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Role of K^+ Channels to Resting Membrane Potential of Rabbit Middle Cerebral Arterial Smooth Muscle Cells

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The aim of the present study is to investigate the contribution of Ca^{2+}-activated K^+ (K_{Ca}) channels and delayed rectifier K^+ (K_V) channels to the resting membrane potential (RMP) in rabbit middle cerebral arterial smooth muscle cells. The RMP and membrane currents were recorded using the whole-cell patch configuration and single K_{Ca} channel was recorded using the outside-out patch configuration. Using the pipette solution containing 0.05 mM EGTA, the RMP was -25.76 ± 5.08 mV (n=12) and showed spontaneous transient hyperpolarizations (STHPs). The membrane currents showed time- and voltage-dependent outward currents with spontaneous transient outward currents (STOCs). When we recorded the membrane potential using the pipette solution containing 10 mM EGTA, the RMP was depolarized and did not show STHPs. The membrane currents showed no STOCs but only showed slowly inactivating outward currents. External TEA (1 mM) reversibly inhibited the STHPs, depolarized the RMP, reduced the membrane currents, abolished STOCs, and decreased the open probability of single K_{Ca} channel. When K_V currents were isolated, the application of 4-AP (5 mM) depolarized the RMP. The important aspect of our results is that K_{Ca} channel is responsible for the generation of the STHPs in the membrane potential and plays an important role in the regulation of the RMP and K_V channel is also responsible for the regulation of the RMP in rabbit middle cerebral arterial smooth muscle cells.

Key Words: Resting membrane potential, K_{Ca} channel, K_V channel, Rabbit middle cerebral arterial smooth muscle cells

INTRODUCTION

The maintenance of cerebral blood flow is dependent upon a certain level of tone maintained by cerebral arterial muscle cells. The regulation of vascular smooth muscle tone underlies the very close coupling that exists in the brain between neuronal activity and local alterations in blood flow required to meet the consequent changes in metabolic demand. In spite of this fact, the number of studies specifically investigating the mechanisms responsible for the control of contraction and relaxation in cerebral blood vessels is somewhat limited. Underlying mechanisms have been of great interest, and it has been proposed that the membrane potential of arterial smooth muscle cells plays a crucial role: depolarization of the membrane potential opens voltage-operated Ca^{2+} channel, increasing Ca^{2+} entry, which leads to vasoconstriction (Daut et al, 1994; Nelson et al, 1995). In spite of such significance, the ion channel mechanism of regulating the resting membrane potential (RMP) in vascular smooth muscle cells has not been investigated in detail.

The RMP of vascular smooth muscle cells, assessed by intracellular microelectrodes, has reported to vary between values as low as −30 mV to around −70 mV. Data relating specifically to the electrophysiological characteristics of cerebrovascular smooth muscle are somewhat limited, although the passive membrane properties of smooth muscle cells from the rabbit basilar artery have been measured (Surprenant

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et al, 1987). Patch-clamp studies on dispersed cerebrovascular smooth muscle cells are limited, but voltage-sensitive Ca^{2+} channels and large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels have been described recently in cells from rabbit and rat basilar arteries (Worley et al, 1991; Stockbridge et al, 1992). Earlier experiments by Hirst et al (1986) described two separate calcium currents and two outward currents, using single-electrode voltage clamp in cells of the rat middle cerebral artery.

It has been suggested that the membrane properties of the smooth muscle cells in cerebral arteries differ significantly from those in systemic arteries, and that as a consequence, the RMP of the cells in these vessels is more negative than in peripheral arteries (Harder, 1980, 1983). However, there have been few studies directly comparing the membrane properties of smooth muscle in cerebral and systemic arteries in the same species and under the same experimental conditions, which would allow this assertion to be made with any confidence. In a comparison of feline middle cerebral arteries with mesenteric and coronary arteries, the former were reported to contain cells with a membrane potential close to -70 mV, while lower values of -49 mV and -58 mV were reported for cells in the respective peripheral vessels (Harder, 1980, 1983). This difference was ascribed to a greater contribution to the RMP in the cerebral arteries from the electrogenic sodium-pump, assessed by the magnitude of smooth muscle depolarization induced by ouabain, and to a higher K^+ conductance derived from the relative input resistance. These differences were suggested to underlie the steeper slope for plots of E_m vs. log $[K^+]_O$ in cerebral compared to peripheral arteries. This may render the contractile state of cerebrovascular smooth muscle cells more sensitive to small changes in the extracellular concentration of K^+ (Harder, 1980, 1983).

Contribution of K^+ channels to the RMP has been widely investigated in many different type of cells. In arterial smooth muscle cells, inward rectifier K^+ channels are apparently absent or very small in the normal extracellular K^+ concentration. However, arterial smooth muscle cells have a high density of K_{Ca} channels and K_V channels. It was therefore suggested that these channels are important regulators of the RMP in cerebral arterial smooth muscles, and thus important regulating arterial tone. The aim of the present study is to investigate the contribution of K_{Ca} channels and K_V channels to the RMP in rabbit middle cerebral arterial smooth muscle cells.

METHODS

Single cell isolation

Single vascular myocytes were isolated from rabbit cerebral arteries by enzymatic dissociation, as discussed previously (Quayle et al, 1993). Rabbits (NewZealand white rabbit, 0.8 ~ 1.2 Kg) were anaesthetized with sodium pentobarbital (10 mg/Kg i.v.) and exsanguinated. Cerebral arteries were removed and cleaned of extraneous connective tissue in a dissecting solution containing (mM): 137 NaCl, 5.6 KCl, 0.42 Na_2HPO_4, 0.44 NaH_2PO_4, 4.17 $NaHCO_3$, 1 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES, pH 7.3 with NaOH. The arteries were then transferred to a isolation solution (mM: 55 NaCl, 6 KCl, 88 L-glutamic acid, 10 HEPES, 10 glucose, pH 7.3 with NaOH) for 20 min. Cerebral arteries were digested for 10 min in a isolation solution containing (mg/ml): 1 albumin, 1 papain, and 1 dithioerythritol, then for 10 min in a isolation solution containing (mg/ml): 1 albumin, 1 collagenase F, and 1 hyaluronidase type I-S. Single smooth muscle cells were obtained by gentle trituration with a wide-bore pipette in fresh a isolation with albumin (1 mg/ml), stored at 4°C and used within 12 hours.

Electrophysiological methods

Whole-cell K^+ currents and membrane potentials were measured in the conventional configurations of the patch-clamp technique (Hamil et al, 1981). The illustrated recordings were started soon after going into the whole-cell configuration. Single-channel currents were measured in outside-out patch configurations of the patch-clamp technique (Han et al, 1993). Membrane potential and channel activity were measured using a patch-clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, CA, USA). Pipettes of 5 ~ 10 MΩ resistance were pulled from borosilicate glass capillaries (Clark Electrochemical, Pangbourne, England) using a vertical puller (Narishige PP-83, Japan). Their tips were coated with Sylgard and fire polished. Membrane potentials and whole-cell currents were filtered at 5 kHz and stored in digitized format on digital audio tapes using a Biologic DTR-1200 recorder (Grenoble, France). Single-channel cur-

rents were digitized at a sampling rate of 48 kHz and stored in digitized format on digital audio tapes using a Biologic DTR-1200 recorder. For the analysis, the data were transferred to a computer with pCLAMP v 6.0 software (Axon Instruments, Burlingame, CA, USA) through an analogue-to-digital converter interface (Digidata-1200, Axon Instruments Inc.).

Data analysis and quantification of single channel activity

The threshold for judging the open state was set at half of the single-channel amplitude (Colquhoun & Sigworth, 1983). The open time histogram was formed from continuous recordings of more than 60 sec. The open probability (P_O) was calculated using the formula:

$$P_O = (\sum_{j=1}^{N} t_j j)/(T_d N)$$

where t_j is the time spent at current levels corresponding to j = 0, 1, 2, ... N channels in the open state, T_d is the duration of the recording and N is the number of channels active in the patch. The number of channels in a patch was estimated by dividing the maximum current that observed by the mean unitary current amplitude. P_O was calculated over 30 sec records.

Solutions and drugs

The solutions used in whole-cell experiments were (in mM): 133 K-Aspartic acid, 7 KCl, 2.5 Mg-ATP, 2.5 Na-ATP, 2.5 tris-creatine phosphate, 2.5 Na-creatine phosphate, 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.05 or 10 ethylene glycol-bis(β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA), pH 7.3 for the pipette and 143 NaCl, 5.4 KCl, 5 HEPES, 0.33 NaH_2PO_4, 1 $MgCl_2$, 16.6 glucose, 0 or 1.8 $CaCl_2$, pH 7.4 for the bath. The solutions used in outside-out patch experiments were (in mM): 140 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 5 HEPES and pH 7.3 for the bath; the composition of pipette solution was similar, except that the Ca^{2+}, EGTA ratio was adjusted to give a pCa of 6×10^{-7} at which simultaneous opening of 1 to 3 channels could be typically observed. All chemicals and drugs were obtained from Sigma Chemical (St. Louis, MO, USA). Experiments were done at a room temperature of $25\pm2^{\circ}C$.

Statistics

Data are presented as mean $\pm$ SE when appropriate. Student's *t* test was used to calculate statistical significance. A probability of 0.05 or less was considered significant.

RESULTS

Recording of the RMP and membrane currents

Membrane potentials were recorded with whole- cell clamp using the pipette solution containing 0.05 mM EGTA. In current clamp mode, membrane potentials

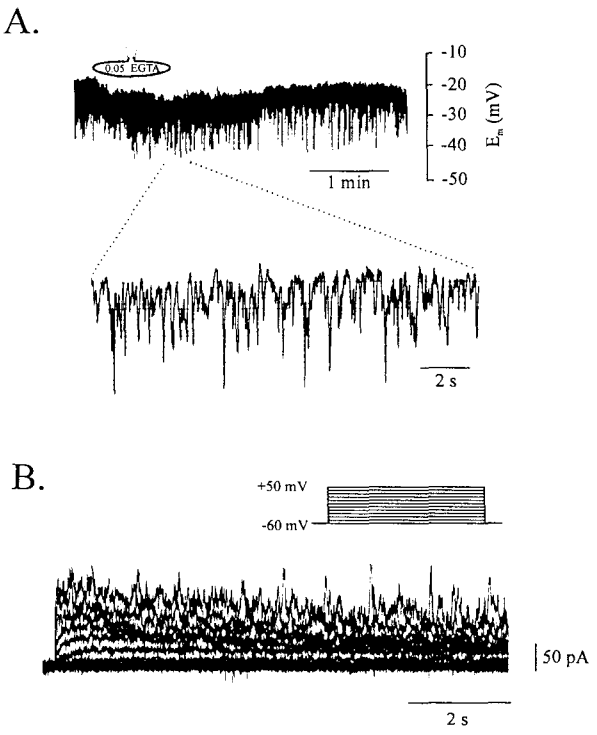

Fig. 1. Recording of the RMP and membrane currents in the rabbit middle cerebral arterial smooth muscle cells. A. Representative trace of the RMP. Using the pipette solution containing 0.05 mM EGTA, the RMP was not quiescent, but showed STHPs. B. Representative family of superimposed membrane current traces elicited in 10 mV increments. The holding potential was -60 mV and the test potentials ranged from -60 mV to $+50$ mV. The bath contained the normal Tyrode solution and the pipette contained the 0.05 mM EGTA. Membrane currents showed time- and voltage-dependent outward currents. STOCs were superimposed on the slowly inactivating outward currents.

were not quiescent, but showed spontaneous hyperpolarizations as was shown in Fig. 1A. In the following, we refer to the minimum negative level of potential as a RMP, and spontaneous transient hyperpolarizations as STHP. The RMP was -25.76 ± 5.08 mV (n=12). STHPs were irregular in frequency and amplitude. Fig. 1B shows membrane currents obtained using step protocol. A step of voltage from -60 mV to $+50$ mV in 10 mV increments was applied from a holding potential of -60 mV. Membrane currents showed time- and voltage-dependent outward currents. Stepping to positive potentials evoked much noiser outward current upon which spontaneous transient outward currents (STOCs) (Knot & Nelson, 1995) were often superimposed on the slowly inactivating outward currents.

Since Ca^{2+}-activated K^+ current is known to produce STOCs (Benham & Bolton, 1986), we tested the effect of EGTA on the membrane potential in order to know the dependence of STHPs on intracellular Ca^{2+}. It is known that EGTA chelates intracellular Ca^{2+}. When we recorded the membrane potential in the whole-cell mode using the pipette solution containing 10 mM EGTA to lower intracellular Ca^{2+}, the RMP was depolarized and did not show STHPs (Fig. 2A). In voltage clamp mode with 10 mM EGTA-containing pipette, membrane currents elicited by the same step depolarization protocol showed no STOCs but only showed slowly inactivating outward currents (Fig. 2B). These results indicate that intracellular Ca^{2+}-dependent currents play an important role in STHPs and the RMP in rabbit middle cerebral arterial smooth muscle cells.

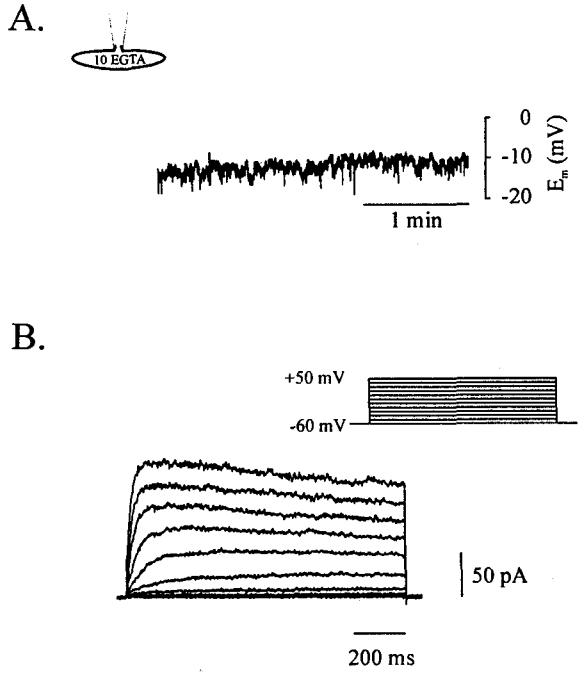

Fig. 2. Effect of 10 mM EGTA on the RMP and membrane currents in the rabbit middle cerebral arterial smooth muscle cells. A. Using the pipette solution containing 10 mM EGTA, the RMP was depolarized and STHPs were disappeared. B. Membrane currents elicited by step depolarization protocol showed no STOCs, but only showed slowly inactivating outward currents.

The RMP and K_{Ca} channels

In order to know whether the Ca^{2+}-depenent current is through the K_{Ca} channel, we tested the effect of 1 mM TEA. TEA inhibit K_V channels, although

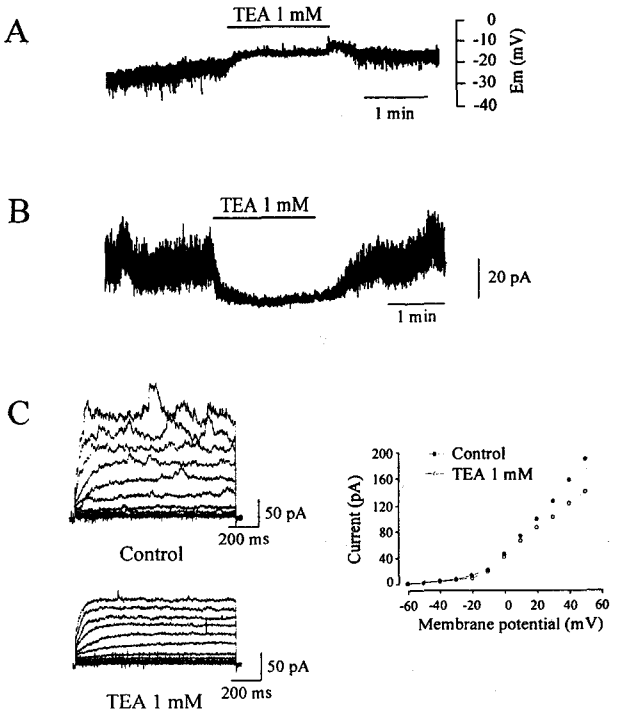

Fig. 3. Effect of K_{Ca} channel blocker on the RMP and membrane currents in the rabbit middle cerebral arterial smooth muscle cells. A. Effect of extracellular TEA on the RMP. TEA (1 mM) reversibly inhibited the STHPs and slightly depolarized the RMP. B. Effect of TEA on the STOCs recorded at the membrane potential of $+50$ mV. TEA reversibly inhibited the STOCs. C. Effect of TEA on the membrane currents. *Left panel*: TEA markedly reduced the current noise and abolished STOCs; *Right panel*: The current-voltage relationship in the absence and presence of TEA.

at higher concentrations than they inhibit K_{Ca} channels (one- half inhibition constant (K_i) > 5 mM; see Nelson & Quayle, 1995). 1 mM TEA reversibly inhibited the STHPs and slightly depolarized the RMP (Fig. 3A). When the cells were voltage-clamped at +50 mV, STOCs were recorded. Application of TEA to the bath solution inhibited the STOCs, similar to their effect on the STHPs (Fig. 3B). These results show that K_{Ca} channel is responsible for the generation of the STHPs in the membrane potential and plays an important role in the regulation of the RMP in cerebral arterial smooth muscle cells (Bae et al, 1999). Stepping to positive potential evoked a much noisier outward current upon which STOCs were often superimposed. A large component of the outward current evoked at positive potentials was blocked by TEA (Fig. 3C), which also markedly reduced the current noises and abolishes STOCs. This suggested that this outward current component was carried by the K_{Ca} channel, which are prevalent in these cells and are blocked by external TEA (Beech, 1988).

When the seal resistance was favorable, current fluctuations attributable to single-channel events were observed, a mode of recording designated the "outside-out configuration". Fig. 4 shows an experiment illustrating the basic property of K_{Ca} channels. From a holding potential of +50 mV, depolarization elicited single-channel events that were clearly distinguishable from noise, demonstrating the underlying unitary currents responsible for the macroscopic currents obtained under the same conditions (Fig. 4A). The mean unitary currents at +50 mV with physiological K^+ gradient was 11.1±0.1 pA (n=4) (Fig. 4B). The open-time and the closed-time histograms were calculated at a membrane potential of +50 mV. The open-time histogram, which was analyzed from the current record filtered at cutoff frequency of 5 kHz, revealed a double exponential distribution. The time constant of the fast component ($\tau_{o,f}$) was 1.0 ms (mean 0.9±0.3, n=5) and that of the slower component ($\tau_{o,s}$) was 15.3 ms (mean 14.8±0.8, n=5) in the open-time histogram (Fig. 4C). The closed time histogram analysis using records filtered at cutoff frequency of 0.2 kHz was fitted using a biexponential function, with time constants of a fast ($\tau_{c,f}$) and a slow ($\tau_{c,s}$) component. $\tau_{c,f}$ was equivalent to τ_c filtered at cutoff frequency of 5 kHz, which was distorted, by heavy filtering. $\tau_{c,f}$ was 0.5 ms (mean 0.45±0.03, n=5) and $\tau_{c,s}$ was 2.0 ms (mean 2.67±0.65, n=5) in the closed-time histogram (Fig. 4D).

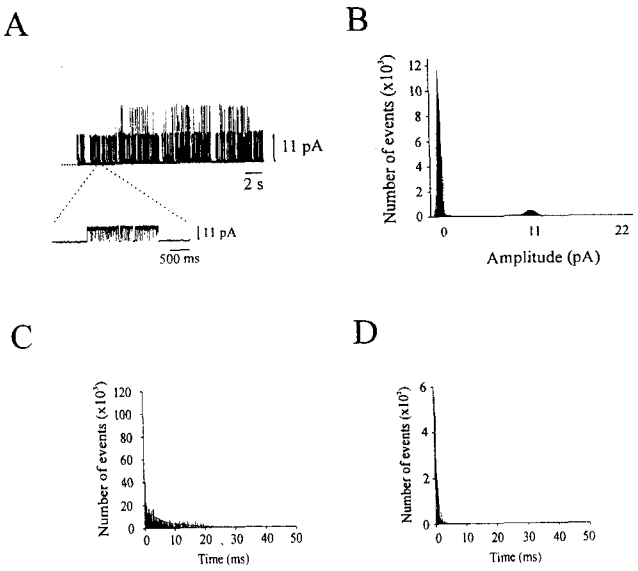

Fig. 4. K_{Ca} channel recorded by outside-out patch configuration in the rabbit middle cerebral arterial smooth muscle cells. A. Representative recording of single K_{Ca} channel at holding potential of +50 mV. Dashed line indicates current level when channel is close. B. Amplitude histogram. Gaussian curves were fitted to the peaks of the amplitude histogram to yield a unitary current of 11 pA. Histograms of open-time (C) and closed-time (D) were analyzed at a membrane potential of +50 mV. The current records were filtered at cutoff frequency of 5 kHz and 0.2 kHz. Smooth curves were fitted by biexponential function.

Fig. 5 shows records during application of 1 mM TEA. External TEA decreased the open probability (P_o) from 0.25±0.03 to 0 at +50 mV (n=4).

The RMP and K_V channel

When we recorded the membrane potentials using the pipette solution containing 10 mM EGTA and bathing solution containing 1 mM TEA in Ca^{2+}-free Tyrode solution, membrane potentials were depolarized, STHPs were inhibited, and the K_V channel was isolated. In this condition, the bath application of 4-AP (5 mM) depolarized the RMP (Fig. 6A). 4-AP is perhaps the most selective known inhibitor of K_V channels in vascular smooth muscle (Beech & Bolton, 1989; Robertson & Nelson, 1994; Knot & Nelson, 1995). For this reason, 4-AP has been used to separate K_V currents from K_{Ca} currents. Therefore, this result show that K_V channel is also responsible for the

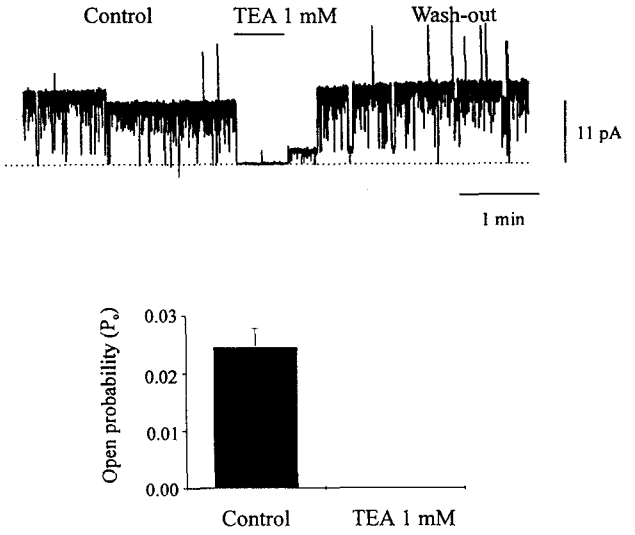

Fig. 5. Complete block of K_{Ca} channels by external 1 mM TEA in the rabbit middle cerebral arterial smooth muscle cells. Unitary K^+ currents were recorded in an outside-out patch held at +50 mV. Upward deflection indicates channel opening and outward current. Dashed line indicates current level when channel is close. Records were digitized at 5 kHz. External TEA decreased the open probability (P_o) from 0.25±0.03 to 0 at +50 mV (n=4). Channel openings were not observed when in TEA but were similar to control after wash-out.

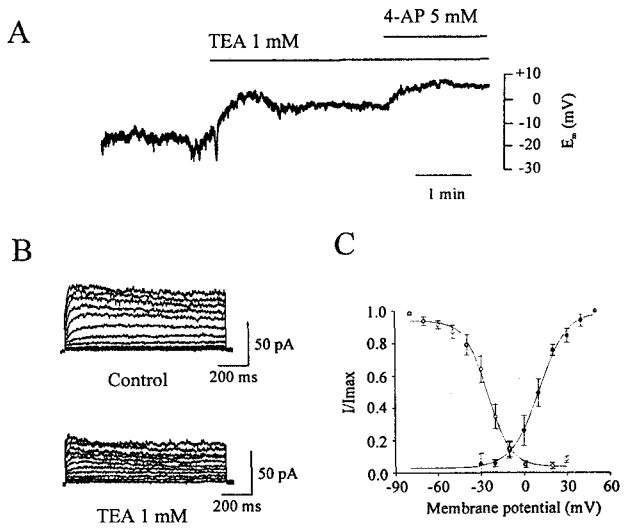

Fig. 6. Effect of the K_V channel blocker on the RMP and basic property of K_V channel in the rabbit middle cerebral arterial smooth muscle cells. A. Using the pipette solution containing 10 mM EGTA and bathing solution containing 1 mM TEA in Ca^{2+}-free Tyrode solution, 4-AP, K_V channel blocker, depolarized the RMP. B. Representative traces of K_V current were elicited by voltage step to +50 mV in 10 mV increments from a holding potential of −60 mV in the presence of TEA. C. The activation/inactivation kinetics of K_V channel. The inactivation points (o) are mean SE (n = 4) and represent normalized peak outward current at −35 mV after holding at various potentials until this current stabilized. The smooth curve through these points is the best fit to the Boltzmann distribution equation, where $V_{1/2}$ = 10.57±1.03 mV and k = 9.54±1.02 mV. The activation points (●) are mean± SE (n = 4) and were determined by fitting deactivation tails at +40 mV after voltage steps to various potentials. The voltage steps that preceded deactivation varied in length so as to return to +40 mV at the peak of the current. The zero time (t_0) amplitudes of the two components of deactivation were combined for each tail and then normalized using the largest tail. The square root was taken to correct for the second power relationship found for this current. These points were also fitted with the Boltzmann distribution equation where $V_{1/2}$ = −25.37 ±0.99 mV and k = 7.93±0.88 mV.

regulation of the RMP in rabbit middle cerebral arterial smooth muscle cells. Fig. 6B shows representative traces of K_V channel evoked by step depolarization to +50 mV in 10 mV increments from a holding potential of −60 mV in the presence of TEA. The inhibitory effects of TEA and 4-AP indicate that the outward currents are through K^+-selective channels. We also analyzed the activation/inactivation kinetics of K_V channel. The activation and inactivation curves were well fitted with the Boltzmann distribution equation (Fig. 6C). In activation curve, half-maximal activation voltage and slope factor were 10.57±1.03 mV and 9.54±1.02 (n=4). In inactivation curve, half-maximal inactivation voltage and slope factor were −25.37±0.99 mV and 7.93±0.88 (n=4).

DISCUSSION

Our experiments were to examine the involvement of K_{Ca} channels and K_V channels in regulating of the RMP in rabbit middle cerebral arterial smooth muscle cells.

The present study demonstrates that the activity of K_{Ca} channels contribute to the RMP and to STHPs in rabbit middle cerebral arterial smooth muscle cells. Although K_{Ca} channel can be recorded in most types of smooth muscle cells, its role in membrane potential has not well been recognized until recently (Nelson & Quayle, 1995; Bae et al, 1999). At concentrations

below 1 mM, TEA preferentially blocks K_{Ca} channels, although at much higher concentration (> 5 mM), it blocks other types of K^+ channels in smooth muscle (Nelson et al, 1990). In present experiment, TEA inhibited the STOCs and STHPs. This result shows that K_{Ca} channel is responsible for the generation of the STHPs in the membrane potential and plays an important role in the regulation of the RMP in rabbit middle cerebral arterial smooth muscle cells. It has been known that K_{Ca} channel is an ideal candidate for a negative feedback (vasodilating) pathway because both membrane depolarization and intracellular Ca^{2+} activate it.

The conclusion from the results presented above is that K_V channels affect the RMP. K_V channels have been postulated to play a role in determining the level of the RMP in a number of different vascular smooth muscle preparations (Gelband & Hume 1995; Post et al, 1995; Yuan, 1995). In support of this proposal we found that 4-AP was capable of depolarizing the RMP in the rabbit middle cerebral artery.

Over this range of voltages (i.e., −60 to −30 mV) a small steady-state level of K^+ current (i.e., window current) is predicted. The K^+ channels responsible for this current are likely to be K_V channels since a blocker of K_V channel, 4-AP abolished currents. The voltage dependence of K_V channels therefore suggests that these channels are likely to contribute to the steady-state regulation of the RMP. This conclusion was supported by measurements in the intact tissue that demonstrated that the RMP was significantly depolarized upon application of the K_V channel blocker, 4-AP.

Recently several studies have suggested that K^+ channels other than K_V channels also regulate membrane potential of vascular smooth muscle cells. Inward rectifier K^+ (K_{ir}) channels and ATP- sensitive K^+ (K_{ATP}) channels have each been implicated in regulating tone (Nelson & Quayle 1995). However, neither K_{ir} nor K_{ATP} channels appeared to contribute to the membrane potentials and membrane currents observed in the present study. This conclusion is based upon several different observations. The voltage dependence of membrane currents that we measured differs from that described for either K_{ir} or K_{ATP} channels, which each exhibit increasing amplitude current as membrane potential is made progressively more negative (Kleppisch & Nelson, 1995; Quayle et al, 1993). The absence of K_{ir} in these experiments is not entirely surprising since these channels may be limited to smaller diameter arteries (Bonev et al, 1994; Quayle et al, 1993), while the present study utilized cells taken from larger conduit arteries. In addition since 5 mM ATP was induced in the pipette solution, the resting activity of K_{ATP} channels was likely to be very small. Intracellular ATP in this concentration range largely suppresses the activity of these channels (Xu & Lee, 1994).

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