Alteration of the Transmembrane K⁺ Gradient During Development of Delayed Rectifier in Isolated Rat Pulmonary Arterial Cells

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ABSTRACT The properties of the tail current associated with the delayed rectifier K⁺ current (Iₖ) in isolated rat pulmonary artery smooth muscle cells were examined using the whole cell patch clamp technique. The tail currents observed upon repolarization to -60 mV after brief (e.g., 20 ms) or small (i.e., to potentials negative of 0 mV) depolarizations were outwardly directed, as expected given the calculated K⁺ reversal potential of -83 mV. The tail currents seen upon repolarization after longer (e.g., 500 ms) and larger (e.g., to +60 mV) depolarizations tended to be inwardly directed. Depolarizations of intermediate strength and/or duration were followed by biphasic tail currents, which were inwardly directed immediately upon repolarization, but changed direction and became outwardly directed before deactivation was complete. When cells were depolarized to +60 mV for 500 ms both Iₖ and the subsequent inward tail current at -60 mV were similarly blocked by phencyclidine. Both Iₖ and the inward tail current were also blocked by 4-aminopyridine. Application of progressively more depolarized 30 s preconditioning potentials inactivated Iₖ and reduced the inward tail current amplitude with a similar potential dependency. These results indicated that the inward tail current was mediated by Iₖ. The reversal potential of the tail current became progressively more positive with longer depolarizations to +60 mV, shifting from -76.1 ± 2.2 mV (n = 10) after a 20-ms step to -57.7 ± 3.5 mV (n = 9) after a 500-ms step. Similar effects occurred when extracellular K⁺ and Na⁺ were replaced by choline. When extracellular K⁺ was raised to 50 mM, the tail current was always inwardly directed at -60 mV, but showed little change in amplitude as the duration of depolarization was increased. These observations are best explained if the dependencies of tail current direction and kinetics upon the duration of the preceding depolarization result from an accumulation of K⁺ at the external face of the membrane, possibly in membrane invaginations. A mathematical model which simulates the reversal potential shift and the biphasic kinetics of the tail current on this basis is presented.

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INTRODUCTION

The delayed rectifier K⁺ current (I_K) in rat pulmonary artery (PA) smooth muscle cells is likely to play an important role in maintaining the resting membrane potential in these cells (Smirnov, Robertson, Ward, and Aaronson, 1994). In characterizing this current, we found that the tail current observed during the deactivation of I_K at ~60 mV was often inwardly directed (Smirnov and Aaronson, 1993). These observations were unexpected, because under the conditions utilized, the reversal potential for K⁺ was near ~83 mV, and the tail current for I_K should thus have been outwardly directed.

There are two possible explanations for the presence of an inward tail current following I_K. Firstly, a current with a reversal potential positive of ~60 mV may have developed at the same time as I_K, and given rise to an inward tail current superimposed upon the outward tail current caused by I_K. An effect of this kind has been proposed to occur in dog coronary artery smooth muscle cells (Wilde and Lee, 1989), where the reversal potential of the tail current associated with the outward current was ~30 mV positive of its value calculated on the basis of the K⁺ gradient.

A second possibility is that the K⁺ reversal potential is shifted towards more positive voltages by a reduction of the transmembrane K⁺ gradient associated with the efflux of K⁺ occurring during K⁺ channel activation. There is evidence that an extracellular accumulation of K⁺ occurs during depolarization in voltage-clamped neurones and cardiac Purkinje fibers (e.g., Frankenhaeuser and Hodgkin, 1956; Neher and Lux, 1973; Cleemann and Morad, 1976; Baumgarten, Isenberg, McDon-ald, and Ten Eick, 1977). The kinetic evidence for extracellular K⁺ accumulation in these multicellular preparations is supported by the existence of a morphological basis for restricted diffusion (e.g., Schwann cells surrounding axons and narrow extracellular clefts in Purkinje fibers). Diffusion barriers of this type would be absent in isolated cells. Recently, however, Yasui, Anno, Kamiya, Boyett, Kodama, and Toyama (1993) have found that activation of the ATP-sensitive K⁺ current in single guinea-pig ventricular cells is associated with the development of an inward tail current, the characteristics of which are best explained by a reduction in the K⁺ gradient. They proposed that this effect was due to the accumulation of K⁺ in transverse tubules.

In this report, we characterize the inward tail current associated with I_K in single rat PA smooth muscle cells. We find that the properties of the tail current can be best explained if it is due to a flux of K⁺ through I_K channels, the direction of which is altered by a reduction of the transmembrane K⁺ gradient occurring during development of I_K. A mathematical model which is able to simulate closely the observed properties of the tail current on the basis of an extracellular near-membrane compartment from which K⁺ diffusion is slowed, is also presented. A preliminary report of this work has been presented (Smirnov and Aaronson, 1993).

METHODS

The methods and solutions used for cell isolation and electrophysiology were similar to those recently described (Smirnov and Aaronson, 1992; Smirnov et al., 1993). Rats were anesthetized with ether, the heart and lungs were removed, and small PA (300–700 μm i.d.) were isolated,
dissected free of connective tissue, cut into pieces (~1 x 1 mm) and allowed to recover for 30 min in a physiological saline solution (PSS). Tissue was then placed in nominally Ca\(^{2+}\)-free PSS for 20 min at 37°C, before enzymatic digestion at 37°C for 20 min in low Ca\(^{2+}\) PSS (15 \(\mu\)M added Ca\(^{2+}\)) containing 1 mg/ml collagenase (Type XI, 1750 U/mg), 1 mg/ml papain (9.5 U/mg), 2 mg/ml bovine serum albumin and 1 mM dithiothreitol (all from Sigma, Poole, Dorset, UK). Tissue was then transferred to enzyme-free Ca\(^{2+}\)-free PSS and triturated with a flame-polished Pasteur pipette in order to disperse cells. The suspension was stored in low-Ca\(^{2+}\) PSS at 4°C for use within 6 h. Membrane currents were recorded using the conventional whole cell patch clamp technique. PSS contained (in mM): 130 NaCl, 5 KCl, 1.2 MgCl\(_2\), 1.5 CaCl\(_2\), 10 HEPES, 10 glucose; pH was adjusted to 7.2 by NaOH. The pipette solution contained (in mM): KCl 110, 2.5 MgCl\(_2\), 2.0 Na\(_2\)ATP, 10 HEPES, and 10 EGTA; buffering to pH 7.2 with KOH brought the total K\(^+\) concentration to 135 mM. The pipette solution also contained either 0.5 or 7.5 mM Ca\(^{2+}\), to bring the free Ca\(^{2+}\) concentration to either 8 or 486 nM (calculated according to Fabiato and Fabiato, 1979). For electrophysiological recording, cells were placed in a chamber of volume 50–100 \(\mu\)l and were continually superfused with PSS or a test solution via a 5-barrel pipette at a rate of ~0.9–1 ml/min. Experiments were carried out at room temperature. At the beginning of each experiment, the cell membrane capacitance was calculated from the area under the capacitive artifact (filtered at 20 kHz and sampled at 50 kHz) elicited by a 10-mV hyperpolarizing step. Data analysis, including fitting linear and exponential expressions to data points, was carried out using SigmaPlot 4.01 (Jandel Scientific, California) software, on an Elonex PC-455 microcomputer. Modeling and simulation of currents was performed using QBASIC. Data values are recorded as mean ± SEM.
Because we were primarily interested in studying tail currents in the present study, it was important to determine the degree to which these might be influenced by possible sources of error such as pipette series resistance. Analysis of capacitive artifacts showed that these decayed monoexponentially with a mean time constant of 100 ± 5 μs (n = 75). Series resistances estimated from the rate of decay of the capacitance artifact ranged between 4 and 20 MΩ (mean 11.7 ± 0.5 MΩ, n = 75). These values were much smaller than the cell input resistance measured during a voltage step from −60 to −90 mV (which ranged from 1.3 to several tens of gigaohms with a mean of 12.7 ± 0.3 GΩ, n = 40). This level of series resistance would be expected to reduce the amplitude of voltage steps by ~10% during the generation of the largest outward currents at very positive test potentials. To examine this possibility more closely, membrane potentials during depolarizing steps were measured using two independent patch pipettes in the same PA cell. After the establishment of the whole cell patch clamp mode with a patch pipette connected to the Axopatch 1B, a second patch pipette, connected to a separate patch clamp amplifier (a Biologic RK-300), was sealed onto each cell. After rupture of the membrane patch at its tip, this second pipette was used to measure the voltage step evoked by a test pulse from −60 to +60 mV applied to the first pipette. Fig. 1 illustrates a comparison of the voltage step imposed on the first pipette, and the actual transmembrane voltage step recorded using the second pipette. The mean voltage step measured in the five cells was 104.3 ± 5.2 mV, a value which was 13% smaller than that of the applied voltage step. This value was virtually identical to that predicted on the basis of the series resistance and current amplitude measured in these cells (104.7 ± 5.3 mV). The small initial sag in the measured voltage step (trace 2) probably reflected the slightly delayed increase in membrane conductance due to the activation of the current. The measured voltage fell monotonically to the holding potential with a time constant of 0.9 ± 0.3 ms (n = 5). Given the relatively slow decay of the tail currents (typical time constants of 10–40 ms), it was unlikely that the pipette resistance and capacitance were markedly distorting the kinetics of these currents. In addition, the reduction in the size of the voltage step caused by the series resistance was essentially irrelevant to the analysis carried out in this study. For these reasons, compensation of pipette resistance and cell capacitance was not deemed necessary.

RESULTS

Morphology and Passive Membrane Properties of Freshly Isolated PA Cells

Enzymatic dissection yielded partially contracted, elongated and round PA cells. The partially contracted and elongated cells were spindle shaped. Their lengths ranged between 24 and 61 μm with mean of 36.5 ± 1.2 and the average width, measured in the region of cell nucleus, was 7.8 ± 0.2 μm in 53 PA cells. Assuming a spindlelike cell shape, the average surface area was calculated to be 450 μm². Partially contracted and elongated PA cells reversibly contracted in response to isotonic KCl and were similar in their electrophysiological properties. The round cells were not used for experiments.

Membrane potential recorded immediately after rupture of the cell membrane in 57 PA cells ranged between −31 and −87 mV with mean value of −54.3 ± 2 mV. The mean cell membrane capacitance measured as described in the Methods was 12.7 ± 0.3 pF (n = 199). Assuming a specific membrane capacitance of 1 μF/cm², the total surface area calculated from the mean value of the cell membrane capacitance was 1,270 μm², a value 2.8 times that estimated from the cell dimensions.
This may be due to the presence of invaginations or infoldings on the surface of the cell membrane (Gabella, 1981). Multiplication of the input resistance (12.7 MΩ, see Methods) and the capacitive membrane area gave a value for the specific membrane resistance of 160 kΩ cm².

**Separation of Outward Potassium Currents**

With a patch pipette filled with the high Ca²⁺ solution and PSS in the bath a step membrane depolarization to -30 mV from the holding potential -60 mV elicited a slowly activating outward current. Its amplitude and rate of activation increased with further depolarization. At membrane potentials positive of +30 mV large fluctuations were superimposed on the main current (Fig. 2A, left). These fluctuations were suppressed by 10 mM TEA, which also reduced the current amplitude (Fig. 2A, middle). Further addition of 5 mM 4-aminopyridine (4-AP) caused almost complete block of the outward current (Fig. 2A, right). Note that a large inward tail current developed after 500-ms depolarization to positive membrane voltages, which was completely blocked by 4-AP (Fig. 2A).

**Figure 2.** Effects of TEA and 4-AP on K⁺ currents in PA cells dialyzed with high- and low-Ca²⁺ pipette solutions. (A) Outward currents at the test potentials indicated in a cell dialyzed with a pipette solution containing a calculated [Ca²⁺] of 486 nM (high Ca²⁺), under control conditions (left), in the presence of 10 mM extracellular TEA (center) and in the presence of 10 mM TEA and 5 mM 4-AP (right). (B) Outward currents in a cell dialyzed with a pipette solution containing a calculated [Ca²⁺] of 8 nM (low Ca²⁺), measured under the same conditions listed for A. (C) Mean current-voltage relationships for 6 high Ca²⁺ perfused cells in the absence of drugs (○), in the presence of 10 mM TEA (□), and in the presence of 10 mM TEA and 5 mM 4-AP (◇). (D) Mean current-voltage relationships for five low Ca²⁺ perfused cells, with conditions and symbols as in C.
A different situation was observed when Ca\(^{2+}\) concentration in the pipette solution was set to 8 nM. The outward current recorded under this condition appeared to be smoother and the effect of TEA was less significant (Fig. 2 B). However, 5 mM 4-AP still blocked the remaining current to an extent similar to that in cells perfused with the high Ca\(^{2+}\) solution (Fig. 2 B, right). A 4-AP-sensitive inward tail current was also observed under these conditions. The results are summarized in C and D where current-voltage (I-V) relationships recorded over a voltage range between -60 and +60 mV are shown. The I-V relationships illustrate that when the high Ca\(^{2+}\) pipette solution was used, the contribution of TEA-sensitive current to the net outward current appeared to be small (16–19%) in the membrane potential range between -30 and -10 mV and increased to 30% at +60 mV (Fig. 2 C). The TEA-sensitive current almost disappeared with the low Ca\(^{2+}\) pipette solution; TEA decreased the current by only 3–4% between -30 and -10 mV and by ~7% at +60 mV (Fig. 2 D).

Therefore, based on the sensitivity to TEA and intracellular Ca\(^{2+}\) concentration, we concluded that the fluctuating component of outward current was due to a Ca\(^{2+}\)-activated K\(^{+}\) current. The other component, as will be shown below, had a high sensitivity to 4-AP and phencyclidine (PCP), and was inactivated by depolarization of the holding potential; on this basis we classified it as a delayed rectifier current (I\(_K\)). From the experiment shown above it is clear that I\(_K\) is the dominant outward current in rat pulmonary arterial cells throughout the physiological range of the membrane potential, while Ca\(^{2+}\)-activated K\(^{+}\) current became obvious only at positive voltages.

The inward tail current which is the subject of the present study was observed under the conditions described below irrespective of the pipette solution used, and whether or not TEA was present (Fig. 2, A and B). To facilitate characterization of the tail current, however, all subsequent experiments were carried out using the low-Ca\(^{2+}\) pipette solution, and in the presence of 10 mM TEA in PSS (TEA-PSS), in order to ensure that I\(_K\) was not superimposed by Ca\(^{2+}\)-activated K\(^{+}\) current.

**Inward Tail Current after Development of I\(_K\)**

Fig. 3 A illustrates a typical set of I\(_K\) currents elicited during a sequence of 500-ms step depolarizations from -40 to +60 mV. The tail currents associated with this family of currents are shown using expanded coordinates in Fig. 3 B. In the negative voltage range I\(_K\) was followed by an outward tail current when the potential was returned to -60 mV. However, at membrane potentials positive of +20 mV the tail current changed its direction and become inward. The size of this inward tail current (hereafter referred to as I\(_t\)) increased progressively with further depolarization, as did the amplitude of the outward current elicited during the voltage step. I-V relationships for the current measured at the end of 500 ms depolarization (○) and the tail current measured 2.5–3 ms after the offset of the voltage step at the holding potential -60 mV (●) are presented in Fig. 3 C and using an expanded scale in D. These are average results obtained from 31 PA cells. A net inward tail current of variable amplitude up to 680 pA was observed in these cells following the step to +60 mV, while in nine other PA cells studied, the tail current had an outward direction after this step. The kinetics of inward tail currents measured at -60 mV after a 500-ms voltage step to +60 mV could be satisfactorily fitted by single exponential
(data not shown). The average time constant was $28.1 \pm 5.2$ ms measured in 18 cells which had only inward tail currents.

**Time-dependent Alterations in $I_{it}$**

The results described above show that in the majority of cells an inward tail current become increasingly prominent as the test potential was progressively made more positive. Further experiments revealed that increasing the duration of the voltage pulse at a given level of depolarization also promoted the appearance of an inward tail current. Fig. 4 represents two typical examples of this phenomenon observed when the duration of the depolarization to $+60$ mV was incremented in successive steps. The family of outward currents evoked in one cell are shown superimposed in Fig. 4Aa, as are the tail currents elicited when the cell was stepped back to $-60$ mV. These latter are expanded in Fig. 4Ab. The tail current observed after a 6-ms step to $+60$ mV was outwardly directed. As the duration of depolarization was incremented in 20-ms steps, the tail current became biphasic; an initial inward component rapidly gave way to a flattened and more slowly decaying outward component. The former component increased, and the latter decreased, as the duration of the preceding depolarization was increased, such that only the inward component could be seen.

**FIGURE 3.** Delayed rectifier current and subsequent tail currents. (A) A family of delayed rectifier currents activated by 500 ms depolarising voltage steps in 10-mV increment between $-40$ and $+60$ mV. (B) Selected tail currents from A shown using an expanded scale; mV values refer to the test potential during the preceding 500-ms step. (Dashed line) Zero current level. (C) Mean $I-V$ relationship of the amplitudes of $I_K$ (measured at the end of 500-ms steps) (○) and of the subsequent tail current (●) measured between 2.5 and 3 ms after the end of the test pulse in 31 PA cells. (D) The same $I-V$s but at an expanded scale.
after depolarizations of more than \( \sim 200 \) ms. The amplitude of the inward tail current then continued to increase with longer depolarizations. Tail currents in 13 of 19 cells studied exhibited this type of behavior. The tail currents observed in a second cell subjected to an identical voltage stepping protocol are shown in Fig. 4 B. In this cell, which was typical of 6/19 cells examined, the tail current was outwardly directed after brief voltage pulses, but became biphasic with longer pulses, such that an initial inward component became progressively larger as the duration of the pulse was increased.

The relationship between the initial amplitude of the tail current, expressed as current density, and the duration of the preceding depolarizing step to +60 mV, was determined in the 13 cells exhibiting tail currents similar to those shown in Fig. 4 A,

![Graph showing tail current development](image)

using the voltage protocol described above. The averaged data, shown in Fig. 5, indicate that the tail current amplitude tended to saturate within 300 ms.

A qualitatively similar result was obtained in four PA cells where the cell membrane was stepped to 0 mV instead of +60 mV. The membrane depolarization elicited outward currents of between 240 and 1,200 pA which were followed by tail currents which were outwardly directed after the short (6 ms) pulse, but which changed direction and became progressively more inwardly directed with more prolonged test pulses. In four other cells studied, increasing the duration of the voltage step to 0 mV resulted in a decrease in the outward tail current amplitude without an appearance of a net \( I_{\text{a}} \) (data not shown).
Effects of Drugs and Conditioning Potential on $I_K$ and $I_{it}$

The appearance of an inward tail current when cells were repolarized to $-60 \text{ mV}$ was unexpected because the calculated reversal potential for $K^+$ was $-83 \text{ mV}$, such that $I_K$ should give rise to only an outward tail current. One explanation for the existence of $I_{it}$ was that an additional conductance which had a reversal potential positive of $-60 \text{ mV}$ was being activated at positive potentials. Based on the results shown in Figs. 4 and 5, this conductance would be expected to be time-dependent, i.e., more

\[ I = \frac{(1 - C)}{1 + \frac{IC_{50}}{\text{[Drug]}}} + C, \]

where $I$ represents the ratio of currents in the presence and absence of the drug. $IC_{50}$ is an apparent dissociation constant equal to 41.1 and 31.8 $\mu\text{M}$ and $C$ is a constant equal to 0.09 and 0.04 for $I_K$ at 500 ms and $I_{it}$, respectively.
current would be activated with longer depolarizations. The change in kinetics of the tail current shown in Fig. 4 might therefore be the result of the superimposition of the tail current contributed by this putative conductance upon the tail current contributed by $I_K$.

If $I_g$ were indeed due to a current other than $I_K$, pharmacological blockade of $I_g$ and the outwardly directed tail current caused by its deactivation, should accentuate the inward tail current. PCP and 4-AP have been shown to be effective blockers of $I_K$ in rabbit portal vein (Beech and Bolton, 1989) and pulmonary artery (Okabe, Kitamura, and Kuriyama, 1987), human mesenteric artery (Smirnov and Aaronson, 1992) and canine renal artery (Gelband and Hume, 1992). Moreover, we found that 4-AP and PCP also effectively suppressed the outward current in PA cells.

Figs. 6 and 7 show the effects of PCP and 4-AP upon the amplitude of the current during 500-ms depolarizing steps to +60 mV, and upon the amplitude of $I_g$ after the membrane potential was stepped back to −60 mV. Cells with a large $I_g$ were selected for these studies to facilitate its measurement.

Fig. 6 (A and B) illustrates that PCP blocked both the outward current and the subsequent $I_g$ in a concentration-dependent manner. The effect of PCP was completely reversible (not shown). The concentration-dependencies of the blockade of the outward current and $I_g$ were very similar. Concentration-response curves were well fitted by the Langmuir equation with apparent $IC_{50}$ of 41.1 and 31.8 μM for the current at the end of the 500-ms pulse, and the subsequent $I_g$, respectively (Fig. 6 C).
A similar effect was observed in the presence of 4-AP. Both the current at the end of 500 ms, and \( I_n \), were reversibly blocked by 4-AP in a concentration-dependent manner by 4-AP in the cell shown in Fig. 7, A and B. An IC_{50} value of 110 \( \mu \)M was measured for \( I_K \) in five cells (Fig. 7 C). Blockade of \( I_n \) at -60 mV was even more sensitive to low doses of 4-AP than was the outward current. In the cell illustrated, as well as in six other cells, application of 0.1 mM 4-AP blocked the \( I_n \) by 50–80%.

These experiments show that the outward current and \( I_n \) were both sensitive to two pharmacological blockers of \( I_K \), suggesting therefore that both the outward current

\[ I = \frac{1 - C}{1 + \exp \left( \left( V - V_{0.5} \right) / k \right)} + C, \]

with half-inactivation potentials \( (V_{0.5}) \) of 24.8 and -26.4 mV; slope factors \( (k) \) of 9.1 and 6.1 mV and fraction of noninactivating current \( (C) \) of 0.16 and 0.09 for 500-ms pulses and \( I_n \), respectively.

\[ I \] and \( I_n \) were mediated by \( I_K \). Although the concentration-response relationships for the block of the current and \( I_n \) by 4-AP were somewhat different, it has been shown in squid axon (Yeh, Oxford, Wu, and Narahashi, 1976), and in other tissues including smooth muscle cells (Smirnov, Zholos, and Shuba, 1992), that 4-AP blocks the outward current in a potential-dependent manner. It was found that the extent of the blockade of the outward current by 4-AP increased with hyperpolarization of the cell membrane and decreased with depolarization. This would be expected to promote the action of 4-AP at -60 mV relative to +60 mV, and might therefore lead to the more potent effect of 4-AP on \( I_n \) compared to \( I_K \).

Additional evidence supporting the hypothesis that \( I_n \) was mediated by \( I_K \) channels was obtained by examining the potential-dependency of the inactivation of \( I_K \) and \( I_n \).
It is well known that $I_K$ is inactivated by depolarization of the holding potential (reviewed by Rudy, 1988). This effect has been described in a variety of types of smooth muscle cells (Hume and Leblanc, 1986; Beech and Bolton, 1989; Yamamoto, Hu, and Kao, 1989; Volk, Matsuda, and Shibata, 1991; Smirnov and Aaronson, 1992). We attempted to separate $I_K$ from other conductance(s) which might contribute to $I_u$ on this basis. An experimental protocol analogous to that used to study the pharmacological sensitivity of $I_K$ and $I_u$ was applied. Current amplitude was measured at the end of a 500-ms test pulse to +60 mV, and 2.5–3 ms after repolarization. In all cases, however a 30-s conditioning prepulse to various membrane potentials preceded the test pulse. As can be seen from Fig. 8 (A and B) both $I_K$ and $I_u$ were similarly depressed as the conditioning potential was made more positive. As illustrated in Fig. 8 C, the half-inactivation potentials obtained from the fit of the steady state inactivation dependencies by the Boltzmann function were similar for the outward current measured after 500 ms (−24.8 mV), and for $I_u$ (−26.4 mV).

Additionally, both the outward and inward tail currents were not affected by application of 50 μM niflumic acid, or 0.3 mM Cd²⁺, or by Ca²⁺-free external solution. After replacement of K⁺ by Cs⁺ ions both in the external and pipette solutions the outward current was blocked and the inward tail current was not observed. Under these conditions a small inward current through Ca²⁺ channels could be recorded in the presence of 10 mM Ba²⁺ (data not shown).

**Alteration of the Reversal Potential During the Development of $I_K$**

The results presented above indicate that both the outward current activated by depolarization and the subsequent inward tail current are mediated by delayed rectifier K⁺ channels. Although the presence of an inward tail current might then be explained if this channel is poorly selective for K⁺, such a lack of selectivity would not explain why the direction of the tail current is dependent both upon the magnitude and duration of depolarization. All of these observations can be explained, however, if the development of the current is accompanied by a shift in the reversal potential of a K⁺ selective $I_K$ channel, due to a reduction in the K⁺ gradient. This might occur if [K⁺] near either the intra- or extracellular face of the cell membrane is progressively altered due to K⁺ efflux during the development of $I_K$.

To examine this possibility, $I_K$ was activated by voltage steps to +60 mV of different durations (15–500 ms). The cell was then repolarized to different membrane potentials in the absence and presence of 5 mM 4-AP. The upper set of traces in Fig. 9 illustrate the tail currents observed in one cell after depolarizing steps of 20 (Fig. 9 A), 100 (B), and 500 ms (C). When this procedure was repeated in the presence of 5 mM 4-AP, the tail current was almost abolished, showing the time course of the capacitive artifact, which decayed much more rapidly than the tail currents (middle traces). Subtraction of the tail currents measured in the presence of 4-AP from those in its absence revealed the tail currents with minimal distortion by the capacitive artifacts (bottom traces).

These sets of tail currents were used to evaluate the effect of depolarizing pulse duration upon the reversal potential of $I_K$, as illustrated in Fig. 10 A. The amplitudes of the tail currents in the absence of 4-AP (open symbols), and of the 4-AP-sensitive tail
currents (solid symbols) were measured 2.5–3 ms after repolarization. These amplitudes are plotted against repolarization potential for the steps of 20 (circles), 100 (squares), and 500 ms (diamonds). Straight lines were fitted to each set of data in order to estimate the reversal potential. The estimates of the reversal potential derived from the tail currents themselves, and from the 4-AP-sensitive tail currents, were indistinguishable for the 100- and 500-ms steps. For the 20-ms step, the reversal potential measured using the 4-AP-sensitive tail current was 5–6 mV negative of that estimated using the tail current itself, probably because of contamination of the tail current, which was quite small at this time, by the capacitive artifact. The 4-AP-

Figure 9. Alteration in the reversal potential during development of $I_K$. (A–C) Tail currents recorded upon repolarization from $+60$ mV to a series of negative potentials, varied in 5 mV increments over the voltage range indicated, after depolarizations of 20, 100, and 500 ms, respectively. In each panel, the upper trace represents the tail current in the absence of 4-AP, the middle trace shows the tail current in the presence of 5 mM 4-AP, and the bottom trace shows the 4-AP-sensitive tail current. Dotted lines show the zero-current level.

sensitive tail current, which should not be contaminated by the capacitive artifact, was therefore used for estimates of the tail current amplitude in this set of experiments.

It is clear that increasing the pulse duration resulted in a progressive positive shift of the reversal potential estimated using either approach. With the shorter pulses, the reversal potential of the 4-AP-sensitive current was close to its predicted value (−83 mV). Increasing the pulse duration to 100 and 500 ms resulted in a progressive positive shift of the reversal potential to −54.1 (▼) and −51.3 mV (▲), respectively. As shown in Fig. 10 B, the average values of the reversal potential obtained using this protocol were −83 (n = 1), −75.3 ± 2.4 mV (n = 9), −71.1 ± 7 (n = 4), −60.4 ± 4.1
that shift in the reversal potential was largely completed within the first 100 ms of depolarization (Fig. 10 B).

$I_h$ in Elevated External $K^+$

The shift in the reversal potential during the development of $I_h$ shown above suggests the existence of some compartment adjacent to the cell membrane where $[K^+]$ is altered during $I_h$, leading to a reduction of the $K^+$ gradient. In this case, it would be predicted that smaller effects of pulse duration upon the reversal potential, and thus upon tail current amplitude and kinetics should occur if extracellular $K^+$ was raised, both because net $K^+$ flux should be smaller, and because redistribution would lead to a smaller change in the ratio of extracellular to intracellular $K^+$ concentrations. To study the effect of elevated $K^+$ on the tail currents the external [K$^+\text{] was raised from 5 to 50 mM K$^+$ (using an equimolar substitution of 45 mM NaCl by KCl). Fig. 11 A illustrates tail currents recorded at $-60 \text{ mV}$ after a test depolarization of various durations in 5 mM K$^+$ TEA-PSS. A short (6 ms) depolarization was followed by a small outward tail current, which become progressively inward and gradually increased in its amplitude with longer depolarizations applied in 80-ms increments. The average amplitude of the tail current, measured in five cells as
already described above, was equal to $-28 \pm 13$ pA after 6 ms depolarization and increased to $-129 \pm 40$ and $-203 \pm 61$ pA after 86 and 326 ms pulses, respectively. Application of 50 mM K$^+$ TEA-PSS reduced the amplitude of $I_K$ measured at the end of 100-ms pulse to $+60$ mV by $12 \pm 1\%$ ($n = 5$, data not shown) and increased the amplitude of $I_h$ to $-431 \pm 62$, $-799 \pm 107$ and $-903 \pm 124$ pA after 6, 86, and 326 ms, respectively (Fig. 11 B). These results are summarized in Fig. 11 C. Between 6 and 86 ms $I_h$ increased by 4.6 times in the normal K$^+$ and by 2.1 times in the high K$^+$ solution. In both cases, this increase could be partially explained by the progressive activation of the current; the amplitude of $I_K$ increased by an average of 1.67 and 1.57 times between 6 and 86 ms in the normal and elevated K$^+$ solutions, respectively. To reveal time-dependent changes in the amplitude of the tail current which were independent of activation, we corrected the amplitude of $I_h$ for $I_K$ activation by dividing the $I_h$ amplitude after each pulse by the amplitude of $I_K$ measured at the end of that pulse. Current ratios thus obtained were normalized to that measured after the 6-ms pulse, and are plotted in Fig. 11 D. It is clearly seen from the figure that normalized tail currents corrected for $I_K$ activation increased to a much greater extent over 326 ms in 5 mM K$^+$ ($5.6 \pm 1.1\text{-fold increase}$) than in 50 mM K$^+$ ($1.4 \pm 0.1\text{-fold increase}$) in the five cells studied in both solutions. These results demonstrate that increasing the external K$^+$ concentration to 50 mM reduced
the time-dependent increase in the amplitude of \( I_h \), consistent with the possibility that this increase was due to a reduction of the K\(^+\) gradient.

**Effects of Removal of Extracellular K\(^+\) and Na\(^+\)**

The previous results could be explained either by an intracellular depletion of K\(^+\) or by an extracellular accumulation of K\(^+\) (or by both). The possibility of extracellular accumulation, which seemed more likely based on previous reports, was investigated using the following approach. The removal of all K\(^+\) from the external solution should result, at least theoretically, in a shift of the equilibrium K\(^+\) potential to an infinitely negative potential. Therefore, if \( I_K \) was completely selective for K\(^+\) over Na\(^+\), it should be impossible to observe \( I_h \) at any potential when the cell is bathed in a K\(^+\)-free medium, unless accumulation of extracellular K\(^+\) occurs. The possibility of K\(^+\) accumulation would be even more strongly indicated if \( I_h \) were observed in the absence of both extracellular Na\(^+\) and K\(^+\). PA cells superfused with a nominally K\(^+\)-free TEA-PSS and held at -60 mV were depolarized to +60 mV for various times, and then repolarized to -60, -80, -100, and -120 mV. Fig. 12 illustrates results obtained from one PA cell where the tail current is shown after repolarization to -80, -100, and -120 mV. After repolarization to -80 mV, an outward tail current was observed, which became increasingly flattened as the duration of the depolarizing step was increased (Fig. 12 A). An outward tail current was also observed at -100 mV.
after a 6-ms depolarizing step (Fig. 12B). Progressively longer depolarizing steps led, however, to the development of an increasingly prominent $I_a$. Although the tail current at $-120$ mV after a 6-ms step was difficult to resolve, longer depolarizing steps led to the development of $I_a$ (Fig. 12C). Similar results were obtained in three other PA cells studied. Moreover, in one of those cells an inward tail current developed even at $-60$ mV, while in others only an outward tail current could be recorded at this potential (data not shown). Estimates of the reversal potential during the development of $I_K$ in K$^+$ free TEA-PSS showed that even after the shortest (6 ms) voltage step the tail current reversed between $-100$ and $-120$ mV (mean value $-105 \pm 5$ mV in four cells studied). The reversal potential was shifted to $-74 \pm 14$ mV ($n = 4$) after 326-ms step depolarization.

$I_a$ was also observed in the absence of both extracellular Na$^+$ and K$^+$ in TEA-PSS, using choline chloride (135 mM, pH was adjusted by TEA-OH) as a substitute for NaCl and KCl. A cell was stepped for various time intervals to $+60$ mV from $-60$, $-80$, $-100$, and $-120$ mV (Fig. 13). The pattern of tail current configurations observed upon repolarization was similar to that obtained in the absence of K$^+$ alone, as shown in A–C. Panel D illustrates the tail currents observed during repolarization to each potential following 6-ms steps. Reversal of the tail current occurred between $-100$ and $-120$ mV. Further experiments were carried out to estimate the reversal potential of the tail current under these conditions in six other cells. In each case, it
proved difficult to hold cells at -120 for any length of time, and the reversal potential was estimated by linear extrapolation of tail current amplitudes measured between -60 and -100 mV. Using this approach, the reversal potential after a 6-ms step was estimated to be -109 ± 1 mV (n = 7), a value which did not differ significantly from that measured in the absence of extracellular K+ alone. The reversal potential after a 326-ms step was significantly shifted to -88 ± 7 mV.

Taken together, the data obtained in the elevated K+ and two types of K+ free solution suggest several conclusions. Firstly, it is clear that the inward tail current is not carried by extracellular Na+. Secondly, the similarity of the reversal potentials in the presence and absence of Na+ indicates that the K+ channel mediating I\(_{\text{it}}\) (and I\(_{\text{g}}\)) must be negligibly permeable to Na+. It is also improbable that this channel is significantly permeable to Cl\(^-\), Mg\(^{2+}\), or Ca\(^{2+}\), because the reversal potential after a short pulse is close to that predicted on the basis of the K+ gradient alone (Fig. 10 B), although we cannot exclude a small contribution to I\(_{\text{K}}\) by these ions. These factors suggest, however, that the existence of a reversal potential is indicative of the presence of extracellular K+. The reversal potential of -105 mV measured for the tail current in the nominally K+ free solution after a 6-ms depolarization to +60 mV implies an actual K+ concentration at the external face of the membrane of 2 mM, if it is assumed that no depletion of intracellular K+ has occurred. After 326 ms, this value would have had to increase to 7 mM to give the observed reversal potential of -74 mV. Conversely, if it assumed that extracellular K+ remained constant at 2 mM over this period, K+ at the intracellular side of the membrane would have had to fall to 38 mM to shift the reversal potential to -74 mV.

It is theoretically possible that accumulation of K+ between cells and the glass floor of the recording chamber might have occurred, leading to the development of inward tail currents and shifts in the K+ reversal potential. However, inward tail currents were not significantly altered in 4 PA cells which were lifted from the floor of the chamber, indicating that such an accumulation was not an important factor in the results described above (data not shown).

It should be noted here that the removal of external K+ could also inhibit the Na+ pump and thus facilitate or even initiate K+ accumulation observed in K+-free solutions. To estimate the degree of contribution of Na+ pump to redistribution of the nearmembrane [K+] we examined the effect of ouabain (100 \(\mu\)M) upon I\(_{\text{K}}\) and I\(_{\text{g}}\). The amplitude of I\(_{\text{K}}\) measured at the end of 500-ms voltage steps decreased by 5 ± 2% in four cells, while the amplitude of I\(_{\text{g}}\) increased by 23 ± 10% (data not shown). Both results are consistent with a small enhancement of the extracellular K+ accumulation by ouabain, suggesting that the Na+ pump acts, as expected, to maintain the K+ gradient during depolarization.

**DISCUSSION**

The observations presented above suggest that K+ efflux during activation of I\(_{\text{K}}\) in rat PA cells results in a diminution of the K+ gradient which is sufficient to shift the reversal potential for this ion by many millivolts during strong and/or prolonged activation of I\(_{\text{K}}\). The results shown in Figs. 12 and 13 demonstrate that an accumulation of extracellular K+ must contribute to this effect. The fact that quite profound changes in intracellular K+ would be necessary to obtain the reversal
potential shifts observed similarly suggests, but does not prove, that extracellular K$^+$ accumulation is likely to play the major role in the diminution of the K$^+$ gradient.

The accumulation of extracellular K$^+$ in multicellular preparations, where there is an obvious morphological basis for the retardation of diffusion of ions in the extracellular space, has been characterized (Frankenhaeuser and Hodgkin, 1956; Neher and Lux, 1973; Cleemann and Morad, 1976; Baumgarten et al., 1977). Recently, a large inward tail current following the activation of ATP-sensitive K$^+$ channels by nicorandil has also been described in single, enzymatically isolated guinea-pig ventricular cells (Yasui et al., 1993). The time-dependent shift of the reversal potential of this inward tail current was similar to that described in the present manuscript, although biphasic tail currents such as those shown in Fig. 4 were not described. The authors proposed that the appearance of the inward tail current was due to accumulation of K$^+$ in the narrow T-tubules present in these cells; a similar phenomenon was not observed in atrial cells, where T-tubules are poorly developed. A quantitative model describing time-dependent alterations in T-tubular [K$^+$] and the amplitude of the tail currents was developed.

A discrepancy between theoretical and experimentally measured reversal potential for K$^+$ currents has been reported in a variety of single smooth muscle cells studied with the whole cell patch clamp technique. The K$^+$ reversal potential was found to be 10–15 mV positive of its predicted value in smooth muscle cells isolated from rabbit pulmonary (Okabe et al., 1987) and coronary (Volk et al., 1991) artery, and from human cystic artery (Akharali, Wyse, and Giles, 1992), guinea-pig ureter (Imaizumi, Murake, and Watanabe, 1990) and rat ileum (Smirnov, Zholos, and Shuba, 1992). In some cases this discrepancy was maintained in Na$^+$- or Cl$^-$-free solutions (Imaizumi et al., 1990; Volk et al., 1991). In rabbit PA, however, the difference between the reversal potentials decreased in Cl$^-$-free solution (Okabe et al., 1987). Yuan and co-workers found a 21-mV discrepancy between the measured and calculated reversal potential in cultured rat PA cells, although they did not report the presence of inward tail currents in their study (Yuan, Goldman, Tod, Rubin, and Blaustein, 1993). Large amplitude inward tail currents qualitatively similar to those described in the present paper, as well as a > +50 mV displacement of reversal potential from the predicted value, were observed following the very large outward currents measured in dog coronary arteries (Wilde and Lee, 1989). The authors concluded that these inward currents resulted from the activation of Ca$^{2+}$ channels in these cells, although they did not investigate this question in detail. Thus, the phenomenon observed in rat PA cells in the present study might be present to a varying extent also in other smooth muscle cells, although it has not heretofore been identified. It should be noted, however, that in some studies a good correlation between the theoretical and experimental reversal potentials was found (Beech and Bolton, 1989; Cole and Sanders, 1989; Yamamoto et al., 1989).

That these tail currents demonstrated the type of biphasic kinetics illustrated in Fig. 4 was unexpected in light of the evidence that they were caused by the deactivation of a single type of K$^+$ current. In the majority of cells, such as that shown in Fig. 4A, the tail currents were clearly biphasic after short depolarizations, but progressively became completely inwardly directed with longer depolarizations. In other cells (e.g., Fig. 4B), the biphasic kinetic profile was retained even after longer
depolarizations, although a tendency for the initial inward component of the tail currents to become more pronounced was invariably present. The time-dependent change in the shape of these tail currents over the duration of depolarizations during which the K⁺ reversal potential was observed to shift suggested strongly that these were related phenomena. This possibility was further supported in that the shift in the reversal potential, as well as the presence of biphasic tail currents and the manner in which they changed shape as depolarization was prolonged, could be simulated in a mathematical model based upon the single assumption that some of the K⁺ leaving the cells during $I_K$ accumulated at the extracellular surface of the cell.

To explore the consequences of such an arrangement, we chose to use a three-compartment scheme similar to that described by Yasui et al. (1993). This model assumes the existence of an extracellular compartment adjacent to the cell membrane from which the diffusion of K⁺ is greatly slowed. The morphological basis of such a compartment is unknown. One possibility is that membrane invaginations or caveolae are involved. Ultrastructural studies of isolated smooth muscle cells have established that caveolae survive the process of enzymatic dispersion (Wadsworth, Berezin, Crankshaw, Kwan, and Daniel, 1988; Kwan, Gaspar, Berezin, Low, and Daniel, 1992). These studies also demonstrated the presence of deep and extensive membrane invaginations in isolated vascular smooth muscle cells. A comparison of our estimates of the membrane surface area based on measurements of membrane capacity with those based on measuring cell dimensions suggested that ~64% of the membrane surface area was invisible. This figure does not differ greatly from similar estimates which have been made in other types of isolated smooth muscle cells (Singer and Walsh, 1980; Klöckner and Isenberg, 1985). The model described below therefore assumes that 64% of the K⁺ channels lie in areas of the plasma membrane which line some type of sequestered compartment. It is noteworthy that Singer and Walsh (1980) have previously considered the possibility that a K⁺ gradient might develop across the mouth of plasmalemmel caveolae during changes in the K⁺ current. Their theoretical calculation, which was based on the assumption that the diffusion constant for K⁺ in the caveolae was equal to that measured in aqueous solution, showed that only a negligible K⁺ gradient could be generated in this manner. The proposed scheme therefore requires either that a diffusion barrier exists within or at the mouth of caveolae, or that other structures, such as the membrane invaginations referred to above form the morphological basis for the barrier to free K⁺ diffusion away from the membrane. Alternatively, an extracellular unstirred layer might contribute to K⁺ accumulation (Barry and Diamond, 1984). The model does not incorporate intracellular K⁺ depletion in order to reduce the number of terms, and because the basic principle involved in such a depletion, that of slowed K⁺ diffusion near the membrane, is similar.

A fraction of the K⁺ leaving the cell was envisioned to enter a compartment from which its diffusion into the bulk extracellular space was retarded. The slowed diffusion from this compartment caused its K⁺ concentration to be elevated above that of the bulk extracellular solution concentration during and immediately after the development of a K⁺ current. The extent of this accumulation, and its time course, were dependent upon the volume of the compartment ($V_c$), the fraction of the total $I_K$ current which entered it ($f = 0.64$) and the rate of K⁺ diffusion from the
compartment to the bulk solution. The differential equation governing the concentration of K\(^+\) in this compartment was then:

\[
\frac{d[K]_{C,t}}{dt} = \frac{f_i K}{F \cdot V_C} - \frac{1}{\tau} ([K]_{C,t} - [K]_e),
\]

where \([K]_{C,t}\) is the concentration of K\(^+\) in the compartment at time \(t\), \([K]_e\) is the concentration of K\(^+\) in the bulk extracellular solution (= 5 mM), \(\tau\) is the time constant for the diffusion of K\(^+\) between the compartment and the bulk extracellular solution, and \(F\) is the Faraday constant. To simplify the solution of this equation, we followed Yasui et al. (1993) in assuming that the current activated instantaneously and had an invariant amplitude \(i_K\) (1.7 \(\times\) 10\(^{-9}\) A, based on the mean peak current amplitude observed at +60 mV in a large number of cells). In this case:

\[
[K]_{C,t} = \left[ \frac{f_i K \tau}{F \cdot V_C + [K]_e} \right] - \frac{f_i K \tau}{F \cdot V_C} \exp\left(-\frac{t}{\tau}\right),
\]

where the term in the brackets represents the K\(^+\) concentration in the compartment at steady state, given current \(i_K\). These equations are analogous to Eqs. 7–9 presented by Yasui et al. (1993). The overall K\(^+\) reversal potential, \(E_{r,t}\), was the weighted average (with \(f\) the weighting factor) of the K\(^+\) reversal potentials across that area of the cell membrane lining the compartment, and that not lining the compartment:

\[
E_{r,t} = \frac{RT}{F} \ln \left( \frac{[K]_{C,t}}{[K]_i} \right) + (1 - f) E_r,
\]

where \(E_r\) was the reversal potential across the area of the plasmalemma not bordering the compartment, \([K]_i\) was the intracellular K\(^+\) concentration of 0.135 M, and \(R, T,\) and \(F\) have their usual meanings. The unknown variables \(V_C\) and \(\tau\) were allowed to vary to generate estimates of \(E_{r,t}\) values (using Eqs. 2 and 3) which were fitted to the measured reversal potentials shown in Fig. 10 B using a least squares algorithm. The best fit was obtained with \(\tau = 100\) ms and \(V_C = 61.1\) \(\mu\)m\(^3\), resulting in the curve illustrated in Fig. 10 B.

Upon the termination of the depolarizing step, \([K]_{C,t}\) would decrease back towards \([K]_e\) as the diffusion-driven loss of K\(^+\) from the layer would exceed its entry. Depending upon the relative rates of this process and the deactivation of the K\(^+\) channel, this decrease in \([K]_{C,t}\) could lead to the biphasic kinetic profile of the tail currents observed, since the K\(^+\) reversal potential would be continually shifting in a negative direction as \([K]_{C,t}\) approached \([K]_c\). To simulate this process as simply as possible, we neglected the contribution of the tail current itself to \([K]_{C,t}\), (which was found to be negligible in a preliminary simulation) and thus described \([K]_{C,t}\), the compartmental K\(^+\) concentration during the tail current, as follows:

\[
[K]_{C,t} = [K]_c + ([K]_{C,t=1} - [K]_c) \exp \left(-\frac{t}{\tau}\right),
\]

where \([K]_{C,t=1}\), the K\(^+\) concentration in the compartment at the moment of
repolarization after voltage step of \( t \) duration was simulated using Eq. 2 and a value for \( \tau \) similar to that derived above.

Finally, \( i_{\text{tail},t} \), the amplitude of the tail current at time \( t \) was calculated as:

\[
i_{\text{tail},t} = G_K'n^t f(E - E_{*,t}) + (1 - f)t(E - E_r),
\]

where \( E_{*,t} \), the reversal potential for the \( K^+ \) channels spanning the area of the membrane adjacent to the compartment of accumulation, was calculated using the Nernst equation and \( [K]_{C,0} \), as determined by Eq. 4. \( E_r \), the membrane potential following repolarization, was set to \(-60\) mV, and \( G_K \) was set to \( 10^{-3} \) S/cm\(^2\) based on the average current density at +60 mV. The expression \( n = \exp(-t/0.03) \) describes the deactivation of the \( I_K \) channel with a time constant equal to 30 ms.

Fig. 14 illustrates that this scheme was able to mimic the range of tail current configurations that we observed, when \( f \) and \( \tau \) were varied over relatively restricted ranges. Increases in both \( f \) and \( \tau \) caused the calculated tail current to become more inwardly directed, because \( K^+ \) accumulation was favored, while decreases in these variables resulted in outwardly directed tail currents, because \( K^+ \) accumulation became less important. The high sensitivity of \( K^+ \) accumulation, and consequently the alteration in the direction and kinetics of the tail current, to these variables, which might for example reflect the extent to which the cell membrane is invaginated, provides a basis for the variability in tail current behavior which was observed.
Although this model is both speculative and simplistic, it has the virtue of simulating all of the results presented above. It also provides a plausible explanation for the significant variability between the calculated and measured K⁺ reversal potentials which has been observed in different types of smooth muscle cells, because it is a feature of this scheme that the predicted shape of the tail currents is extremely sensitive to small changes in the variable which represents the extent of the membrane area involved in the formation of invaginations. If such structures do in fact retard K⁺ diffusion away from cells, it may be that the degree to which they are preserved, or created, by the specific conditions used in isolating cells will determine whether K⁺ can accumulate.

The physiological significance of K⁺ accumulation, and the extent to which it might occur in intact arteries, remains to be established. It is possible, however, that K⁺ accumulation might occur at times when K⁺ efflux is enhanced, for example during hyperpolarizations following action potentials in intestinal muscle, or during the myogenic response in vascular smooth muscle, when Ca²⁺-activated K⁺ channels open (Brayden and Nelson, 1992). Extracellular K⁺ accumulation would be expected to promote depolarization by reducing the K⁺ gradient. Conversely, an increase in K⁺ at the outer mouth of K⁺ channels may increase their conductance, thus leading to a hyperpolarizing influence. If K⁺ accumulation does occur in vivo, it is therefore likely make a complex and subtle contribution to cellular electrical activity.

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