Classification of ion channels in the luminal and abluminal membranes of guinea-pig endocardial endothelial cells

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1. The ion channels on both the luminal and abluminal membranes of endocardial endothelial (EE) cells were separately recorded using the patch clamp technique in the guinea-pig heart.

2. The major population consisted of two types of non-selective cation channels, which showed open probabilities of 0·21 and 0·33 at the resting potential, and conductances of 36 and 11 pS, respectively.

3. The next major class was Cl^- channels with an ohmic conductance of 409 pS. The channel was quiescent in the cell-attached mode but was activated by strong depolarization after excising the patch membrane.

4. The channels activated by intracellular Ca^{2+} were mainly K^+ channels showing a 34 pS slope conductance and, less frequently, Ca^{2+}-dependent K^+ channels having a large conductance (210 pS). The inward rectifier K^+ channel (32 pS) was also observed.

5. The non-selective cation channels were recorded on the luminal membrane, but scarcely on the abluminal membrane, suggesting an active transport of K^+ and Na^+ across the endocardium.

6. The resting membrane conductance of the EE cells may be provided mostly by non-selective cation channels and 34 pS Ca^{2+}-dependent K^+ channels.

The accompanying paper (Manabe, Ito, Matsuda & Noma, 1995) demonstrated that the membrane potential of the intact guinea-pig endocardial endothelial (EE) cells was relatively low (around −45 mV) in the absence of vasoactive substances. The application of ATP, ADP, AMP, adenosine, histamine and substance P hyperpolarized the membrane by activating the 34 pS Ca^{2+}-activated K^+ channels. The hyperpolarization of the membrane may provide the driving force for the Ca^{2+} influx through the plasmalemma (EE cells: Laskey, Adams, Johns, Rubanyi & van Breemen, 1990; vascular endothelial cells: Cannel & Sage, 1989), which is required for the release of various active substances from the endothelial cells (for review see Adams, Barakeh, Laskey & van Breemen, 1989; Takeda & Klepper, 1990). To get more insight into the regulation of the membrane potential of EE cells, the present study explores the ion channel activities in the absence of the vasoactive substances.

The ion channels in the polarized epithelial cells are also related to the transepithelial ion transport as in the blood–brain barrier (Betz & Goldstein, 1986). Transepithelial ion transport is generally considered to be accomplished by an asymmetrical distribution of both the ion channels (Gögelein, 1990; Geibel, Zweifach, White, Wang & Giebisch, 1990) and the Na^+–K^+ pumps (Dibona & Mills, 1979) between the luminal and abluminal membranes. In cardiac tissue a similar mechanism is suggested to prevent an accumulation of interstitial K^+ which is critical in maintaining the function of cardiac myocytes (Brutsaert & Meulemans, 1988; Brutsaert, 1989; Brutsaert & Andries, 1992).

In the present study, a new method of recording ion channels separately from the luminal or abluminal membrane of intact EE cells was developed. A similar technique was used in confluent endothelial cells (Colden-Stanfield, Cramer & Gallin, 1992). At least six classes of ion channels, including two types of non-selective cation channels, a large conductance Cl^- channel, two types of Ca^{2+}-dependent K^+ channels and an inward rectifier K^+ channel, were distinguished by comparing their ion selectivities, conductances and gating properties. In agreement with the studies on ion channels of intact endothelial cells (Yamamoto, Chen, Miwa & Suzuki, 1992; Ito, Matsuda & Noma, 1993), non-selective cation channels represented the major population of ion channels which were spontaneously open on the luminal

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membrane. On the other hand, these channels were scarcely observed in the abluminal membrane.

METHODS

Preparation

The procedures to prepare both the small tissue specimen with intact endocardium (*in situ* endothelium) and EE cells isolated with the stamp method from guinea-pig hearts are the same as described in the accompanying paper (Manabe *et al.* 1995). The stamp method for obtaining the endothelial cells with their abluminal side up was similar to that used in cultured endothelial cells (Colden-Stanfield *et al.* 1992). A poly-L-lysine-coated (0·5%; Sigma Chemical Co., St Louis, MO, USA) coverslip was attached to the endocardium and then removed from the tissue (Fig. 1*C*). The isolation of EE cells was facilitated by the pretreatment of the heart with collagenase (for detail of sequential procedures, see the accompanying paper, Manabe *et al.* 1995).

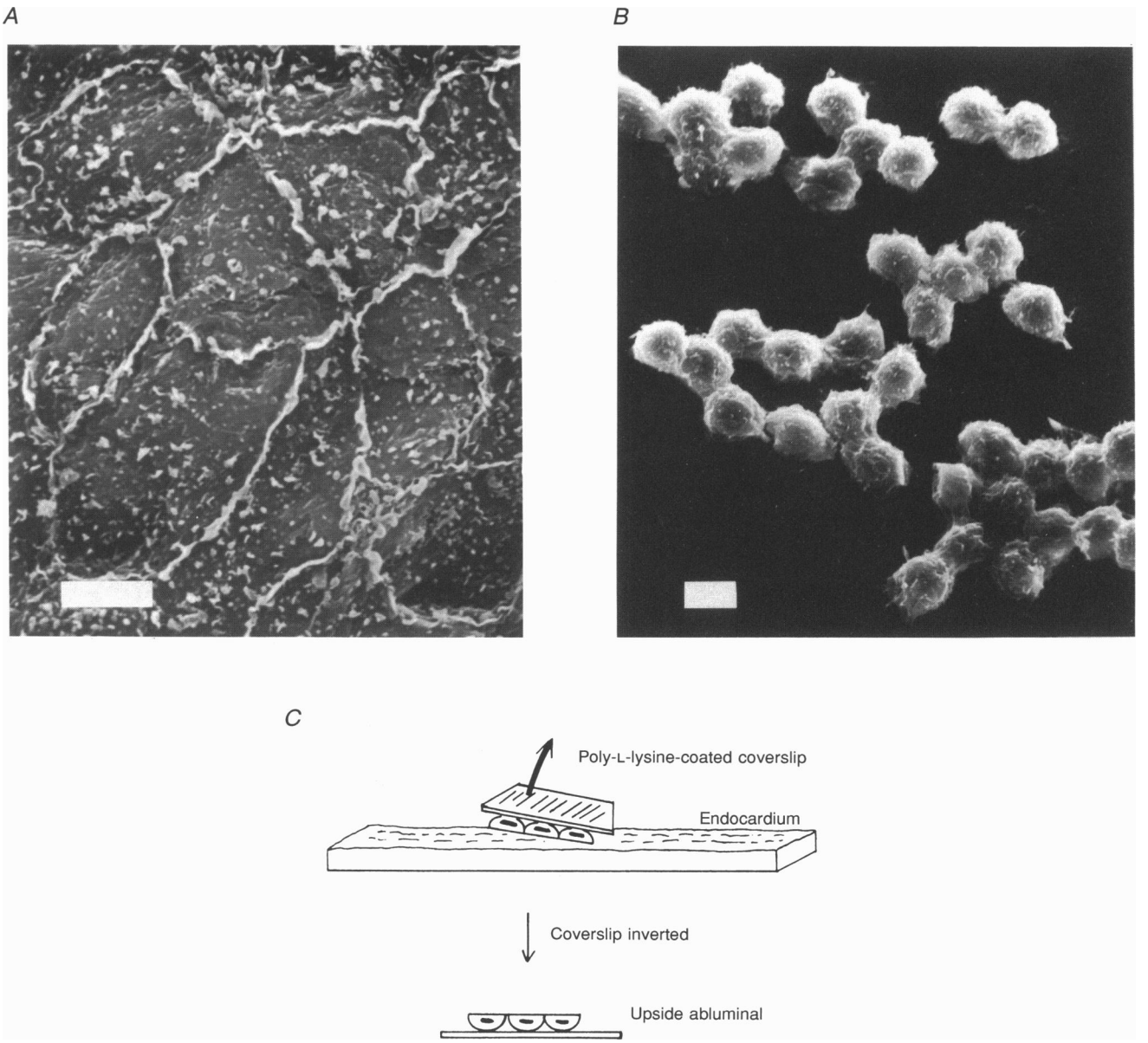

Figure 1. Scanning electron microscopy of EE cells

A, a small tissue specimen was dissected from the atrium as used for electrophysiology. The endocardium is continuously covered by polygonal endothelial cells separated by marginal folds. The cell surface is covered by scattered microvilli. *B*, endothelial cells attached to the surface of a coverslip. Cells were detached from the atrial endocardium mostly in groups of 2–15 cells. The smaller diameter of the cells is due to fixation and drying. Scale bar in *A* and *B*, 5 μm. *C*, schematic presentation of the method to isolate EE cells with their abluminal membranes up. After pretreatment of the heart with collagenase, a poly-L-lysine-coated coverslip was attached to the endocardium and removed from the tissue.

Table 1. Composition of solutions (mM)

Solution	NaCl	NaOH	NaH_2PO_4	KCl	KOH	NMDG	HCl	Aspartate	$CaCl_2$	$MgCl_2$	Hepes	Glucose	V_j (mV)
Tyrode	140	—	0·33	5·4	—	—	—	—	1·8	0·5	5	5·5	—
A	—	—	—	150	—	—	—	—	—	—	5	—	—
B	75	—	—	75	—	—	—	—	—	—	5	—	—
C	—	—	—	20	—	130	130	—	—	—	5	—	—
D	—	—	—	300	—	—	—	—	—	—	5	—	—
E	—	145	—	—	5·4	—	20	130	—	—	5	—	−7
F	—	—	—	50	100	—	—	100	—	—	5	—	−8

NMDG, *N*-methyl-D-glucamine (Tokyo Kasei, Tokyo, Japan); Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (Sigma). All solutions were adjusted to pH 7·4 with KOH or NaOH. V_j is the liquid junction potential with reference to the control Tyrode solution.

Since preserving the polarity of the membrane is essential in the present study, the EE cells in these two types of preparations were examined electron microscopically. Figure 1*A* shows that the endocardium of the small tissue specimen is continuously covered by polygonal endothelial cells. The tip of the patch electrode, if attached to the endocardial surface, may be tight-sealed onto the luminal membrane. The cells isolated with the stamp method were present usually in clusters of two to fifteen cells on the glass surface (Fig. 1*B*). We assume that the tip of the patch electrode is tight-sealed onto the abluminal membrane, if attached to one of the cells within the cluster, keeping the side-to-side contact. The intactness of the isolated cells could be verified by the findings that: (1) most of the isolated cells were not stained with Trypan Blue (DeRenzis & Schechtman, 1973); (2) the success ratios of forming a gigaseal were not significantly different between the *in situ* endothelium (60%) and the isolated cells (70%), although cells stained with Trypan Blue were rarely tight-sealed; and (3) the isolated cells had a resting potential (V_r) quite similar to that of the small tissue preparations (−42·6 ± 3·0 and −44·4 ± 0·8 mV (means ± S.E.M.), respectively; see the accompanying paper, Manabe *et al.* 1995).

Solutions

The bath was perfused with control Tyrode solution (Table 1) or 150 mM KCl solution containing 1·8 mM $CaCl_2$. In the inside-out patch recordings, the free Ca^{2+} concentration of the bath solution was buffered by 0·3 or 1·0 mM ethyleneglycol-bis-(β-amino-ethylether)*N,N,N',N'*-tetraacetic acid (EGTA; Sigma). The concentration of free Ca^{2+} was calculated by a computer program (Fabiato & Fabiato, 1979; Tsien & Rink, 1980). The ion selectivity of the channels was examined by applying internal solutions containing various concentrations of ions as listed in Table 1. The pipette solution used for single channel recording contained (mM): 150 KCl, 1 $CaCl_2$ and 5 Hepes–KOH (pH 7·4) or was the control Tyrode solution.

Recording and data analysis

Single channel recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were performed using a patch clamp amplifier (EPC-7 List Electronic, Darmstadt, Germany). The electrode resistance was 10–12 MΩ when filled with the pipette solutions. The membrane potential was corrected for the liquid junction potential at the tip of the pipette in the control Tyrode solution when it was more than 5 mV. All experiments were performed at 32–34 °C.

The single channel currents were filtered using a four-pole Bessel-type low-pass filter (E-3201A, NF, Yokohama, Japan) with a −3 dB corner frequency of 1 kHz and sampled at 2 kHz unless otherwise indicated, using a data acquisition program (Shioya, 1990). Membrane potentials are expressed in the conventional way, inside relative to outside, and outward currents are ascribed a positive sign. In the cell-attached mode membrane potentials are deviations from resting potential. The amplitude of a unitary current was measured as the distance between two horizontal lines set by eye at the closed and open levels. In several experiments, the variance–mean analysis method (Patlak, 1988) was used. There was no marked difference in the unitary amplitudes obtained from these two methods. The open probability of the channel was obtained by setting a threshold midway between the open and closed levels and calculating the sum of open times and closed times.

The average results in this text are given as the mean ± S.E.M.

RESULTS

Non-selective cation channels

Figure 2 shows a representative record of two types of non-selective cation channels which were most frequently observed on the luminal membrane in the cell-attached mode. In both cases, the channel currents were inward at the resting potential under the cell-attached condition with the KCl pipette and Tyrode bath solutions. In the case of the channel with a larger conductance (*A*), the inward open channel events were interrupted by frequent short gaps during hyperpolarization. On depolarization, the open channel events reversed their polarity and the mean lifetime was prolonged. The arithmetic mean open time was 22·4 ± 8·3, 14·7 ± 3·8, 3·9 ± 0·7, 3·0 ± 0·4 and 1·9 ± 0·3 ms measured at $V_r + 120$, $V_r + 80$, V_r, $V_r - 40$ and $V_r - 80$ mV, respectively ($n = 7$). The channel with a smaller conductance (*B*) showed no obvious outward currents at positive potentials. The current–voltage (*I–V*) relationships for both types of channel crossed the abscissa at around $V_r + 40$ mV (*C*). The measurement of V_r (−44 mV) in the whole-cell mode (Manabe *et al.* 1995) suggests a reversal potential of approximately 0 mV.

Inward rectification was evident for both types of channel, and the slope conductances measured at $V_r - 60$ mV were 38 and 10 pS, respectively.

The slope conductance for the larger channel averaged 36·3 ± 1·4 pS ($n = 22$). Non-selectivity for physiological cations such as Na^+ and K^+ was examined by excising the patch membrane from the cell body after replacing the bathing Tyrode solution with the Ca^{2+}-free 20 mM Cl^- solution (solution E). In five experiments the reversal potential was about $V_r + 40$ mV in the cell-attached recordings and about 0 mV in the inside-out mode with the asymmetrical ion distribution of external 150 mM K^+, internal 145 mM Na^+ plus 5·4 mM K^+ (Fig. 3*A*). It should be noted that the equilibrium potential for Cl^- for the excised patch is −54 mV. In nine additional inside-out experiments the reversal potential was measured in solution E ($n = 6$) or Ca^{2+}-free Tyrode solution with 1 mM EGTA ($n = 3$) without recording in the cell-attached mode. For each case the permeability ratio, P_{Na}/P_K, was determined from the Goldman–Hodgkin–Katz equation. In the total of fourteen experiments, the value of P_{Na}/P_K was 0·97 ± 0·02. The reversal potential close to 0 mV was also obtained in the inside-out patches exposed to 75 mM Na^+, 75 mM K^+ solution (solution B, $n = 3$) (Fig. 3*B*). Usually the inward rectification observed in the cell-attached condition was attenuated in the excised patch, maintaining an almost constant slope conductance for the inward currents (Fig. 3*C*). The permeability for monovalent cations other than Na^+ and K^+ or the permeability for divalent cations through the 36 pS channel was difficult to examine because of relatively rapid run-down within 1 min of excising the patch membrane and because of difficulty in forming outside-out patches in the small tissue preparation.

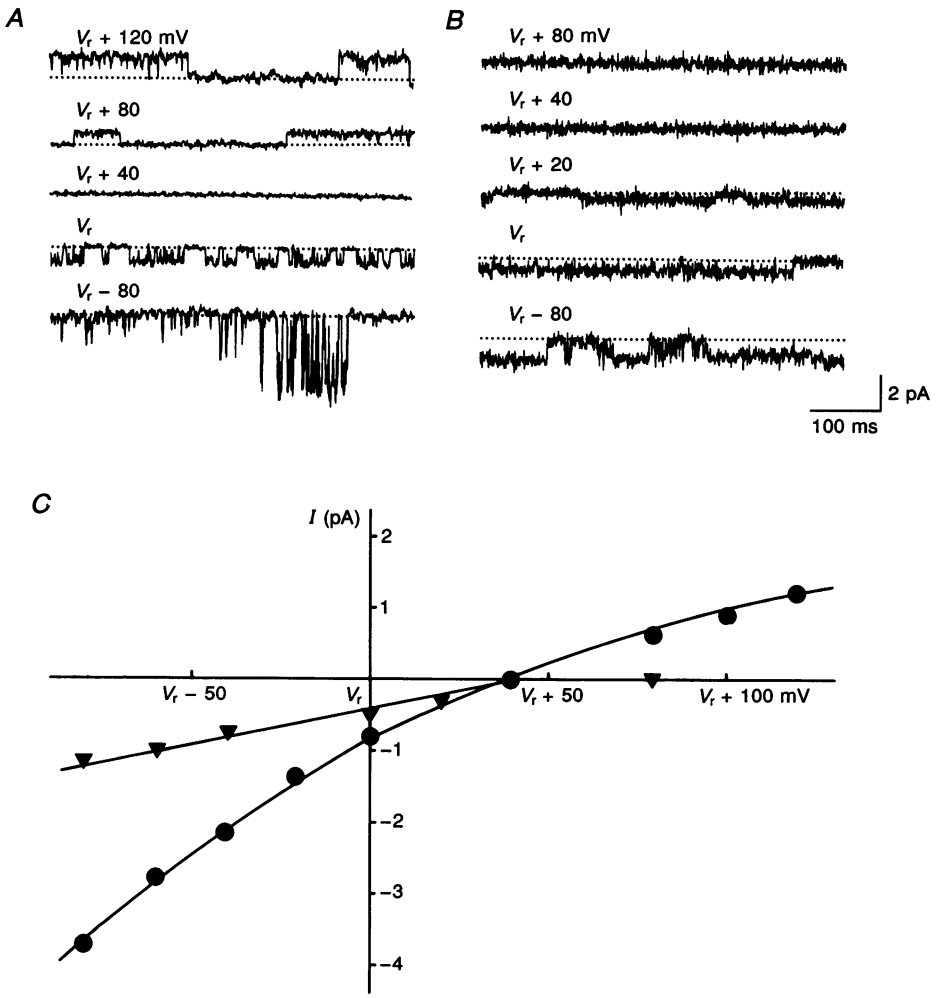

Figure 2. Two types of non-selective cation channels observed on the luminal membrane of the EE cells

A and *B*, original current recordings from the cell-attached patch at membrane potentials indicated. The dotted lines indicate the closed level. Solutions: pipette, 150 mM KCl; bath, control Tyrode solution. *C*, *I–V* relationships determined from experiments in *A* (●) and *B* (▼). The slope conductances at $V_r - 60$ mV were 38 and 10 pS.

The mean open probabilities of the 36 pS channel measured in fifteen cell-attached experiments were plotted against membrane potentials (Fig. 4). The open probability was 0·21 ± 0·04 at the V_r and increased with depolarization. This voltage-dependent activation of the channel is similar to that of the EE cells in the frog heart (Ito *et al.* 1993). The various concentrations of free Ca^{2+} (0, 10 and 1800 μM) on the intracellular side of the membrane did not affect the channel activity ($n = 7$). About a quarter of the ion channels recorded on the luminal membrane were this type of non-selective cation channel (Table 2).

The slope conductance for the smaller channel was 11·2 ± 0·5 pS ($n = 12$) measured in inside-out patches with 150 mM K^+ pipette solution and 145 mM Na^+, 5·4 mM K^+ bath solution. The cation selectivity of this channel was examined by recording the current in low-Cl^- solution (solution E; Fig. 5*A*) and in Na^+-free solution (solution C; Fig. 5*B*). In both conditions, no outward events were detected. If the reversal potential was estimated by extrapolating the slope for the inward currents, it was about 0 mV in solution E ($n = 6$). This value is different from the Cl^- equilibrium potential of −54 mV. The value of P_{Na}/P_K was 1·01 ± 0·05 ($n = 11$) when calculated from the reversal potentials obtained with 150 mM K^+ in the pipette and 145 mM Na^+, 5·4 mM K^+ in the bath. Although extrapolating the slope for the inward currents in solution C crossed the abscissa at 23·3 ± 1·7 mV ($n = 4$), this value may not reflect the true reversal potential, since the asymmetrical distribution of K^+ might cause a strong curvature of the *I–V* curve near the reversal potential.

The 11 pS channel activities described above were observed in the absence of intracellular Ca^{2+} (0·3 or 1 mM EGTA), and no Ca^{2+}-dependent activation of the channel was observed in a further three experiments. The open

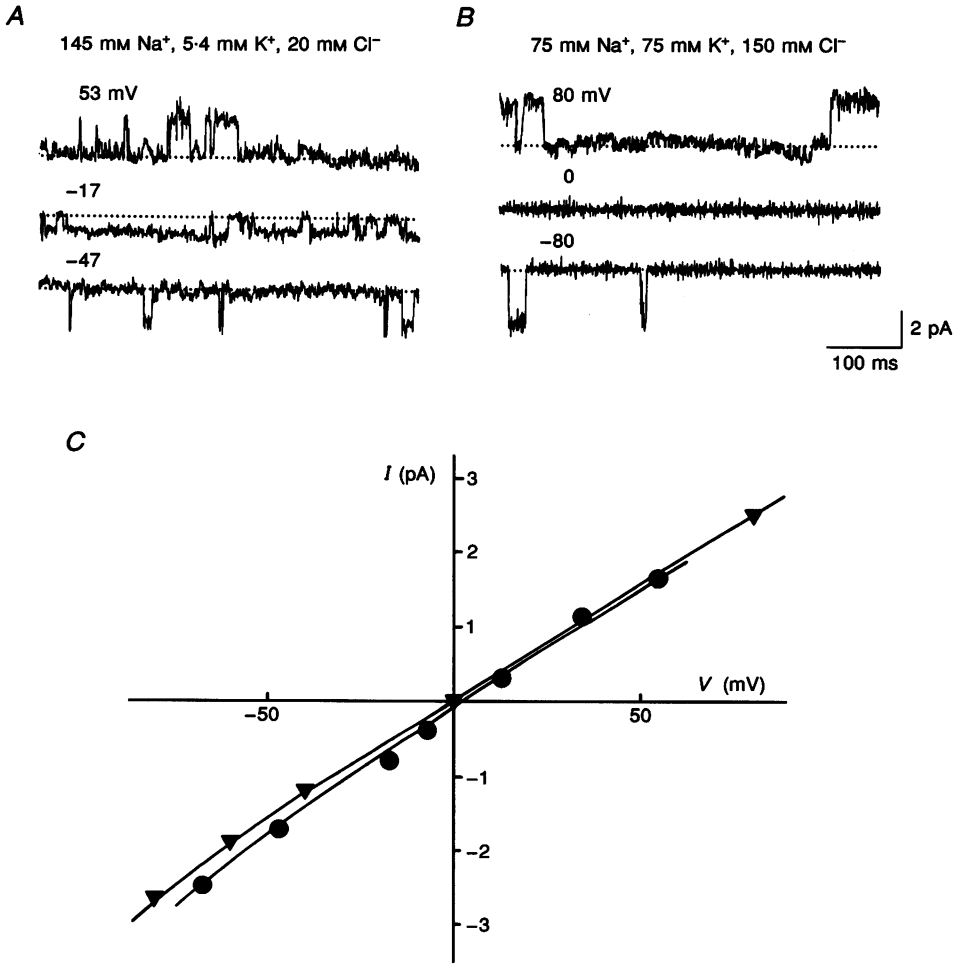

Figure 3. Ion selectivity of the 36 pS channel

A and *B*, representative recordings from the inside-out patches of luminal membrane. The ionic compositions of the bath are indicated, and Ca^{2+} was removed with 1 mM EGTA (*A*) or buffered to 10 μM (*B*). The pipette solution contained 150 mM KCl. The dotted lines indicate the closed level. *C*, *I–V* relationships determined from experiments in *A* (●) and *B* (▼).

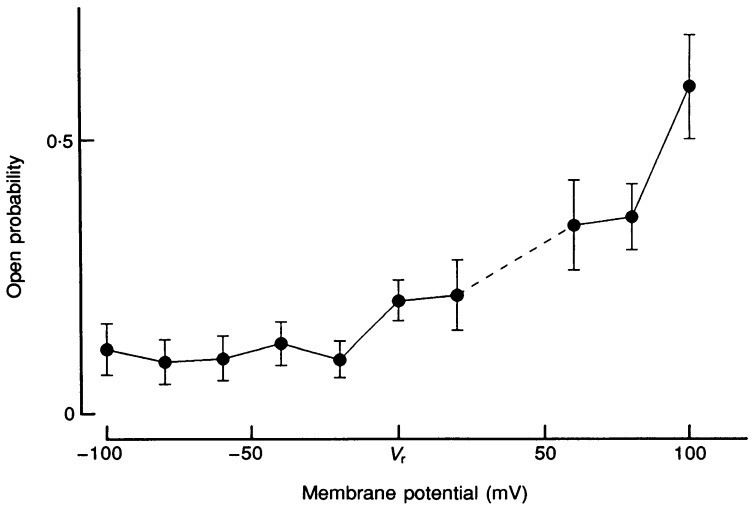

Figure 4. The open probability (means ± S.E.M., $n = 15$) of the 36 pS non-selective cation channel at different membrane potentials

Cell-attached patch recordings in the control Tyrode solution. The pipette contained 150 mM KCl. No data were available at around the reversal potential ($V_r + 40$ mV).

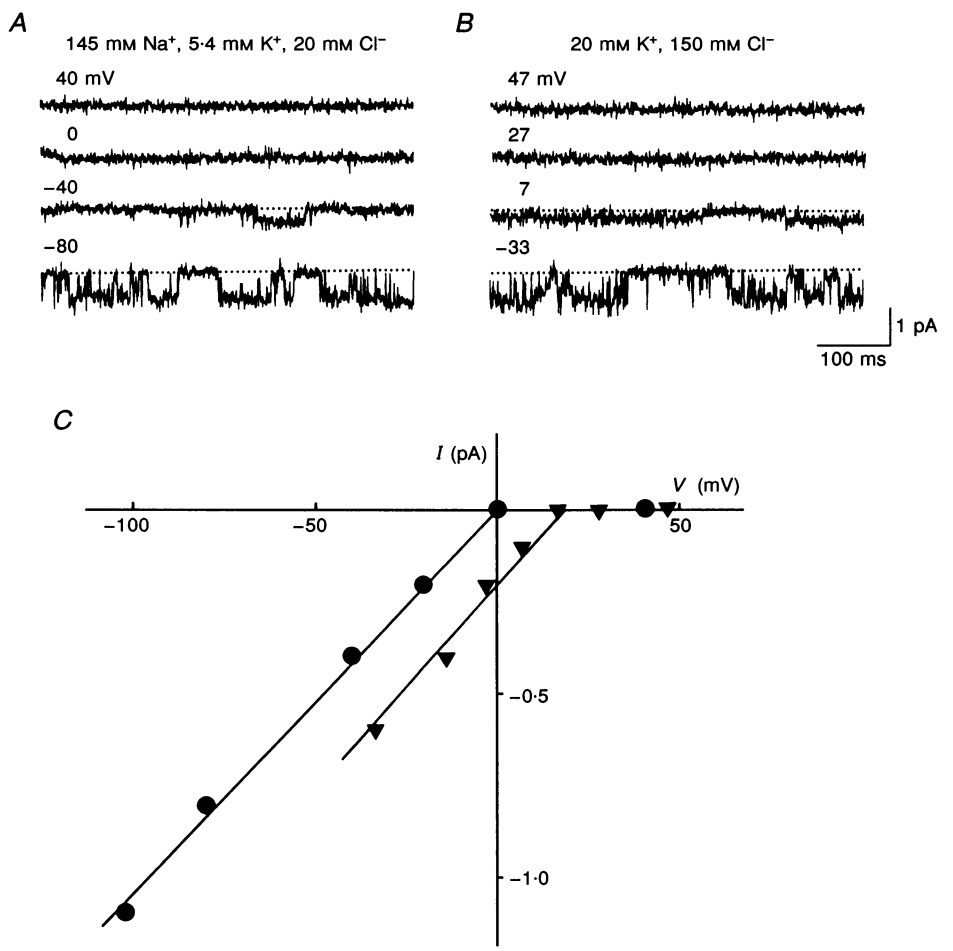

Figure 5. Ion selectivity of the 11 pS channel

A and *B*, representative recordings from inside-out patches of luminal membrane. The ionic compositions of the bath are indicated, and Ca^{2+} was chelated with 1 mM EGTA. The pipette contained 150 mM KCl. The dotted lines indicate the closed level. *C*, *I–V* relationships from records in *A* (●) and *B* (▼).

probability of this channel was not affected by clamping to negative potentials. In nine inside-out experiments with 150 mM K^+ pipette solution and 145 mM Na^+, 5·4 mM K^+ bath solution containing 0·3 or 1 mM EGTA, the open probability was 0·33 ± 0·05, 0·46 ± 0·07 and 0·48 ± 0·09 at −40, −80 and −100 mV, respectively. Both types of non-selective cation channels were rarely found on the abluminal membrane (Table 2).

Cl^- channels

The second class of ion channels observed frequently in the EE cells was the Cl^- channel. This channel was activated by depolarization positive to +40 mV in the excised patch membranes but was scarcely observed in the cell-attached mode. As shown in the inset of Fig. 6, the channel rapidly closed on hyperpolarization and did not open before the application of a subsequent depolarizing pulse. We did not observe the decrease of open probability with stronger depolarization which has been reported in the analogous channels in endothelial cells (Olesen & Bundgaard, 1992; Groschner & Kukovetz, 1992). The Cl^--selective nature was examined by measuring the *I–V* relationships at different internal Cl^- concentrations (Fig. 6). At symmetrical Cl^- concentrations of 150 mM, the *I–V* relationship of the channel was ohmic with a slope conductance of 408·6 ± 24·4 pS ($n = 32$) and reversed at 0 mV. The reversal potential shifted to −23·4 ± 1·0 mV ($n = 5$) with 50 mM Cl^- (solution F) and 14·6 ± 1·8 mV ($n = 9$) with 300 mM Cl^- (solution D) at a constant intrapipette Cl^- concentration of 150 mM. These values are close to the theoretical Cl^- equilibrium potential of −29·3 and 18·5 mV at 50 and 300 mM Cl^-, respectively. Varying the intracellular Ca^{2+} concentration between 0 and 10 μM did not affect the channel activity in three inside-out patches.

K^+ channels

The K^+ channels reported so far in vascular endothelial cells were also observed in the intact EE cells. The accompanying paper describes in detail the Ca^{2+}-activated

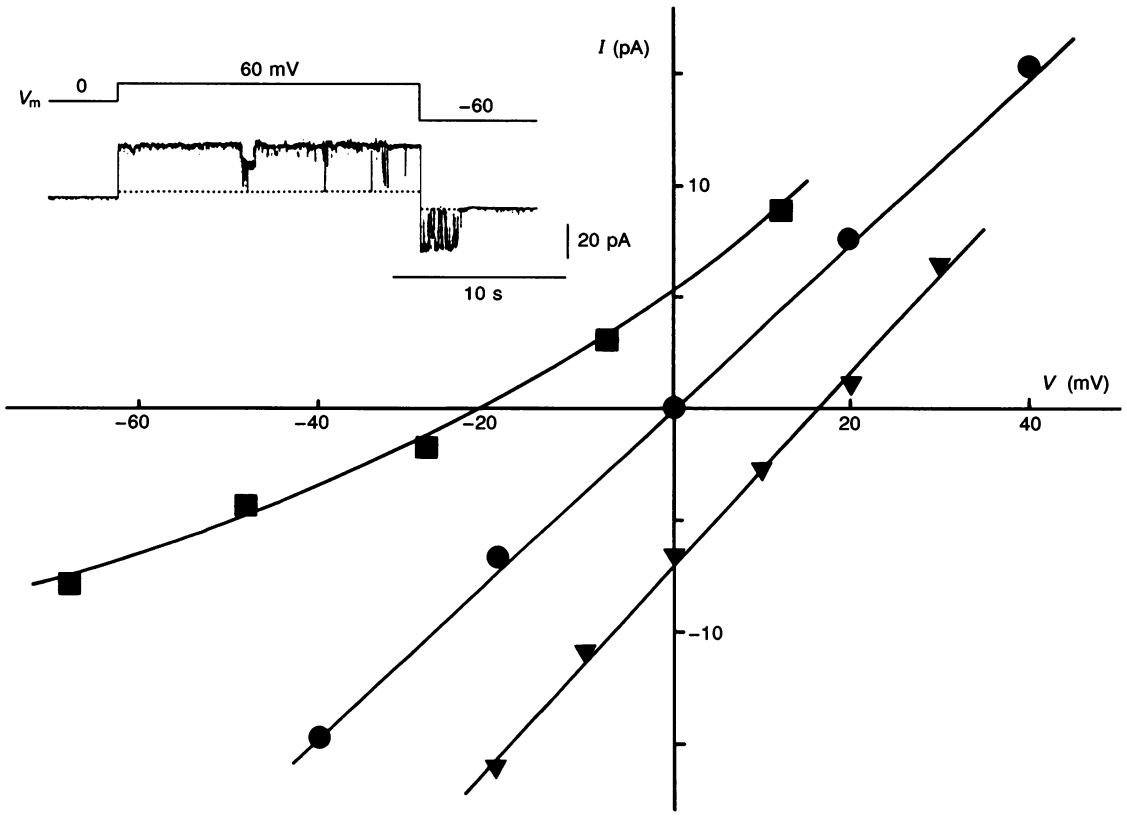

Figure 6. *I–V* relationships of the 409 pS Cl^- channel obtained from an inside-out patch excised from the abluminal membrane

The inward current was measured by repolarizing to various negative potentials after activating the channel by depolarization as demonstrated in the inset. The internal solution contained 150 mM Cl^- (●; solution A); 300 mM Cl^- (▼; solution D); 50 mM Cl^- (■; solution F). The concentration of free Ca^{2+} was adjusted to 10 μM using 1 mM EGTA in solutions D and F or to zero in solution A. The pipette contained 150 mM KCl. Inset, original trace from the chart recorder recorded in an inside-out patch from the abluminal membrane at the symmetrical Cl^- concentration of 150 mM. The dotted lines indicate the closed channel current level for each potential. V_m, membrane potential.

K^+ channel with a slope conductance of 34 pS, which is responsible for the hyperpolarization induced by various vasoactive substances (Manabe *et al.* 1995). In addition to this 34 pS K^+ channel, a Ca^{2+}-activated K^+ channel having a larger conductance was observed as shown in Fig. 7*A*, where the internal Ca^{2+} concentration was increased from 0 to 10 μM in an inside-out patch. Multiple outward channel events appeared after a perfusion delay of about 3 s, and disappeared on washing out Ca^{2+}. In Fig. 7*B*, the channel was recorded at symmetrical 150 mM KCl in the presence of an intracellular Ca^{2+} concentration of 10 μM. The channel current reversed at around 0 mV and its open probability showed little voltage dependence. The *I–V* relationship was linear and the slope conductance was 330 pS in this patch (Fig. 7*C*) and 380 pS in another patch. In these two patches, the decrease in the internal K^+ concentration from 150 to 75 mM shifted the reversal potentials to +16 and +18 mV, which are in good agreement with the K^+ equilibrium potential of +18·5 mV. In six other experiments, the *I–V* relationship showed an inward rectification at 150 mM external and 5·4 mM internal K^+ concentrations, and the averaged slope conductance was decreased to 210·7 ± 8·0 pS measured at −20 mV (not shown).

Inward rectifier K^+ channels similar to those described in cultured vascular endothelial cells (Takeda & Klepper, 1990; Colden-Stanfield *et al.* 1992) were also observed, but less frequently, in the intact EE cells. Figure 8*A* illustrates a representative recording of the channel from an inside-out patch in the Ca^{2+}- and Mg^{2+}-free internal solution (solution E). Characterization of the single channel

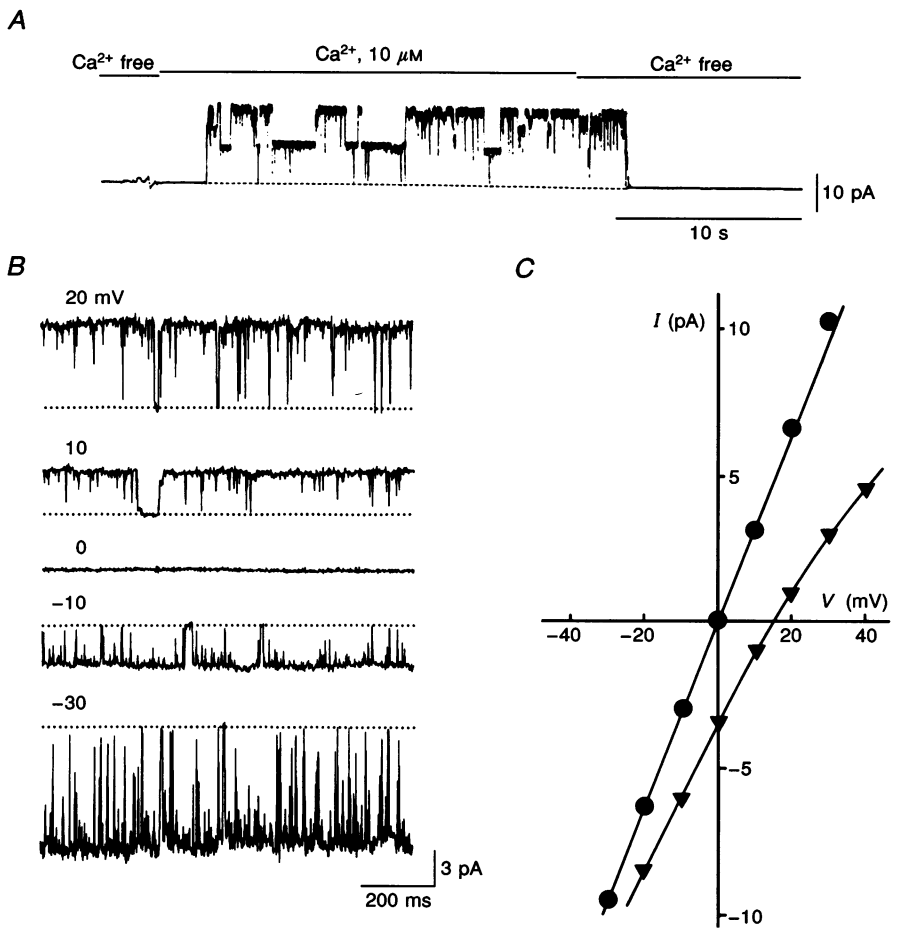

Figure 7. 210 pS Ca^{2+}-dependent K^+ channel observed on the luminal membrane

A, the internal Ca^{2+} concentration for an inside-out patch was changed as indicated above the current recording. The holding potential was +20 mV. The pipette contained control Tyrode solution and the bath was perfused with solution A. Assuming two channels within the patch, the open probability was 0·88 during the period of channel activation. *B*, current traces from another inside-out patch at various potentials in the presence of 10 μM Ca^{2+}. The pipette and the bath contained 150 mM KCl. The sampling frequency was 1 kHz. The dotted lines indicate the closed level. *C*, *I–V* relationships at internal K^+ concentrations of 150 mM (●; solution A) and 75 mM (▼; solution B). Both solutions contained 10 μM Ca^{2+}. Same patch as in *B*.

Table 2. Number of observations for different channel types

Channels	Conductance (pS)	Appearance rate (%)	
		Luminal	Abluminal
NSC	36·3 ± 1·4	11·6 (43/371)	2·6 (4/154)
NSC	11·2 ± 0·5	4·9 (18/371)	0·6 (1/154)
Cl^-	408·6 ± 24·4	8·6 (32/371)	13·6 (21/154)
K_{Ca}	33·5 ± 3·8	5·7 (21/371)	3·9 (6/154)
K_{Ca}	210·7 ± 8·0*	1·9 (7/371)	4·5 (7/154)
K_{ir}	31·7 ± 2·2	1·6 (6/371)	0 (0/154)
Unclassified	—	11·1 (41/371)	3·9 (6/154)
Total	—	45·3 (168/371)	29·2 (45/154)

Numbers in parentheses indicate actual number of experiments. Overlap of multiple channel openings of a given type channel was neglected in calculating the appearance rate. NSC, non-selective cation channel; K_{Ca}, Ca^{2+}-dependent K^+ channel; K_{ir}, inward rectifier K^+ channel. * The slope conductance was measured with a K^+ gradient of 150/5·4 mM.

events revealed relatively long open time and spontaneous sublevels. The *I–V* relationship showed a clear inward rectification, and the reversal potential may be more positive than +60 mV (●; Fig. 8*B*). When the K^+ concentration in the bath solution was increased to 75 mM (solution B), the *I–V* relationship became almost ohmic over the potential range examined, and the reversal potential was around +18 mV (▼). In the cell-attached condition (○), the *I–V* relationship showed a clear inward rectification, and no outward currents were recorded. The average slope conductance for the inward currents at V_r − 60 mV was 31·7 ± 2·2 pS in three experiments.

Distribution of ion channels

In order to estimate the distribution of ion channels in the luminal and abluminal membranes, the number of patches exhibiting the activities of each channel type was summarized for each membrane in Table 2. Both the 36 pS

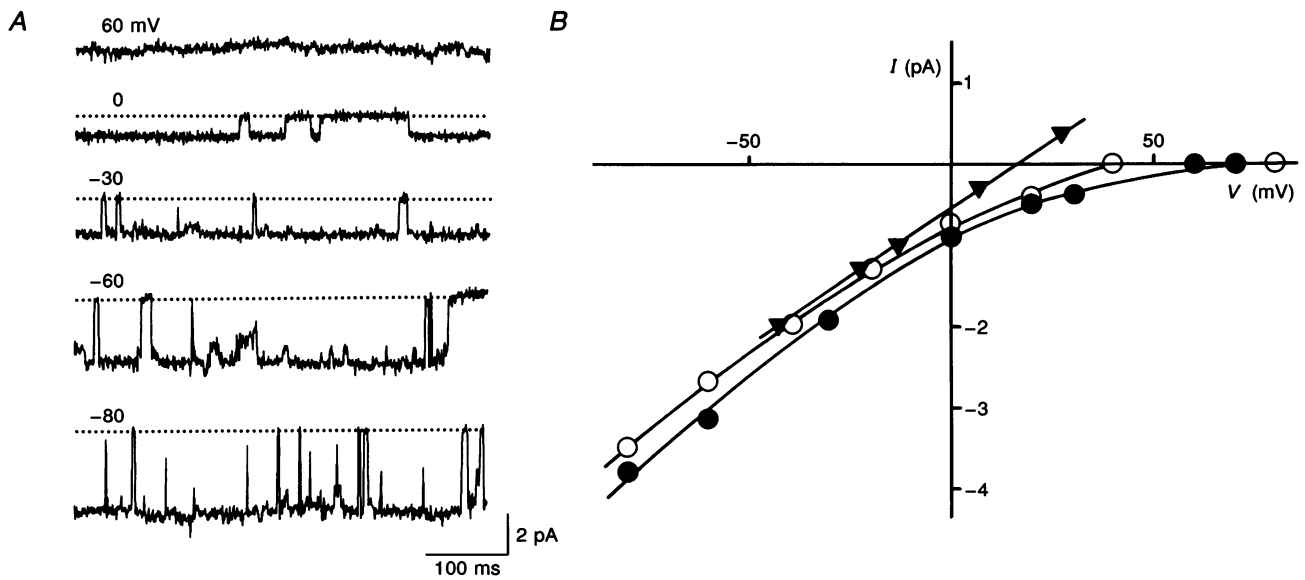

Figure 8. Inward rectifier K^+ channel observed in the luminal membrane

A, original currents recorded in an inside-out patch with 150 mM K^+ in the pipette and 145 mM Na^+, 5·4 mM K^+ in the bath (solution E). The dotted lines indicate the closed level. *B*, the *I–V* relationship indicated by ○ was obtained in the cell-attached mode. After excising the patch membrane, *I–V* relationships were obtained in 145 mM Na^+, 5·4 mM K^+ solution (●; solution E) and 75 mM Na^+, 75 mM K^+ internal solution (▼; solution B). In these bath solutions Ca^{2+} was removed with 1 mM EGTA. The pipette contained 150 mM KCl. The zero potential on the abscissa represents either the resting potential in the cell-attached mode or absolute potential in the inside-out mode.

and 11 pS non-selective cation channel types were located predominantly in the luminal membrane. In a small proportion of the experiments, clear open channel events were observed at several potentials, but their activity ceased before ion selectivity could be tested. The unclassified channels in Table 2 include these channels.

DISCUSSION

At least six classes of ion channels were identified in the intact guinea-pig EE cells: two types of non-selective cation channels (36 pS and 11 pS), a large conductance Cl^- channel (409 pS), two types of Ca^{2+}-dependent K^+ channels (34 pS and 210 pS) and an inward rectifier K^+ channel (32 pS). Corresponding types of ion channels have been described in vascular endothelial cells as follows.

Non-selective cation channels recorded in the luminal membrane of different intact endothelial cells share several common properties (Yamamoto *et al.* 1992; Ito *et al.* 1993; this study). The channels showed significant open probability at the resting membrane potential and were activated on strong depolarization. Ca^{2+}-mediated activation was observed in none of these channels. The *I–V* relationship showed a weak inward rectification with a slope conductance of 26 pS for the inward currents in the rat intrapulmonary artery and 36 pS in the guinea-pig EE cells. The non-selective cation channel of the frog EE cells only showed slightly different conductance properties: it showed slight outward rectification with a slope conductance of 36 pS at positive potentials (Ito *et al.* 1993).

The activation of the non-selective cation channel or current (Bregestovski, Bakhramov, Danilov, Moldobaeva & Takeda, 1988; Adams *et al.* 1989; Nilius, 1990; Nilius & Riemann, 1990) by vasoactive substances has been demonstrated in various tissues. The accompanying paper (Manabe *et al.* 1995) studied the agonist-induced responses of the EE cells, and showed that the membrane was hyperpolarized through the activation of the Ca^{2+}-dependent K^+ channels, and concluded that the contribution of the non-selective cation channel should be minor, if any. The present single channel recordings showed that the non-selective cation channels are insensitive to intracellular Ca^{2+}. However, these findings do not necessarily exclude the possibility that the channels are activated by various agonists independent of a rise in the intracellular Ca^{2+} concentration, as reported by others (Adams *et al.* 1989; Mendelowitz, Bacal & Kunze, 1992; Yamamoto *et al.* 1992).

The non-selective cation channel activated by mechanical stretch in vascular endothelial cells (Lansman, Hallam & Rink, 1987; Popp, Hoyer, Meyer, Galla & Gögelein, 1992; Nilius, Droogmans, Gericke & Schwarz, 1993) showed a slight inward rectification with a slope conductance of 37–40 pS. The channel activity increased with depolarization. In the present study, however, we failed to observe obvious stretch-induced activation of the channel (authors' unpublished observation). Most of the endothelial non-selective cation channels have been shown to have significant Ca^{2+} permeability (Adams *et al.* 1989; Nilius, 1990; Nilius & Riemann, 1990). In the present study, however, multiple types of ion channels were recorded and therefore it would be difficult to examine the Ca^{2+} permeability of one particular channel type by comparing the current recordings obtained using different patch pipettes.

In addition to the major group of non-selective cation channels, the guinea-pig EE cells also had smaller conductance (11 pS) channels. This channel is similar to that of aortic endothelial cells with regard to single channel conductance of 16 pS, larger open probability with hyperpolarization and Ca^{2+}-independent activation (Fichtner, Fröbe, Busse & Kohlhardt, 1987).

The vascular endothelial cells may share a common large-conductance Cl^- channel, which has ohmic conductance of about 300 pS, closes at the resting potential and is activated by depolarizing the excised patch membrane independently from intracellular Ca^{2+} (Shapiro & Decoursey, 1991; Olesen & Bundgaard, 1992; Groschner & Kukovetz, 1992). The voltage range of activation, however, seems variable between different tissues. The inactivation on stronger depolarization reported in the arterial Cl^- channels was not observed in the endocardial Cl^- channel.

The accompanying paper demonstrated that the resting membrane potential of the EE cells was approximately −45 mV (Manabe *et al.* 1995). This low resting membrane potential is partly due to the significant open probability (0·21 for the 36 pS channel and 0·33 for the 11 pS channel) of the non-selective cation channels, which were the major population of ion channels opening at the resting potential. The 34 pS Ca^{2+}-dependent K^+ channel may provide a counterbalancing outward current at the resting potential of the EE cells, since this channel shows an open probability of 0·05 at the physiological intracellular Ca^{2+} concentration of 0·1 μM (accompanying paper, Manabe *et al.* 1995). The contribution of the 34 pS K^+ channel to the resting potential is supported by our previous experiments using the ruptured patch clamp technique; the zero-current potential in the *I–V* relationship was $-29{\cdot}0 \pm 1{\cdot}2$ ($n = 66$) and $-9{\cdot}9 \pm 1{\cdot}8$ mV ($n = 41$) when intracellular Ca^{2+} was buffered with 0·05 and 10 mM EGTA in the pipette solution (accompanying paper). It seems that the 210 pS Ca^{2+}-dependent K^+ channel requires a slightly higher intracellular Ca^{2+} concentration for its activation (>0·3 μM, authors' unpublished observation) than the analogous channel reported in aortic endothelial cells (Rusko, Tanzi, van Breemen & Adams, 1992). A negligibly small contribution of the 210 pS K^+ channel to the resting conductance of the

EE cells was also suggested by the absence of large current noise in the whole-cell current on depolarization, which was observed in the aortic endothelial cells (Rusko *et al.* 1992). It should be noted that the input conductance of single EE cells is less than 300 pS (see the accompanying paper, Manabe *et al.* 1995). Although the open probability of the inward rectifier K^+ channel was quite high at potentials more negative than the K^+ equilibrium potential (Fig. 8), the contribution of the channel to the resting K^+ conductance may be negligible. This is because the channel may be almost completely closed at the resting potential of −45 mV. The Cl^- channel was observed at a relatively high rate and showed a large single channel conductance (Table 2), but its contribution to the resting membrane conductance may also be negligible. This is because opening of the channel was scarcely observed in the cell-attached mode and the channel was activated only on strong depolarization of the excised patch membrane (>+40 mV). We conclude that the resting membrane conductance of the EE cells is mostly provided both by the non-selective cation channels and by the 34 pS Ca^{2+}-dependent K^+ channel. On this background conductance, the activation of the 34 pS Ca^{2+}-dependent K^+ channel by vasoactive substances may cause a large hyperpolarization of the EE cell membrane.

In the present study, a new method of recording ion channels separately from the luminal or abluminal membrane of intact EE cells was developed. Recording ion channels separately from the luminal or abluminal membrane of cultured aortic endothelial cells failed to detect an asymmetrical distribution of the inward rectifier and Ca^{2+}-dependent K^+ channels (Colden-Stanfield *et al.* 1992). The present study also demonstrated that the 34 pS Ca^{2+}-dependent K^+ channels are present on both sides of the EE cells. The new finding of the present study is that the two types of the non-selective cation channels are predominantly distributed on the luminal membrane (Table 2).

The asymmetrical distribution of ion channels between the luminal and abluminal membranes supplemented by that of the Na^+–K^+ pump is considered to provide a basis for ion transport through epithelia (the leak–pump arrangement). It has been reported that Na^+–K^+-ATPase activity is localized mainly on the abluminal membrane in vascular endothelial cells (Betz, Firth & Goldstein, 1980; Ogawa, Fujimoto & Ogawa, 1986). Thus it seems reasonable to assume a transcellular active transport mechanism for Na^+ and K^+ across EE cells. Through the spontaneous openings of non-selective cation channels, Na^+ is transported into the cells and K^+ out of the cells. If this K^+ efflux is compensated for by the Na^+–K^+ pump on the abluminal membrane, a transcellular K^+ flux of a corresponding magnitude would be expected through the endothelial cell layer from the myocardial side to the cardiac cavity. In the steady state, the diffusion of K^+ through the inter-endothelial space may balance this transcellular K^+ flux. Transendothelial ion transport is well known in the central nervous system, and the K^+ concentration in the cerebrospinal fluid is about 60% of that in the serum (Betz & Goldstein, 1986; Newman, 1987). The present study suggests that essentially the same mechanism as that in the blood–brain barrier is present in the heart. Development of a precise method to measure the interstitial K^+ concentration would be necessary to prove the transendocardial K^+ gradient.

Adams, D. J., Barakeh, J., Laskey, R. & van Breemen, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB Journal* **3**, 2389–2400.

Betz, A. L., Firth, J. A. & Goldstein, G. W. (1980). Polarity of the blood–brain barrier: distribution of enzymes between the luminal and antiluminal membranes of brain capillary endothelial cells. *Brain Research* **192**, 17–28.

Betz, A. L. & Goldstein, G. W. (1986). Specialized properties and solute transport in brain capillaries. *Annual Review of Physiology* **48**, 241–250.

Bregestovski, P., Bakhramov, A., Danilov, S., Moldobaeva, A. & Takeda, K. (1988). Histamine-induced inward currents in cultured endothelial cells from human umbilical vein. *British Journal of Pharmacology* **95**, 429–436.

Brutsaert, D. L. (1989). The endocardium. *Annual Review of Physiology* **51**, 263–273.

Brutsaert, D. L. & Andries, L. J. (1992). The endocardial endothelium. *American Journal of Physiology* **263**, H985–1002.

Brutsaert, D. L. & Meulemans, A. L. (1988). Transendothelial ionic exchange underlies endocardial control of myocardial performance. *Biophysical Journal* **53**, 59a.

Cannel, M. B. & Sage, S. O. (1989). Bradykinin-evoked changes in cytosolic calcium and membrane currents in cultured bovine pulmonary artery endothelial cells. *Journal of Physiology* **419**, 555–568.

Colden-Stanfield, M., Cramer, E. B. & Gallin, E. K. (1992). Comparison of apical and basal surfaces of confluent endothelial cells: patch-clamp and viral studies. *American Journal of Physiology* **263**, C573–583.

DeRenzis, F. A. & Schechtman, A. (1973). Staining by neutral red and trypan blue in sequence for assaying vital and nonvital culture cells. *Stain Technology* **47**, 135–136.

Dibona, D. R. & Mills, J. W. (1979). Distribution of Na^+-pump sites in transporting epithelia. *Federation Proceedings* **38**, 134–143.

Fabiato, M. & Fabiato, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de Physiologie* **75**, 463–505.

Fichtner, H., Fröbe, U., Busse, R. & Kohlhardt, M. (1987). Single nonselective cation channels and Ca^{2+}-activated K^+ channels in aortic endothelial cells. *Journal of Membrane Biology* **98**, 125–133.

Geibel, J., Zweifach, A., White, S., Wang, W. & Giebisch, G. (1990). K^+ channels of the mammalian collecting duct. *Renal Physiology and Biochemistry* **13**, 59–69.

Gögelein, H. (1990). Ion channels in mammalian proximal renal tubules. *Renal Physiology and Biochemistry* **13**, 8–25.

GROSCHNER, K. & KUKOVETZ, W. R. (1992). Voltage-sensitive chloride channels of large conductance in the membrane of pig aortic endothelial cells. *Pflügers Archiv* **421**, 209–217.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

ITO, H., MATSUDA, H. & NOMA, A. (1993). Ion channels in the luminal membrane of endothelial cells of the bull-frog heart. *Japanese Journal of Physiology* **43**, 191–206.

LANSMAN, J. B., HALLAM, T. J. & RINK, T. J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* **325**, 811–813.

LASKEY, R. E., ADAMS, D. J., JOHNS, A., RUBANYI, G. M. & VAN BREEMEN, C. (1990). Membrane potential and Na^+–K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria. *Journal of Biological Chemistry* **265**, 2613–2619.

MANABE, K., ITO, H., MATSUDA, H. & NOMA, A. (1995). Hyperpolarization induced by vasoactive substances in intact guinea-pig endocardial endothelial cells. *Journal of Physiology* **484**, 25–40.

MENDELOWITZ, D., BACAL, K. & KUNZE, D. L. (1992). Bradykinin-activated calcium influx pathway in bovine aortic endothelial cells. *American Journal of Physiology* **262**, H942–948.

NEWMAN, E. A. (1987). Distribution of potassium conductance in mammalian Müller (glial) cells: a comparative study. *Journal of Neuroscience* **7**, 2423–2432.

NILIUS, B. (1990). Permeation properties of a non-selective cation channel in human vascular endothelial cells. *Pflügers Archiv* **416**, 609–611.

NILIUS, B., DROOGMANS, G., GERICKE, M. & SCHWARZ, G. (1993). Nonselective ion pathways in human endothelial cells. In *Nonselective Cation Channels: Pharmacology, Physiology and Biophysics*, ed. SIEMEN, D. & HESCHELER, J., pp. 269–280. Birkhauser Verlag, Basel, Switzerland.

NILIUS, B. & RIEMANN, D. (1990). Ion channels in human endothelial cells. *General Physiology and Biophysics* **9**, 89–112.

OGAWA, K. S., FUJIMOTO, K. & OGAWA, K. (1986). Ultracytochemical studies of adenosine nucleotidases in aortic endothelial and smooth muscle cells. Ca^{2+}-ATPase and Na^+, K^+-ATPase. *Acta Histochemica et Cytochemica* **19**, 601–620.

OLESEN, S. P. & BUNDGAARD, M. (1992). Chloride-selective channels of large conductance in bovine aortic endothelial cells. *Acta Physiologica Scandinavica* **144**, 191–198.

PATLAK, J. B. (1988). Sodium channel subconductance levels measured with a new variance–mean analysis. *Journal of General Physiology* **92**, 413–430.

POPP, R., HOYER, J., MEYER, J., GALLA, H. J. & GÖGELEIN, H. (1992). Stretch-activated non-selective cation channels in the antiluminal membrane of porcine cerebral capillaries. *Journal of Physiology* **454**, 435–449.

RUSKO, J., TANZI, F., VAN BREEMEN, C. & ADAMS, D. J. (1992). Calcium-activated potassium channels in native endothelial cells from rabbit aorta: conductance, Ca^{2+} sensitivity and block. *Journal of Physiology* **455**, 601–621.

SHAPIRO, M. S. & DECOURSEY, T. E (1991). Chloride currents in bovine pulmonary artery endothelial cells. In *Ion Channels of Vascular Smooth Muscle Cells and Endothelial Cells*, ed. SPERELAKIS, N. & KURIYAMA, H., pp. 327–336. Elsevier Science Publishing Company, Inc., New York.

SHIOYA, T. (1990). Development of a fast and large-capacity data sampling program in BASIC. *Journal of the Physiological Society of Japan* **52**, 345–354.

TAKEDA, K. & KLEPPER, M. (1990). Voltage-dependent and agonist-activated ionic currents in vascular endothelial cells: a review. *Blood Vessels* **27**, 169–183.

TSIEN, R. Y. & RINK, T. J. (1980). Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochimica et Biophysica Acta* **599**, 623–638.

YAMAMOTO, Y., CHEN, G., MIWA, K. & SUZUKI, H. (1992). Permeability and Mg^{2+} blockade of histamine-operated cation channel in endothelial cells of rat intrapulmonary artery. *Journal of Physiology* **450**, 395–408.

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