle cells of the canine colon was isolated and gene amplification products encoding K^+ channels (CSMK1 and CSMK2) were identified. CSMK1, which belongs to the $K_V1.2$ class of K^+ channels, when expressed in *Xenopus* oocytes, showed rapid activation (time for half-maximal activation, $t_{½}$, of 7·6 ± 0·2 ms at +20 mV, $n = 6$) and was highly sensitive to 4-AP ($K_D = 75\ \mu$M; Hart *et al.* 1993). 4-AP (10 mM) caused > 90% block of CSMK1 current. These properties are very similar to the rapidly activating K^+ current $I_{dK(f)}$. With 10 mM TEA in the bath, time for half-maximal activation of $I_{dK(f)}$ was 9·3 ± 0·8 ms at +20 mV ($n = 11$). The slope conductance of CSMK1 expressed in *Xenopus* oocytes was 14 pS ($n = 3$) in symmetrical 140/140 mM KCl (Hart *et al.* 1993), a value similar to 4-AP-sensitive delayed rectifier channels observed in excised patches from colonic myocytes (S. D. Koh, unpublished observation). Therefore, it is likely that CSMK1 underlies $I_{dK(f)}$. Block of CSMK1 current by 4-AP (0·1 mM) was time independent over 1 s duration step depolarizations (60 ± 10% block at 10 ms, 59 ± 8% block at 100 ms at +20 mV, $n = 5$; Russell *et al.* 1994) giving further support to the notion that the observed time dependence of 4-AP block of macroscopic whole-cell current in native myocytes is due to block of distinct channel types with different activation kinetics.

Delayed rectifier channels in squid axon are known to be sensitive to TEA applied to the cytoplasmic membrane surface (Armstrong & Binstock, 1965; Armstrong, 1975; Stanfield, 1983). When we included 10 mM TEA in the pipette solution 'delayed rectifier' current was absent during voltage ramps, when membrane potential was slowly (0·05 mV ms^{-1}) depolarized (Du *et al.* 1994). However, when membrane potential was step depolarized from very negative holding potentials (this study), a small-amplitude inactivating current component was still present. This current had properties quite distinct from delayed rectifier current measured in the absence of TEA in the pipette solution: the voltage for half-maximal inactivation (half-maximal availability) was ~13 mV more negative than that of the mixed current. It is very unlikely that the shift in voltage for half-maximal inactivation was due to a voltage-dependent block of current by internal TEA since the interaction of TEA with K^+ channels is fast. In contrast, the difference in current availability was seen even at the end of a 5 s test pulse to +10 mV (Fig. 7). Therefore, it is likely that $I_{dK(n)}$ constitutes a distinct component of 'delayed rectifier' current. This current was completely insensitive to 4-AP up to a concentration of 10 mM but was blocked by external TEA with an EC_{50} of 7·7 mM.

While $I_{dK(f)}$ represents the 4-AP-sensitive current delayed rectifier component and $I_{dK(n)}$ could be easily isolated from the remaining components by adding 10 mM TEA to the pipette solution, we currently have no protocol or pharmacological tool to isolate $I_{dK(s)}$. Some features of $I_{dK(s)}$ and the current observed when 10 mM TEA was included in the pipette solution ($I_{dK(n)}$) are similar; both currents were sensitive to external TEA but not to 4-AP (10 mM). However, $I_{dK(s)}$ appeared to be more sensitive to TEA than $I_{dK(n)}$. It is not clear whether $I_{dK(s)}$ and $I_{dK(n)}$ are carried by different K^+ channel proteins or are current through the same type of channel observed under different conditions. In experiments using voltage ramps, both 4-AP- and TEA-sensitive currents were abolished when 10 mM TEA was included in the pipette solution (Du *et al.* 1994). It seems possible that $I_{dK(s)}$ is blocked by internal TEA, but to a slightly lesser degree than $I_{dK(f)}$ so that the remaining current ($I_{dK(n)}$) would be the residual, unblocked portion of $I_{dK(s)}$. If that is the case, the low inactivation threshold current $I_{dK(n)}$ should provide a substantial contribution to overall outward current and one would predict a biphasic steady-state inactivation curve of the mix current. While in most experiments the steady-state inactivation curve appeared monophasic, i.e. suggesting that $I_{dK(f)}$ and $I_{dK(s)}$ have similar inactivation voltages and implying that $I_{dK(s)}$ and $I_{dK(n)}$ are distinct entities, in some experiments the steady-state inactivation curves of the mixed currents were indeed biphasic (K. Thornbury, unpublished observation). Clearly, more studies, preferably on the

single channel level, are required to determine the number and properties of K^+ channels which, taken together, constitute the delayed rectifier current in colonic myocytes.

The currents identified in this study do not belong to the class of A-currents. A-currents are blocked by millimolar concentrations of 4-AP and are characterized by fast inactivation and half-maximal inactivation at very negative potentials (Beech & Bolton, 1989; Lang, 1989; Vogalis, Lang, Bywater & Taylor, 1993). In contrast, $I_{dK(f)}$ showed slow inactivation and a half-maximal voltage for inactivation of approximately −38 mV (measured from the current in the presence of 10 mM external TEA). The low inactivation threshold current identified by including 10 mM TEA in the pipette solution was completely insensitive to 4-AP. Furthermore, block of A-current by 4-AP in guinea-pig colon was not use dependent (Vogalis *et al.* 1993), while block in canine colonic myocytes shows strong use dependence. A-currents can be isolated by subtracting currents elicited from holding potentials around −40 mV from currents elicited from more negative holding potentials (−100 mV; Beech & Bolton, 1989; Lang, 1989). In canine colon, these difference currents were insensitive to 4-AP (1 mM) (Thornbury *et al.* 1992*a*) suggesting that A-currents are not present in canine colonic smooth muscle cells.

Smooth muscle tissue has been classified into phasic and non-phasic types, and the phasic muscles can be further divided into spiking and non-spiking types (Golenhofen, 1976). In the canine proximal colon, longitudinal tissue is of the spiking type while circular tissue is non-spiking (El-Sharkawy, 1983; Sanders, 1992). Therefore, it may be of value to compare the properties of the delayed rectifier components identified in circular layer myocytes with those previously described in circular and longitudinal layer colonic smooth muscle cells. Thornbury, Ward & Sanders (1992*b*) reported that outward current in circular myocytes was dominated by a 4-AP-sensitive current (EC_{50} = 1–5 mM at 37 °C), while outward current in myocytes from the longitudinal layer was dominated by a TEA-sensitive current (EC_{50} = 2–5 mM) which was more resistant to 4-AP (5 mM caused only 20% block at potentials positive to 0 mV). Interestingly, the TEA-sensitive current in longitudinal myocytes also showed inactivation negative of current from circular myocytes (−63 mV in longitudinal *vs.* −36 mV in circular myocytes, measured at 37 °C), thereby resembling the low inactivation threshold current, $I_{dK(n)}$, identified in this study. Similarly, delayed rectifier current in longitudinal cells also activated as rapidly as delayed rectifier current in circular myocytes. It is possible that longitudinal cells express K^+ channels qualitatively different from those in circular myocytes, or it may be speculated that longitudinal cells express the same types of channels, but in a quantitatively different proportion. Future studies will be necessary to determine the relationship between currents expressed in longitudinal myocytes and $I_{dK(n)}$ of circular myocytes. Differential expression of K^+ channels may be a major determinant of electrical activity types of smooth muscles in the Golenhof scheme.

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