Calcium-activated potassium channels in the endothelium of intact rat aorta

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1. Single K^+ channel currents and membrane potential were recorded in the endothelium of excised intact rat aorta.

2. Two types of K^+ channel were found in excised patches, K_{Ch} and K_{Ap}. With Na^+ and K^+ as the main external and internal cations, outward conductances were 6·7 pS (K_{Ch}) and 2·8 pS (K_{Ap}). In symmetric 150 mM K^+, the inward conductances were 18 and 9·1 pS.

3. Activation by Ca^{2+} was concentration dependent. K_{Ch} channels were activated by $[Ca^{2+}] > 0{\cdot}1$ μM and K_{Ap} by $[Ca^{2+}] > 0{\cdot}5$ μM.

4. Apamin at concentrations > 1 nM inhibited K_{Ap} channels. Block was complete at 10 nM. K_{Ap} channels were insensitive to charybdotoxin. K_{Ch} channels were inhibited by charybdotoxin at concentrations > 50 nM, but were insensitive to apamin.

5. *d*-Tubocurarine (dTC) evoked flickering activity of K_{Ap} channels at concentrations > 5 μM and complete block at 100 μM. At these doses, dTC did not affect K_{Ch} channels, but at concentrations > 1 mM it decreased the single channel amplitude.

6. Hyperpolarization evoked by acetylcholine was unaffected by apamin or dTC at low concentrations ($\leqslant$ 100 μM), but inhibited by high concentrations of charybdotoxin (>50 nM) or dTC (>1 mM).

7. These data suggest that K_{Ch} channels are novel Ca^{2+}-activated K^+ channels responsible for the ACh-evoked hyperpolarization in the endothelium of rat aorta.

Acetylcholine (ACh) and other vasodilators evoke complex electrical responses in the endothelium of intact excised rat aorta, which consist of an initial hyperpolarization, followed by depolarization and in some preparations by oscillations in membrane potential (Marchenko & Sage, 1993). Such responses are also evoked by ACh in endothelium mechanically isolated from underlying smooth muscle (Marchenko & Sage, 1994*a*). Thus the vasodilator-evoked response is generated entirely within the endothelium itself, in contrast to the electrical responses evoked by vasoconstrictors which require the presence of smooth muscle (Marchenko & Sage, 1994*b*).

Simultaneous recording of endothelial membrane potential and cytosolic calcium concentration ($[Ca^{2+}]_i$) shows that the vasodilator-evoked hyperpolarization temporally coincides with a rise in $[Ca^{2+}]_i$ (Usachev, Marchenko & Sage, 1995). Reducing Ca^{2+} entry by block with Ni^{2+} or removal of external Ca^{2+} abolishes a plateau phase of hyperpolarization (Marchenko & Sage, 1993), whilst loading the endothelium with the Ca^{2+} buffer BAPTA, reduces both the initial peak and the plateau of the hyperpolarization (Marchenko & Sage, 1994*a*). Responses similar to those evoked by vasodilators could be evoked by ionomycin or thapsigargin, agents which generate receptor-independent elevations in $[Ca^{2+}]_i$ (Marchenko & Sage, 1994*a*). Hyperpolarizing the endothelial membrane below the K^+ equilibrium potential converts the ACh-evoked response to one of depolarization (Marchenko & Sage, 1993). All these observations suggest the involvement of Ca^{2+}-activated K^+ channels in the ACh-evoked hyperpolarization of endothelium of intact aorta. Such channels have also been suggested or observed in a number of cultured endothelial preparations (Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Sauve, Parent, Simoneau & Roy, 1988; Chen & Cheung, 1992; Sharma & Davis, 1994).

We have now studied K^+ channels in the lumenal surface membrane of the endothelium of excised intact rat aorta. We have identified two types of Ca^{2+}-activated K^+ channel, one sensitive to apamin (K_{Ap}) and one to charybdotoxin (K_{Ch}). The pharmacological properties of the latter were similar to that of the ACh-evoked hyperpolarization.

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Some of these results have been reported in preliminary form (Marchenko & Sage, 1994*c*, 1995).

METHODS

The experiments were carried out on the endothelium of excised intact rat aorta essentially as previously described (Marchenko & Sage, 1993). Briefly, rats aged 1–2 months were anaesthetized with diethylether and killed by cervical dislocation. The thoracic aorta was dissected out, cut into rings of 3–4 mm length and stored in modified Krebs solution of composition (mM): 118·3 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·2 NaH_2PO_4, 1·2 $MgSO_4$, 2·5 $CaCl_2$, 11·1 glucose, 0·01 phenol red; pH 7·4 at 20 °C. The solution was gassed with 95% O_2–5% CO_2 and 50 μg ml^{-1} gentamycin was added. Rings were used for up to 2 days during which time no noticeable changes in responses were observed (Marchenko & Sage, 1993). Before an experiment, an aortic ring was cut open and pinned, lumen face up, to the rubber bottom of a chamber of volume 100 μl and perfused with modified Krebs solution at a rate of 1–2 ml min^{-1}.

Membrane potential was recorded using the whole-cell configuration of the patch clamp technique with an Axopatch-1B patch clamp amplifier (Axon Instruments) in current clamp mode. Electrical contact with the cytosol was established using nystatin, which was added to the filling solution at a concentration of 400 μg ml^{-1}. Pipettes were filled with a solution of composition (mM): 140 KCl, 10 Hepes; pH adjusted to 7·4 with NaOH. Single channel activity was recorded from patches of endothelial membrane from the lumenal face of the vessel in inside-out, outside-out and cell-attached configurations. Patch pipettes were pulled from aluminosilicate glass (Clark Electromedical, Pangbourne, Berks, UK). In the outside-out configuration, the pipettes were filled with a solution of composition (mM): 150 KCl, 3 NaCl, 10 Hepes and 0·5 EGTA. In the inside-out configuration, the pipettes were filled with a solution of composition (mM): 150 NaCl, 3 KCl, 10 Hepes and 0·5 mM EGTA. For both solutions, pH was adjusted to 7·35 with NaOH. $CaCl_2$ was added to the solutions to obtain the required concentration of free Ca^{2+}. $[Ca^{2+}]_i$ in the solutions was measured using fura-2. After formation of an excised patch, the pipette was transferred to a smaller chamber where it was perfused with one of the above solutions.

Experiments were conducted at room temperature (range, 18–20 °C). Pharmacological agents were applied by bath perfusion. Traces shown are typical of at least four experiments. Quantitative data are expressed as means ± S.E.M. of the number (n) of observations indicated.

RESULTS

Potassium channels in endothelium of intact rat aorta

Two types of K^+ channel were found in excised membrane patches from the lumenal surface of intact excised rat aorta (Fig. 1*A* and *B*). In asymmetric solutions (K^+ at internal membrane face, Na^+ at external face), the voltage dependencies of the outward currents carried by both channels were linear and the extrapolated reversal potentials for both currents were close to the K^+ equilibrium potential, which was −98·5 mV in these experiments (Fig. 1*C*). The larger channel (K_{Ch}) had a slope conductance for outward current of 6·7 ± 1·1 pS ($n = 7$). The slope conductance for outward current for the smaller

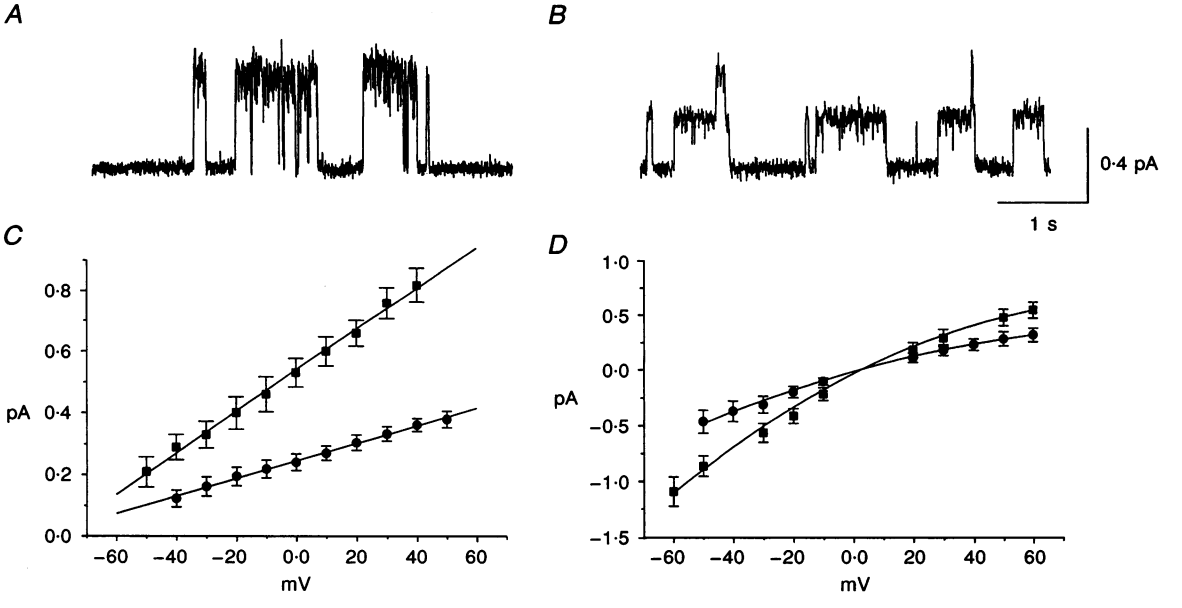

Figure 1. Two types of K^+ channel in outside-out patches from endothelium of intact rat aorta

K_{Ch} channels (*A*) and K_{Ap} channels (*B*), both recorded at a membrane potential of 0 mV. *C* and *D* show current–voltage relationships for K_{Ch} (■) and K_{Ap} (●) channels, respectively. In *A*, *B* and *C*, the pipette contained a K^+ solution and the bath a Na^+ solution. For *D*, symmetrical K^+ solutions were used.

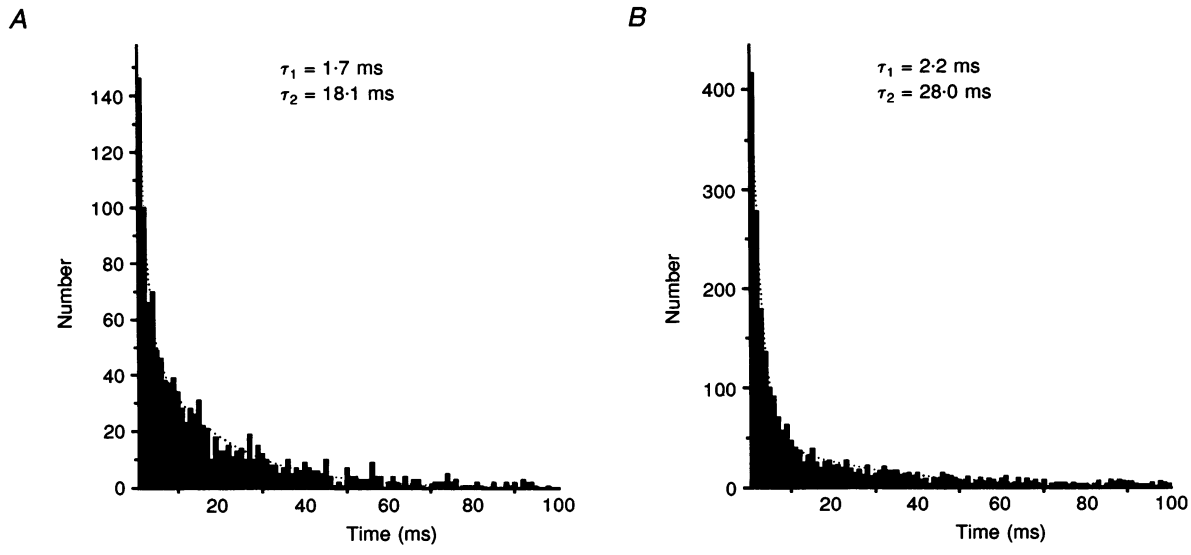

Figure 2. Representative open time distributions of K_{Ch} (*A*) and K_{Ap} (*B*) channels
These histograms were fitted with two exponentials with the time constants (τ) shown.

channel (K_{Ap}) was 2·8 ± 0·7 pS ($n = 8$). In symmetric K^+ solutions, the current–voltage relationships for both channels showed some inward rectification (Fig. 1*D*). The slope conductance for inward current through K_{Ch} channels was 18 ± 3 pS ($n = 5$): that of K_{Ap} channels was 9·1 ± 2·4 pS ($n = 7$).

The open time distribution of K_{Ch} was fitted by two exponentials with $\tau_1 = 1{\cdot}7 \pm 0{\cdot}2$ ms and $\tau_2 = 18{\cdot}1 \pm 2{\cdot}3$ ms ($n = 5$; Fig. 2*A*). For K_{Ap}, the open time distribution was fitted by two exponentials with $\tau_1 = 2{\cdot}2 \pm 0{\cdot}3$ ms and $\tau_2 = 28{\cdot}0 \pm 3{\cdot}7$ ms ($n = 8$; Fig. 2*B*).

K_{Ch} channels were found in 12 out of 231 patches studied. K_{Ap} channels were much more common and found in 94 out of 207 patches. Six patches with both channel types were found (not shown).

Calcium dependence of potassium channels

Depolarization activated neither K_{Ap} nor K_{Ch} channels, but both were activated by a rise in $[Ca^{2+}]$ at the internal membrane face (Fig. 3). K_{Ch} channels were practically silent at a $[Ca^{2+}] < 100$ nM and a rise within the submicromolar range sharply increased the open probability of the channel (Figs 3*A* and 4), with no effect on its amplitude (not shown).

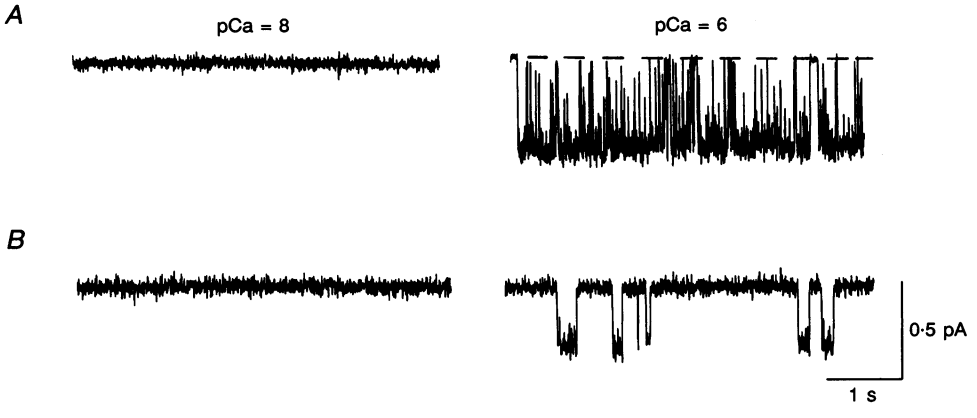

Figure 3. Activation of K_{Ch} (*A*) and K_{Ap} (*B*) channels by an increase in $[Ca^{2+}]$ in the K^+ solution at the internal membrane face
Inside-out patches, openings downward. Both channels were silent at $[Ca^{2+}]$ of 10 nM. K_{Ch} channels were completely activated (*A*, dashed lines indicate baseline current (closed state)) and K_{Ap} channels partially activated (*B*) by an increase in $[Ca^{2+}]$ to 1 μM.

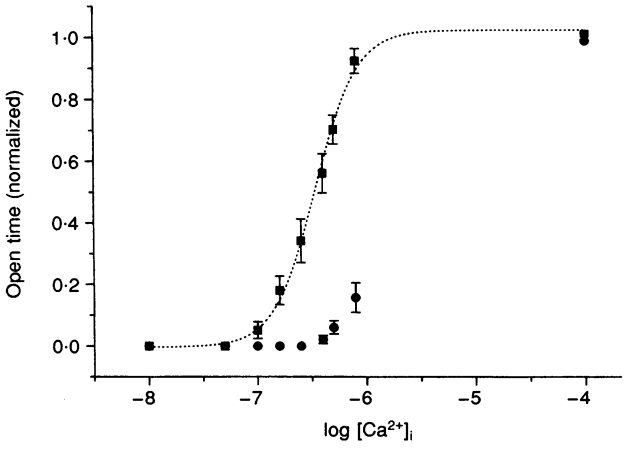

Figure 4. Dependence of the normalized open times of the K_{Ch} (■) or K_{Ap} (●) channels on $[Ca^{2+}]$ at the internal membrane face

K_{Ch} channels were activated by $[Ca^{2+}]$ in the range 0·1–1·0 μM. K_{Ap} channels were activated by $[Ca^{2+}] > 0{\cdot}5$ μM.

The EC_{50} was $0{\cdot}34 \pm 0{\cdot}04$ μM ($n = 5$) and activation was complete at a $[Ca^{2+}]$ of about 1 μM. The Hill coefficient for the activation was $2{\cdot}5 \pm 0{\cdot}3$ ($n = 5$), suggesting the participation of at least three Ca^{2+} binding sites.

K_{Ap} channels were activated by $[Ca^{2+}] > 0{\cdot}5$ μM. Activation was not complete at a $[Ca^{2+}]$ of 1 μM (Figs 3*B* and 4). Elevating $[Ca^{2+}]$ at the external membrane face did not affect the activity of either channel (not shown).

Effects of charybdotoxin on K_{Ch}

Charybdotoxin (10 nM to 1 μM) did not affect K_{Ap} channels (not shown). At low concentrations (1–20 nM), charybdotoxin was without effect on K_{Ch} channels, but at higher concentrations it inhibited them with an EC_{50} of 137 ± 24 nM ($n = 4$; Fig. 5). Block was complete at a charybdotoxin concentration of about 1 μM. The effect was readily reversible (Fig. 5, lower record).

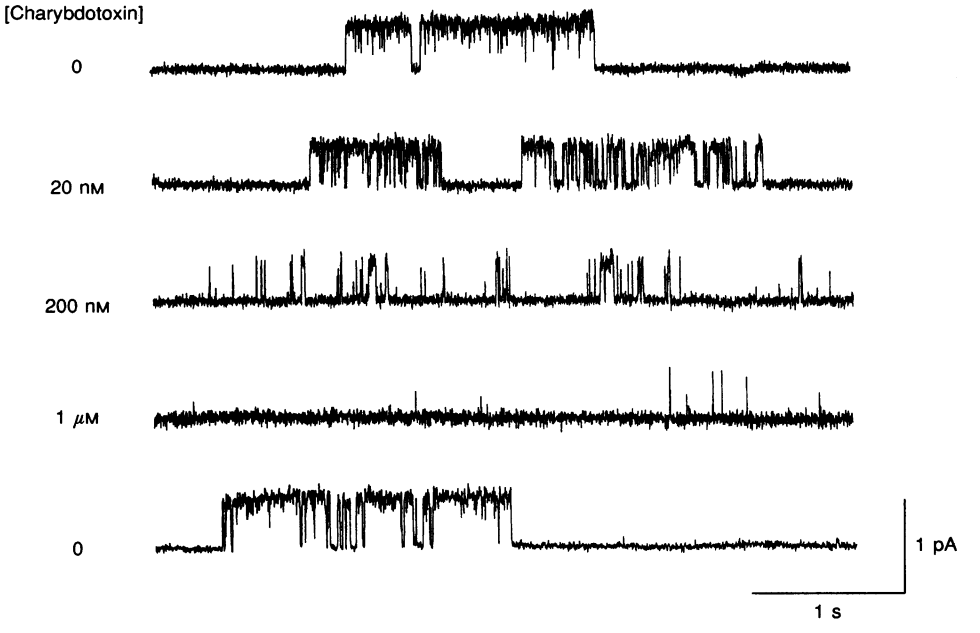

Figure 5. Inhibition by charybdotoxin of K_{Ch} channels in an outside-out patch

The concentration of charybdotoxin indicated at left-hand side of traces. Charybdotoxin reversibly inhibited K_{Ch} channels at concentrations > 50 nM, with complete block at about 1 μM.

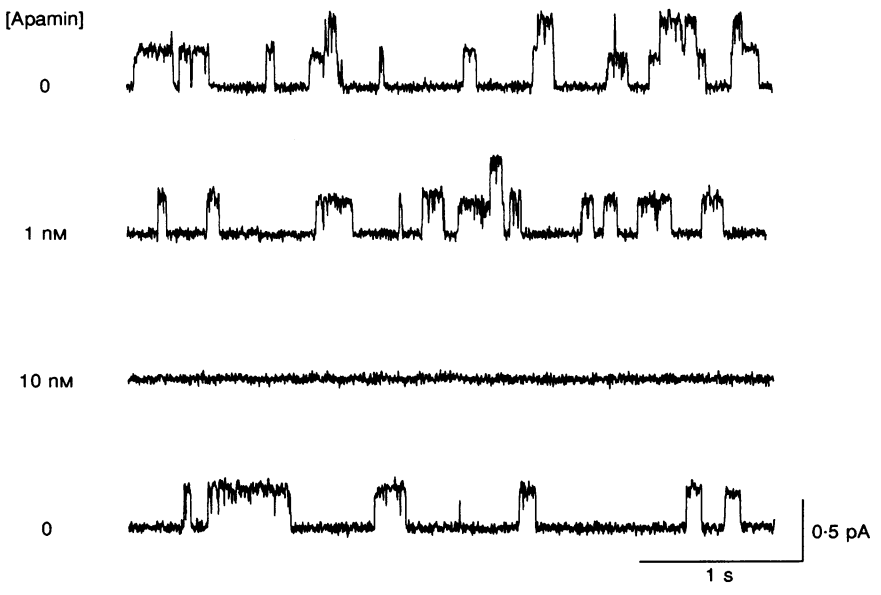

Figure 6. Inhibition of K_{Ap} channels by apamin
Apamin inhibited K_{Ap} channels at concentrations > 1 nM, with complete block at 10 nM. The effect was slowly reversible.

Effects of apamin on K_{Ap} and K_{Ch}

Apamin at concentrations >1 nM inhibited K_{Ap} channels and block was complete at an apamin concentration of 10 nM (Fig. 6). This effect was only slowly reversible. It usually took 20–50 min for the activity to partially recover. K_{Ch} channels were not inhibited by apamin at concentrations of 10 nM to 1 μM (not shown).

Effects of *d*-tubocurarine on K_{Ap} and K_{Ch}

d-Tubocurarine at concentrations > 5 μM induced flickering of K_{Ap} channels and completely blocked their activity at 100 μM (Fig. 7*B*). At these concentrations, *d*-tubocurarine did not affect K_{Ch}, but at concentrations $\geqslant$ 1 mM, K_{Ch} single channel current amplitude was reduced (Fig. 7*A*). The effects of *d*-tubocurarine were reversible (Fig. 7, lower traces).

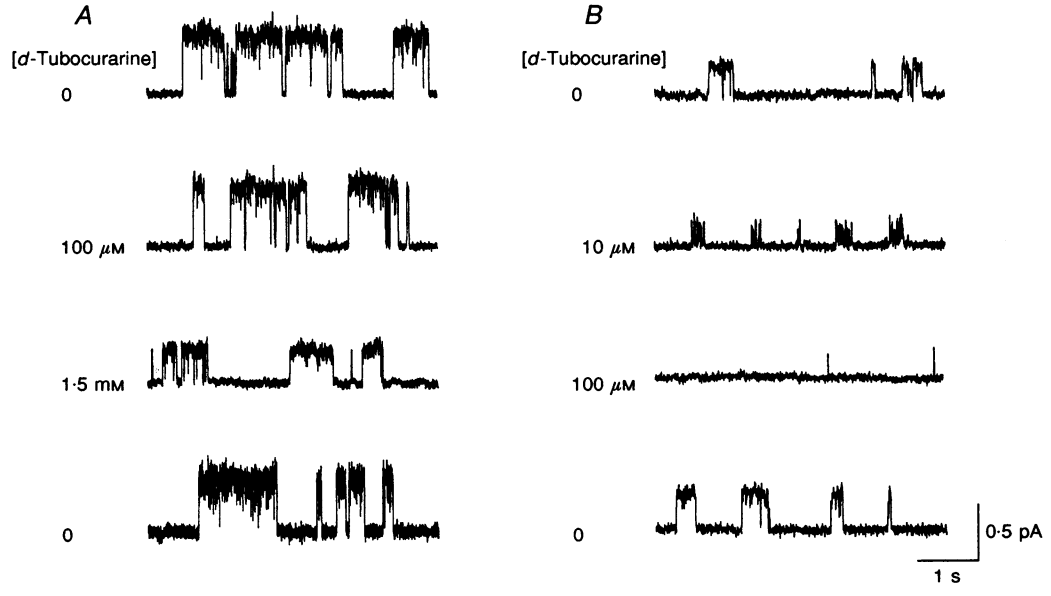

Figure 7. Effects of *d*-tubocurarine on Ca^{2+}-activated K^+ channels in outside-out patches
A, *d*-tubocurarine at 100 μM did not affect K_{Ch} channels, but at 1·5 mM reduced the single channel current amplitude. *B*, *d*-tubocurarine at 10 μM induced burst activity of K_{Ap} channels and at 100 μM completely blocked them. These effects were readily reversible (lower records).

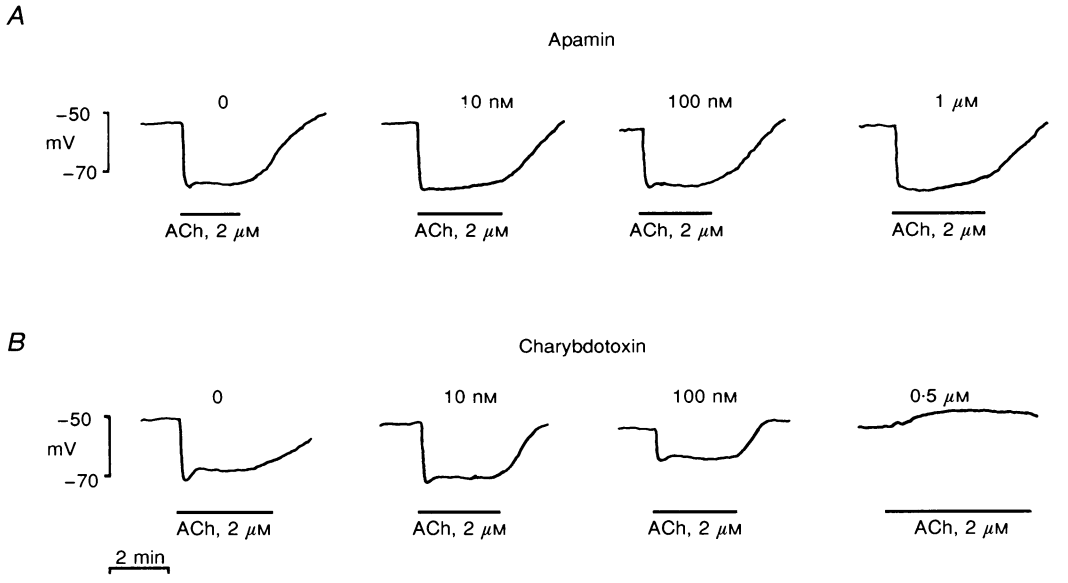

Figure 8. Effects of apamin (*A*) or charybdotoxin (*B*) on ACh-evoked hyperpolarizations of the endothelium

A, apamin at concentrations up to 1 μM was without effect. *B*, charybdotoxin at concentrations > 50 nM reduced the hyperpolarization.

Pharmacology of the ACh-evoked hyperpolarization

As previously reported (Marchenko & Sage, 1993), ACh (0·5–2 μM) evoked a large hyperpolarization in the endothelium of intact rat aorta which lasted >2 min and was usually followed by a depolarization above the resting potential and then, in some preparations, by oscillations in the membrane potential. Apamin (10 nM to 1 μM) affected neither the resting membrane potential nor the ACh-evoked hyperpolarization (Fig. 8*A*). In fourteen out of sixteen preparations, charybdotoxin did not affect the resting membrane potential. In two preparations, it depolarized the membrane by 1–2 mV at a concentration of 1 μM (not shown). Charybdotoxin at lower concentrations (1–20 nM) had little effect on the ACh-evoked hyperpolarization, but at higher concentrations (>50 nM) reduced it in a concentration-dependent manner (Fig. 8*B*). In the presence of charybdotoxin at concentrations of 0·5–1 μM, ACh (2 μM) evoked a depolarization rather than a hyperpolarization (Fig. 8*B*).

d-Tubocurarine at lower concentrations (10–100 μM) did not affect the ACh-evoked hyperpolarization, but at concentrations > 1 mM it reduced the response in a concentration-dependent manner (not shown).

DISCUSSION

Two types of Ca^{2+}-activated K^+ channel were found in the membrane of the lumenal surface of the endothelium of intact rat aorta. Those we have designated K_{Ap} are inhibited by nanomolar concentrations of apamin and micromolar concentrations of *d*-tubocurarine, but are insensitive to charybdotoxin. The channels designated K_{Ch} were, in contrast, insensitive to apamin and micromolar concentrations of *d*-tubocurarine, but were inhibited by charybdotoxin and millimolar concentrations of *d*-tubocurarine.

The classification of Ca^{2+}-activated K^+ channels includes two distinct types (for reviews, see Latorre, Oberhauser, Labarca & Alvarez, 1989; Castle, Haylett & Jenkinson, 1989). Large conductance (100–250 pS), Ca^{2+}-activated K^+ channels (BK channels), are inhibited by nanomolar concentrations of charybdotoxin (Barrett, Magelby & Pallotta, 1982; Miller, Moczydlowski, Lattorre & Phillips, 1985). Small conductance (< 80 pS), Ca^{2+}-activated K^+ channels (SK channels), are inhibited by nanomolar concentrations of apamin (Blatz & Magelby, 1986). A third class of Ca^{2+}-activated K^+ channel is less well studied. It includes Ca^{2+}-activated K^+ channels of intermediate conductance (18–60 pS) which are also inhibited by nanomolar concentrations of charybdotoxin (IK channels) (Grygorczyk & Schwarz, 1983; Ewald, Williams & Levitan, 1985).

The K_{Ap} channels described here have properties similar to the low conductance, apamin-sensitive channels (SK channels) found in other cells (Latorre *et al.* 1989). The K_{Ch} channels have a conductance (18 pS) that falls into the range of both small conductance and intermediate conductance Ca^{2+}-activated K^+ channels. However, unlike SK channels, K_{Ch} channels are not inhibited by apamin,

and they are several hundred times less sensitive to charybdotoxin than the reported IK channels (Castle, Haylett & Jenkinson, 1989). Therefore, K_{Ch} channels are clearly distinct from BK and SK channels and significantly different pharmacologically from reported IK channels. K_{Ch} channels cannot, therefore, be confidently placed in any existing class of Ca^{2+}-activated K^+ channel. Ca^{2+}-activated K^+ channels of relatively low conductance (35 pS) and low sensitivity to charybdotoxin (apparent dissociation constant 30 nM) have also been found in *Aplysia* neurones (Hermann & Erxleben, 1987). These channels may form a new class of Ca^{2+}-activated K^+ channels. However, it may be that they should be classified as IK channels and this class be considered to have a wide range of sensitivities to charybdotoxin.

Ca^{2+}-activated K^+ channels have been reported in cultured and freshly isolated endothelial cells in a number of studies. Large conductance Ca^{2+}-activated K^+ channels (BK channels) were found in cultured bovine and freshly isolated rabbit arterial endothelial cells (Fichtner, Frube, Busse & Kohlhardt, 1987; Cannell & Sage, 1989; Rusko, Tanzi, van Breemen & Adams, 1992). We did not observe BK channels in the endothelium of intact rat aorta and the low sensitivity of the ACh-evoked hyperpolarization to charybdotoxin in the endothelium of this vessel suggests against their participation in this response. Ca^{2+}-activated K^+ channels of lower conductance were observed in cultured bovine aortic and porcine coronary artery endothelial cells (Sauve *et al.* 1988; Sharma & Davis, 1994). The pharmacological properties of these channels were not characterized in enough detail to classify them. They had conductances of 23–40 pS for inward current in symmetric K^+ solutions, were not activated by depolarization, and were activated by $[Ca^{2+}] < 1$ μM. It is possible that these channels are similar to the K_{Ch} channels that we report here. As far as we are aware, no channels similar to K_{Ap} channels have previously been described in endothelial cells (Revest & Abbott, 1992).

Apamin and charybdotoxin had little effect on the membrane potential of unstimulated endothelium in intact rat aorta. This suggests that neither K_{Ap} nor K_{Ch} channels participate in setting the resting potential. K_{Ch} channels were activated by a rise in $[Ca^{2+}]$ at the internal membrane face in the range of 0·1–1 μM, whereas K_{Ap} channels were only activated by a $[Ca^{2+}] > 0{\cdot}5$ μM. Our recent measurements of the cytosolic calcium concentration ($[Ca^{2+}]_i$) in the endothelium of intact rat aorta (made using fura-2) indicated that resting $[Ca^{2+}]_i$ was 95 ± 8 nM (Usachev *et al.* 1995). Therefore, the $[Ca^{2+}]_i$ in the resting endothelium appears too low to activate both K_{Ap} and K_{Ch} channels.

The hyperpolarization evoked by ACh in the endothelium of intact rat aorta was inhibited by high concentrations of charybdotoxin and *d*-tubocurarine, but was unaffected by apamin or lower concentrations of *d*-tubocurarine. Therefore, the pharmacology of this response was similar to that of the K_{Ch} channels but quite different to that of the K_{Ap} channels. This indicates the involvement of K_{Ch} but not K_{Ap} channels in the ACh-evoked hyperpolarization.

Vasodilator-evoked hyperpolarizations have been reported to have a similar pharmacology by other workers. *d*-Tubocurarine (1 mM) reduced bradykinin-evoked hyperpolarization in cultured guinea-pig endothelial cells (Merke & Daut, 1990). Apamin at very high concentrations (1–10 μM) had little or no effect on bradykinin or ACh-evoked hyperpolarization of coronary artery endothelium (Merke & Daut, 1990; Chen & Cheung, 1992), while charybdotoxin inhibited ACh-evoked hyperpolarization at concentrations >10 nM (Chen & Cheung, 1992).

Acetylcholine evokes a rise in $[Ca^{2+}]_i$ in the endothelium of excised intact rat aorta that temporally coincides with the membrane hyperpolarization (Usachev *et al.* 1995). After the initial ACh-evoked rise in $[Ca^{2+}]_i$ in this preparation, the $[Ca^{2+}]_i$ fell to a plateau maintained below 500 nM. Carter & Ogden (1994) measured $[Ca^{2+}]_i$ in a preparation identical to that which we have used, but employed a low affinity fluorescent indicator, furaptra. This allowed the detection of elevations in $[Ca^{2+}]_i > 1$ μM. It was reported that ACh evoked transient spikes in $[Ca^{2+}]_i$ of up to 35 μM, which were followed by a fall in the $[Ca^{2+}]_i$ below the resolution of the indicator. Simultaneous recordings of membrane potential indicated that the ACh-evoked hyperpolarization lasted for longer than the spikes in $[Ca^{2+}]_i$, suggesting that these spikes were not activating the Ca^{2+}-activated K^+ channels responsible for the hyperpolarization. Rather, it suggests that the channels responsible are activated by a $[Ca^{2+}]_i < 1$ μM and below the limit of resolution using furaptra. These data are thus compatible with the hypothesis that the K_{Ch} channels we report here are responsible for the ACh-evoked hyperpolarization. Both resting and stimulated changes in $[Ca^{2+}]_i$ in endothelium are influenced by membrane potential changes which influence the driving force for Ca^{2+} entry (e.g. Cannell & Sage, 1989; Usachev *et al.* 1995). The functions of the K_{Ap} channels are unclear. They may be transiently activated by large spikes in $[Ca^{2+}]_i$, but most of the time $[Ca^{2+}]_i$ would appear to be below the concentration needed for activation of K_{Ap} channels (Carter & Ogden, 1994; Usachev *et al.* 1995).

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