Muscarnin-Activated $K^+$ Current in Bovine Aortic Endothelial Cells

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Isolated, cultured arterial endothelial cells express an acetylcholine (ACh)-activated $K^+$ current in addition to an inward rectifier current whose conductance is unaffected by ACh. The cholinergic $K^+$ current is specifically blocked by atropine (1 μM) and shows single saturation kinetics with ACh (half-maximal stimulation 51 nM ACh). Unlike the cardiac muscarinic receptor-gated $K^+$ channel, its stimulation appears independent of a pertussis toxin-sensitive GTP-binding protein. Activation of the endothelial muscarinic $K^+$ current resulting in hyperpolarization may represent an initial component of the vasodilatory effect of ACh. (Circulation Research 1988;62:1059–1064)

The presence of vascular endothelial cells is essential for acetylcholine (ACh)-induced relaxation of precontracted blood vessels. Endothelial cells possess cholinergic ACh receptors as shown functionally and by receptor binding studies. A humoral mediator of smooth muscle cell relaxation, endothelium-derived relaxing factor (EDRF), has been demonstrated in response to stimulation of the muscarinic receptors of either superfused artery with intact endothelium or microcarrier-borne endothelial cells. Recently, however, Segal and Duling have demonstrated the bidirectional conductance of an ACh-mediated vasodilatory stimulus in arteries that is not accountable by simple diffusion of a humoral mediator such as EDRF and is independent of blood flow. Their data suggest that some form of direct communication between arteriolar cells is necessary for propagation of the stimulus leading to coordinated vasodilation. The initial steps in activation of endothelial cells by ACh are not known. This study describes the activation by ACh of a muscarinic $K^+$ channel in cultured aortic endothelial cells. The resulting hyperpolarization may represent the initial cellular response linked to ACh-induced vasoactivity mediated via direct junctional conductance to smooth muscle cells or by stimulating the release of humoral effectors such as EDRF.

Materials and Methods

Single endothelial cells were isolated from calf aorta (BAEC-CLC1) by collagenase digestion according to Schwartz followed by subculture to passage 7 at which time the cells were frozen in the presence of dimethyl sulfoxide. Vials containing $1 \times 10^5$ viable endothelial cells were thawed and, after subculture, dispersed by trypsin:EDTA in 35-mm dishes (Corning Glass Works, Corning, New York; $5 \times 10^4$ cells/dish) that had been precoated with polyHEMA (an evaporated solution of 250 μl 0.024% polyhydroxyethyl methacrylate in ethanol; Hydron Laboratories, Brunswick, New Jersey). The thin polymer layer promoted slight changes of cell shape, resulting in greater curvature of the apical cell surface and facilitating reproducible approach and attachment by the patch-clamp micropipette. Dulbecco’s Modified Eagle’s Medium containing 10 mM HEPES, 2 μmol glutamine, 100 U penicillin, and 100 μg streptomycin/ml and supplemented with 10% calf serum was used for all endothelial cell cultures. The endothelial cells used for this study were from subcultures 8–16 or were in primary culture.

We observed that fast perfusion of the bath surrounding the endothelial cells activated stretch-sensitive conductances as previously described. To avoid perfusion-related currents, all applications of ACh were made via slow changes in the total bath concentration at low rates (<2 ml/min). The ion composition in the bath solution was as follows (mM): K+ 4, Na+ 150, Cl− 150, Ca2+ 1, Mg2+ 2, HEPES 10, pH was adjusted to 7.4 with HCl. The intracellular solution contained (mM): K+ 145, Cl− 116, Ca2+ 1, Mg2+ 2, EGTA 11, HEPES 10, GTP 0.20, pH was adjusted to 7.2 with HCl.

The cells were voltage-clamped, and whole-cell currents were recorded using the patch-clamp technique. The signals were recorded with a List EPC-7 amplifier and whole-cell recordings sampled at 200 μsec/point by an INDEC computer. The electrode tip resistance was 5–8 MΩ. The average cell capacitance was 23.0 pF (SD = 11.7 pF, $n = 57$) giving an estimated cell surface area of 2,300 μm², assuming a specific membrane capacitance of 1 μF/cm².

Results

The resting membrane potential of endothelial cells, determined immediately after access to the cell interior, was $-77.4$ mV (SD = 3.5 mV, $n = 26$). Depolar-
ization of the cells from $-80$ mV holding potential to potentials ranging from $-40$ to $+50$ mV failed to elicit any detectable inward currents. The endothelial cells had a very small time-independent outward conductance in this range with an average value of $5.0 \text{ pS/pF} (SD = 2.3 \text{ pS/pF}, n = 6) \text{ at } +40 \text{ mV}$. The lack of inward currents activated by depolarization was also observed in two other bovine aortic endothelial cell lines (BAEC-SOC1, BAEC-11), in primary cultures of bovine aortic endothelial cells, and in primary cultures of endothelial cells from human umbilical cord vein.

Hyperpolarizing the cells below $-90$ mV activated a large inward current. The response to a voltage step from a holding potential of $-40$ mV to $-120$ mV is shown in Figure 1A. The current-voltage (I-V) curve, obtained by similar steps to different membrane potentials, shows typical inward rectification (Figure 1B, control). Administration of $10 \mu M$ ACh to the bath activated a second current, which added to the inward rectifier (Figure 1A). The ACh-induced current, named $I_{K,ACH}$, was defined as the difference between the control current and the membrane current in the presence of ACh in the bath. $I_{K,ACH}$ is also an inward rectifier with a slope conductance of $70 \text{ pS/pF} \text{ at } -110 \text{ mV}$ (Figure 1B). The extrapolated reversal potential at $[K^+]_o = 4 \text{ mM}$ was $-79$ to $-85 \text{ mV}$. In primary cultures of bovine aortic endothelial cells, $10 \mu M$ ACh also induced a current with a similar I-V relation and conductance as $I_{K,ACH}$ (reversal potential $= -86 \text{ mV}$ at $[K^+]_o = 4 \text{ mM}$, slope conductance $= 85 \text{ pS/pF} \text{ at } V_h = -110 \text{ mV}, n = 6$).

The amplitude of the whole-cell current induced by ACh was concentration-dependent. The increase in inward current $20$ seconds after application of ACh to the bath was determined at four different concentrations in each cell at a membrane potential of $-120 \text{ mV}$. When ACh in the bath solution was raised from $0.01$ to $0.1$, and $10 \mu M$, the inward current increased in a dose-dependent fashion. The dose-response curve in Figure 2A is roughly approximated by single saturation kinetics, and a half-maximal effect was evoked by $40 \text{ nM}$ ACh. The average concentration of ACh, which induced half-maximal effect, was $51 \text{ nM} (SD = 26 \text{ nM})$ based on six dose-response curves. This compares with the half-maximal activation of the inward-rectifying K$^+$ channel in heart atrial cells induced by $150 \text{ nM}$ ACh.$^{13}$. The current induced by $1 \mu M$ ACh was fully inhibited by $1 \mu M$ atropine (Figure 2B), indicating that the response is mediated via a muscarinic receptor ($n = 11$). The responsiveness of the endothelial cells to ACh was gradually regained after atropine treatment over a period of $-5$ minutes.

Muscarnic receptors are often coupled to cellular responses by GTP-binding proteins (G-proteins).$^{14}$ An inward rectifying K$^+$ conductance gated by muscarinic receptors coupled to G-proteins has been described in cardiac atrial cells.$^{15,16}$ Two G-proteins (G$\alpha_i$ and G$\alpha_o$) can be irreversibly inhibited by pertussis toxin (PT), which uncouples them from the receptor. Endothelial cells were pretreated with up to $1 \mu g/ml$ PT for $16$ hours before the experiment, and $I_{K,ACH}$ was determined at a membrane potential of $-120 \text{ mV}$. Treatment of endothelial cells with PT (100-400 ng/ml) for $1-3$ hours has

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Whole-cell currents through inward rectifying channels in bovine aortic endothelial cells. A: Inward currents elicited by hyperpolarization. The top trace shows the command voltage step from $-40 \text{ mV}$ to $-120 \text{ mV}$, and the lower traces the current records before (control) and $20$ seconds after administration of $10 \mu M$ ACh to the bath solution. Cell capacitance was $18 \text{ pF}$. B: I-V curves of inward rectifier currents. The cells were held at $-40 \text{ mV}$, and a series of command voltage pulses from $-120 \text{ mV}$ to $+20 \text{ mV}$ were applied, each for a period of $200 \text{ msec}$. Currents were recorded at each test potential before ($\times$) and $10-60$ seconds after administration of $10 \mu M$ ACh ($\bigcirc$). The current component induced by ACh, $I_{K,ACH}$, was obtained by subtracting the control current from the current in the presence of ACh at each potential ($\triangle$). Whole-cell currents were divided by cell capacitance (ordinate). Data from six different cells were averaged for each point ($\pm \text{SD}$).
Figure 2. A: Endothelial cell $I_{K,ACh}$ dose-response curve. The membrane potential was held at $-40 \text{ mV}$ and stepped to $-120 \text{ mV}$ for 500 msec periods every 2 seconds. The increased inward current was measured 20 seconds after perfusing the bath solution with various concentrations of ACh. Cell capacitance = 17 pF. Half-maximal effect was induced by 40 nM ACh. The sigmoid curve was fitted by eye. B: Inhibition of $I_{K,ACh}$ by atropine. The membrane potential was held at $-110 \text{ mV}$. ACh (1 pM) and atropine (1 yM) were administered slowly to the bath during the periods indicated by the bars above the current trace. ACh induced an inward current that was totally inhibited by administration of atropine + ACh. When ACh was applied alone immediately after the atropine treatment only a very small current could be activated. The response to ACh gradually increased 90 seconds and 210 seconds later. The recovery time at which half-maximal $I_{K,ACh}$ could be activated was $\sim 110$ seconds after atropine wash. Traced from original strip chart recording.

Previously been shown to ADP-ribosylate a 41–42 kDa protein in the cell membrane. Under these conditions, $I_{K,ACh}$ was 4.1 pA/pF (SD = 1.5 pA/pF, $n = 6$), not significantly different from the 5.5 pA/pF (SD = 2.0, $n = 4$) recorded in nontreated cells. Dialysis of atrial cells with a GTP-free solution blocks muscarinic activation of K$^+$ currents. In endothelial cells, dialysis with GTP-free solutions did not remove the muscarinic current ($I_{K,ACh}$ = 6.4±2 pA/pF, $n = 4$). More importantly, dialysis of the cell interior with a pipette solution containing 20 uM GTPyS (a nonhydrolyzable GTP analogue) for several minutes before ACh challenge did not affect the ACh-induced current ($I_{K,ACh}$ = 4.5±1.5 pA/pF, $n = 3$). Thus, it seems unlikely that this muscarinic receptor is coupled via G-proteins to the channel.

The ACh-induced conductance was K$^+$-selective. I-V relations for $I_{K,ACh}$ were determined at four different extracellular potassium concentrations ([K$^+$]o = 4, 8, 16, and 32 mM), and the extrapolated reversal potentials were plotted as a function of [K$^+$]o (Figure 3). The average change in reversal potential per 10-fold change in [K$^+$]o was 53 mV (SD = 5 mV, $n = 4$), close to the predicted Nernst relation for a purely potassium-selective membrane. In other experiments, Cl$^-$ was replaced by glutamate to a final Cl$^-$ concentration of 15 mM. The reversal potentials of $I_{K,ACh}$ at an extracellular potassium concentration of 145 mM were 3±5 mV at [Cl$^-$]o = 116 mM and 1±4 mV at [Cl$^-$] = 15 mM ($n = 3$).

In an attempt to study the kinetics of $I_{K,ACh}$, we used a fast perfusion method to give a steady-state ACh concentration at the cell surface within 100 msec. At a membrane potential of $-120 \text{ mV}$, with 10 uM ACh applied to the cell, $I_{K,ACh}$ was fully activated within 250 msec. The current did not desensitize within an administration period of 40 seconds ($n = 6$). When ACh was washed away, the inward current returned to control level within 2 seconds. This may mean that $I_{K,ACh}$ does not desensitize, but we cannot exclude the possibility that the increased shear stress induced by the fast application of ACh activated other currents that compensated desensitization.

Single channel currents were recorded in outside-out patches of endothelial cell membrane. Both the bath and the pipette contained the intracellular solution with [K$^+$]o = 145 mM; GTP (200 uM) was either present in or absent from the pipette solution. No single channel openings were encountered at positive membrane potentials, but at negative membrane potentials,
A

\[ V \text{ (mV)} \]

\[ -120 \quad -100 \quad -80 \quad -60 \quad -40 \quad -20 \quad 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

\[ [K^+]_o \text{ (mM)} \]

32

16

8

4

\[ \text{A: } I-V \text{ relation recorded on an endothelial cell at various } [K^+]_o \text{ (4, 8, 16, and 32 mM), obtained by replacing sodium chloride with potassium chloride to the desired concentrations in the bath solution.} \]

\[ \text{B: Reversal potentials extrapolated from the I-V curve in Figure 3A and plotted against } [K^+]_o. \text{ The line through the points is a linear least-squares fit, with slope } = 56 \text{ mV per 10-fold increase in } [K^+]_o. \]

most patches contained a channel with a conductance of \(~25\) pS, which is similar to other time-independent inward rectifier K⁺ channels. Channel activity, measured as the integral of single channel current divided by single channel amplitude (see reference 15), was not changed after administration of \(10 \mu M\) ACh to the bath, indicating that ACh does not significantly modify the kinetics or conductance of this channel (Figure 4). Also, channel activity in cell-attached patches of cells perfused with ACh was not significantly modified. We did not observe ACh-gated single channels in outside-out patches perfused with \(10 \mu M\) ACh. Thus, as we would expect from the small size of the whole-cell current, \(I_{K_{\text{ACh}}}\) channels are sparse, or their conductance is too low to measure.

Acetylcholine at a concentration of \(10 \mu M\) does not increase the free cytosolic Ca²⁺ concentration in suspensions of BAEC-CLC1 cells loaded with the fluorescent Ca²⁺ dye, fura-2 (T. Brock, personal communication), in agreement with results obtained on other bovine aortic endothelial cells. In the present study, the free intracellular Ca²⁺ concentration was buffered to \(~10^{-8}\) M. Removing Ca²⁺ from the bath solution did not attenuate \(I_{K_{\text{ACh}}}\) \((n = 4)\). This indicates that activation of the K⁺ channel by ACh is largely independent of changes in the intracellular free Ca²⁺ concentration.

Discussion

The endothelial ACh current repolarizes the cell toward \(-90\) mV (\(E_\text{K}\)). Since the cell at resting membrane potential is close to \(E_\text{K}\), one might expect \(I_{K_{\text{ACh}}}\) to have little role in a resting endothelial cell. However, endothelial cells are connected by gap junctions to vascular smooth muscle cells that regularly undergo depolarization. Application of ACh to the endothelial cell surface could then open K⁺ channels and aid in smooth muscle repolarization. Even though the potassium current is small in the depolarized range we have found no other significant currents to prevent repolarization of the cell. Furthermore, since \(I_{K_{\text{ACh}}}\) of endothelial cells does not desensitize, in contrast to \(I_{K_{\text{ACh}}}\) of atrial cells, ACh stimulation of endothelium will have long-lasting effects. The presence of gap junction communicating channels between endothelial cells and smooth muscle cells is well documented in the microcirculation and more recently in arteries, although at lower frequency and/or area of membrane contact. In tissue culture, homocellular and heterocellular electrical and metabolic communication via gap junctions occurs read-
ily between arterial endothelial and smooth muscle cells, and some evidence for metabolic communication between endothelium and smooth muscle cells in bovine aorta organ culture has been obtained.

While the significance of such communication for vasorelaxation is unclear in relation to EDRF-mediated responses, a likely possibility is that ACh-mediated hyperpolarization of the endothelial cells acting through the K⁺ channel can be communicated to subjacent smooth muscle cells and may play a role in the subsequent vasoactive response.

Direct hyperpolarization of monkey coronary artery has previously been shown to cause relaxation. Whether the endothelium-derived electrical conductance alone can explain smooth muscle relaxation or whether its local dissipation requires further amplification via other secondary systems in the vessel wall remains to be investigated.

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References


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