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Single Nonselective Cation Channels and Ca^{2+}-Activated K^+ Channels in Aortic Endothelial Cells

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Summary. In cultured bovine aortic endothelial cells, elementary K^+ currents were studied in cell-attached and inside-out patches using the standard patch-clamp technique. Two different cationic channels were found, a large channel with a mean unitary conductance of 150 ± 10 pS and a small channel with a mean unitary conductance of 12.5 ± 1.1 pS. The 150-pS channel proved to be voltage- and Ca^{2+}-activatable and seems to be a K^+ channel. Its open probability increased on membrane depolarization and, at a given membrane potential, was greatly enhanced by elevating the Ca^{2+} concentration at the cytoplasmic side of the membrane from 10^{-7} to 10^{-4} M. 150-pS channels were not influenced by the patch configuration in that patch excision neither induced rundown nor evoked channel activity in silent cell-attached patches. However, they were only seen in two out of 55 patches. The 12-pS channel was predominant, a nonselective cationic channel with almost the same permeability for K^+ and Na^+ whose open probability was minimal near −60 mV but increased on membrane hyperpolarization. An increase in internal Ca^{2+} from 10^{-7} to 10^{-4} M left the open probability unchanged. Although the K^+ selectivity of the 150-pS channels remains to be elucidated, it is concluded that they may be involved in controlling Ca^{2+}-dependent cellular functions. Under physiological conditions, 12-pS nonselective channels may provide an inward cationic pathway for Na^+.

Key Words patch clamp · ionic channels · vascular endothelium

Introduction

During the past few years, it has become apparent that the endothelium plays an important role in regulating vascular smooth muscle tone. Endothelial cells are stimulated, by a variety of endogenous vasoactive compounds, to synthesize and to release a labile endothelial factor (endothelium-derived relaxant factor, EDRF) which seems to be dominant in mediating relaxation of the subjacent vascular smooth muscle (for review *see* Furchgott, 1983; Busse, Trogisch & Bassenge, 1985). Receptor occupation by endothelium-dependent vasodilating compounds, such as acetylcholine, bradykinin and ATP, induces a cascade of biochemical events including an increased production of prostacyclin and a rise in intracellular free calcium (Peach, Singer & Loeb, 1985; Lückhoff & Busse, 1986). It remains to be clarified whether these reactions are causally related to EDRF secretion or not. Receptor occupation could also lead to changes in the cationic permeability of the surface membrane which might in turn influence the formation and release of EDRF. In fact, a close relationship between the bradykinin- or ATP-induced K^+ efflux and the release of EDRF was demonstrated in cultured aortic endothelial cells (Gordon & Martin, 1982). As Ca^{2+} may modulate this K^+ efflux, Ca^{2+}-activatable K^+ channels might exist in the surface membrane of these cells.

In order to study the potassium permeability of endothelial cells in greater detail the present patch-clamp experiments were designed to analyze single K^+ channels in cultured aortic endothelial cells. Besides a nonselective, Ca^{2+}-independent K^+ channel with a small conductance of 12 pS, a Ca^{2+}-activatable K^+ channel with a conductance of 150 pS was demonstrated.

Materials and Methods

Endothelial Cell Culture

Segments of bovine thoracic aortae were obtained from the local slaughterhouse. After incubating the tissue for 15 min in a collagenase- (CLS I Biochrom, Berlin, FRG, 125 U/ml) containing physiological saline solution, endothelial cells were scraped from the intimal surface of the aorta. The cells were cultured in Dulbecco's modified Eagle's/Ham F 12 medium and grown in standard culture dishes. The culture medium was supplemented with 20% fetal calf serum, heparin binding factor (prepared according to Maciag and Weinstein, 1979), streptomycin (50 mg/

liter) and penicillin (5000 U/liter). Within two days, the cultured endothelial cells formed a monolayer. Then, by exposure to a Ca^{2+}- and Mg^{2+}-free, phosphate-buffered solution containing trypsin (0.5 g/liter) and EDTA (0.2 g/liter), the monolayer was disaggregated, yielding single endothelial cells which were subcultured with a split ratio of 1 : 3. Their characteristic "cobblestone" morphology together with the synthesis of factor VIII antigen and of angiotensin-converting enzyme as tested randomly by applying indirect immunofluorescent staining techniques identified the endothelial nature of these cells. Endothelial cells in the second or third subculture passage were used for the patch-clamp experiments. After trypsinization, the cells were kept in a minimal serum medium supplemented by only 1% fetal calf serum in order to reduce the tendency of the cells to become confluent with each other and to form a monolayer. Moreover, the diminished serum concentration preserved the cells in their spherical shape for a relatively long period of time, 1 to 2 hr. Spherical cells were exclusively selected for the patch-clamp experiments as this shape greatly facilitates the seal formation between the patch pipette and the cell membrane.

Patch-Clamp Experiments

Elementary K^+ currents were recorded in the cell-attached configuration or, in most of the cases, in inside-out patches with an L/M-EPC 5 amplifier (List Electronic, Darmstadt, FRG) by using the standard patch-clamp technique (Hamill et al., 1981). Seal resistances of 50 GΩ or larger were easily achieved in short-time cultured spherical endothelial cells with 3 to 5 MΩ (after filling with pipette solution) borosilicate glass pipettes.

The cells were washed with modified, Ca^{2+}-poor (0.2 mM) Tyrode's solution in order to remove a very small cell fraction not yet firmly attached at the bottom and brought to the stage of an inverted Zeiss microscope. The bath temperature was controlled by a Peltier element device and was kept at 19.5 ± 1.0°C in order to improve the time resolution of the single-channel currents. Cell-attached patch-clamp experiments were either performed in Ca^{2+}-poor physiological saline solution (solution C, see below), or after incubating the endothelial cells in intracellular solution (solution B, *see below*). Intracellular solution faced the cytoplasmic side of the membrane in the experiments performed in inside-out patches.

Solutions (Composition in mM)

A. Pipette solution (extracellular solution): KCl 140; MgCl 10; $CaCl_2$ 0.1; HEPES 10; pH 7.4. B. Intracellular solution (facing the cytoplasmic side of the membrane): KCl 140; MgCl 10; $CaCl_2$ 0.0001; EGTA 0.006; HEPES 20; glucose 10; pH 7.4. C. Tyrode's solution: NaCl 120; KCl 5.4; $CaCl_2$ 0.1; $MgCl_2$ 5; glucose 20; HEPES 10; Na^+-pyruvate 5; pH 7.4.

Data Acquisition and Analysis

Samples of 250-msec duration were continuously recorded with a rate of 0.33 Hz during the whole life-time of a patch. These patch-clamp recordings were filtered at 1 kHz using an eight-pole Bessel filter, digitized by a microcomputer with a sampling rate of 5 kHz and stored on floppy discs. Thus, channel detection required a minimal open time of 0.2 msec. Elementary currents were analyzed by setting a threshold for a transition, i.e. opening or closing, at 50% of the unitary current amplitude (Colquhoun & Sigworth, 1983). Open and closed times were analyzed exclusively from nonoverlapping events and are given as mean values calculated from $\bar{t}_o = \Sigma_i n_i T_i / n$ and from $\bar{t}_c = \Sigma_i n_i T_i / n$ (Fenwick, Marty & Neher, 1982), respectively, where T_i is the duration of n_i open or closed channels and n means a count of events. A histogram analysis yielded the open and closed kinetics by fitting the distribution with the least-squares method.

Results

After forming a seal between the pipette containing K^+ as the main cation and the membrane, elementary currents were observed in 20 to 30% of the patches. They were inwardly directed when the membrane potential was set to a level more negative than the resting potential on exposing the endothelial cells to a physiological salt solution.

Under these conditions (120 mM Na^+, 5.4 mM K^+), the resting potential in whole-cell clamp measurements was found to be close to −60 mV. It approached 0 mV on exposing the endothelial cells to intracellular solution containing 140 mM K^+ (solution B) which seemed to abolish the transmembrane K^+ concentration gradient. In the latter experimental conditions, under which most of the cell-attached patches were studied, inward currents were exclusively observed, too, when the membrane potential was more negative than 0 mV. Silent patches without activity were also systematically excised and brought into the inside-out configuration thereby establishing a symmetrical K^+ concentration (140 mM) on both surfaces of the membrane. In about 30% of the silent cell-attached patches, channel activity instantaneously occurred after their dissection and thereafter persisted during the whole lifetime of the inside-out patch. The large percentage of silent patches, namely 50% or more, irrespective of the patch configuration, contrasts with the relatively large patch area which was calculated, for a 3- to 5-MΩ pipette by Sakmann and Neher (1983), to be about 5 μm^2 and could be suggestive of a low density of ionic channels in cultured endothelial cells.

Two different ionic channels were distinguished in the membrane of cultured aortic endothelial cells, a large conductance and a small conductance channel (Fig. 1). Elementary currents through the large conductance channel were found in only two out of 55 patches with activity, one of them studied in the cell-free (Fig. 1*A*) and the other in the cell-attached configuration. In the latter case, the endothelial cell was incubated in 140 mM KCl (solution B). In both patches, the currents reversed at or near 0 mV and were exclusively inwardly directed between −70 and 0 mV. Provided that depolarized endothelial

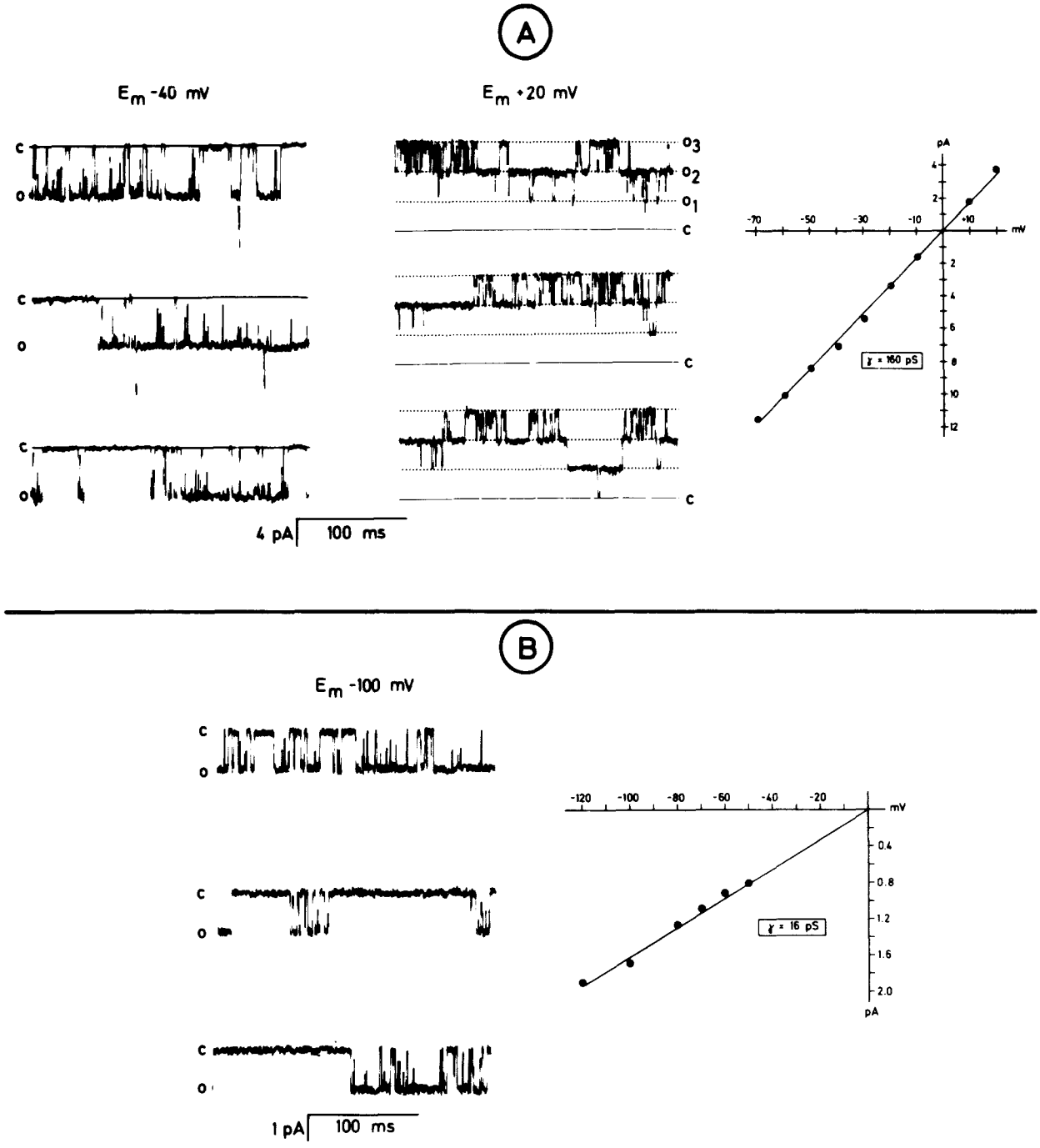

Fig. 1. Voltage-dependence of elementary K^+ currents. (*A*) Consecutive patch-clamp recordings at different membrane potentials from a 160-pS channel. The solid lines in the recording represent zero current (*c*, closed) and the dashed lines indicate the different current levels ($o_1 - o_3$). The graph on the right demonstrates the I/V relation. (*B*) Consecutive patch-clamp recordings and the I/V relation of a 16-pS channel. *c* means closed and indicates zero current and *o* means open and means the unitary current size. The inside-out patches demonstrated in (*A*) and (*B*) of this Figure were exposed to a symmetrical (140 mM) K^+ concentration. Each point of the I/V relation is the mean current amplitude of 50 events

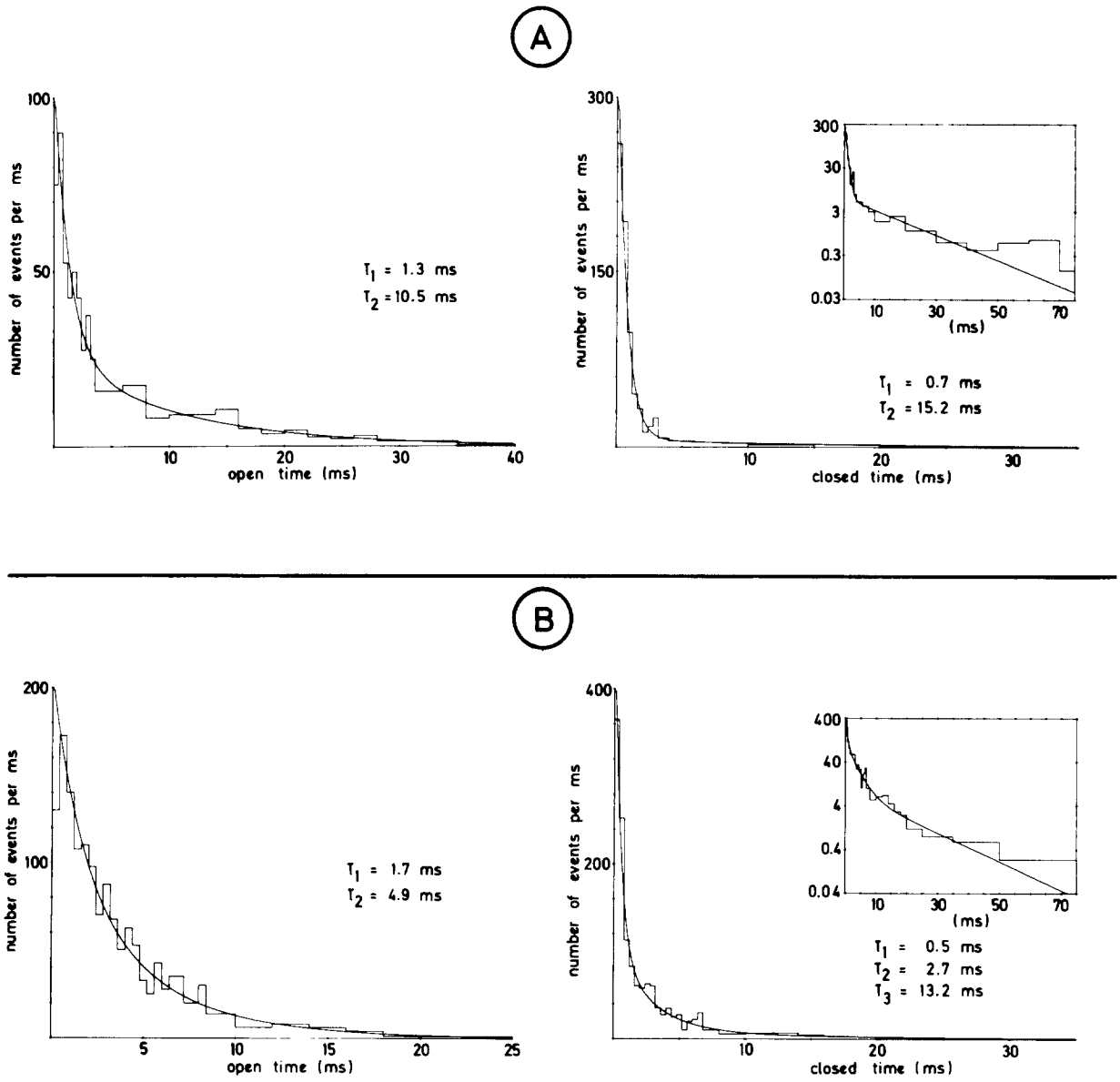

Fig. 2. Open and closed time histograms of a 160-pS channel (*A*) and of a 12-pS channel (*B*). The curve in each histogram was drawn from the best fit as obtained from the least-squares method and represents the sum of two or, for the closed-time histogram in (*B*), three exponentials with time constants as indicated. The insets present the closed-time distributions in a semilogarithmic scale, the ordinate gives the number of closing events per millisecond

cells are not loaded with Cl^- and maintain their physiological intracellular Cl^- concentration which may—in analogy to epithelial cells—be in a range between 20 and 30 mM, the reversal potential found in the cell-attached patch makes Cl^- movements through the large endothelial ionic channel unlikely. When analyzed throughout at an external K^+ concentration of 140 mM, mean values of the unitary conductance (γ) of 150 ± 10 pS (n = 2) were obtained. This is another argument against a Cl^- channel. Cl^- channels were shown in several tissues to be comparatively smaller (White & Miller, 1979; Hamill, Bormann & Sakmann, 1983; Marty, Tan & Trautmann, 1984; Blatz & Magleby, 1985; Krouse, Schneider & Gage, 1986) exhibiting usually a conductance of 70 pS or far below. It is obvious from the I/V relation (Fig. 1*A*) that the large conductance channel probably has no inward-rectifying properties. A mean value for γ of 12.5 ± 1.1 pS (n = 5) was found for the small conductance channel (Fig. 1*B*). Both channels showed grouped openings separated by short gaps in the 1- to 2-msec range indicating burst-like activity (Fig. 1). Substate events not fitting the main current size were occasionally seen but occurred too rarely to be studied systematically.

As 150-pS channels were present in only two

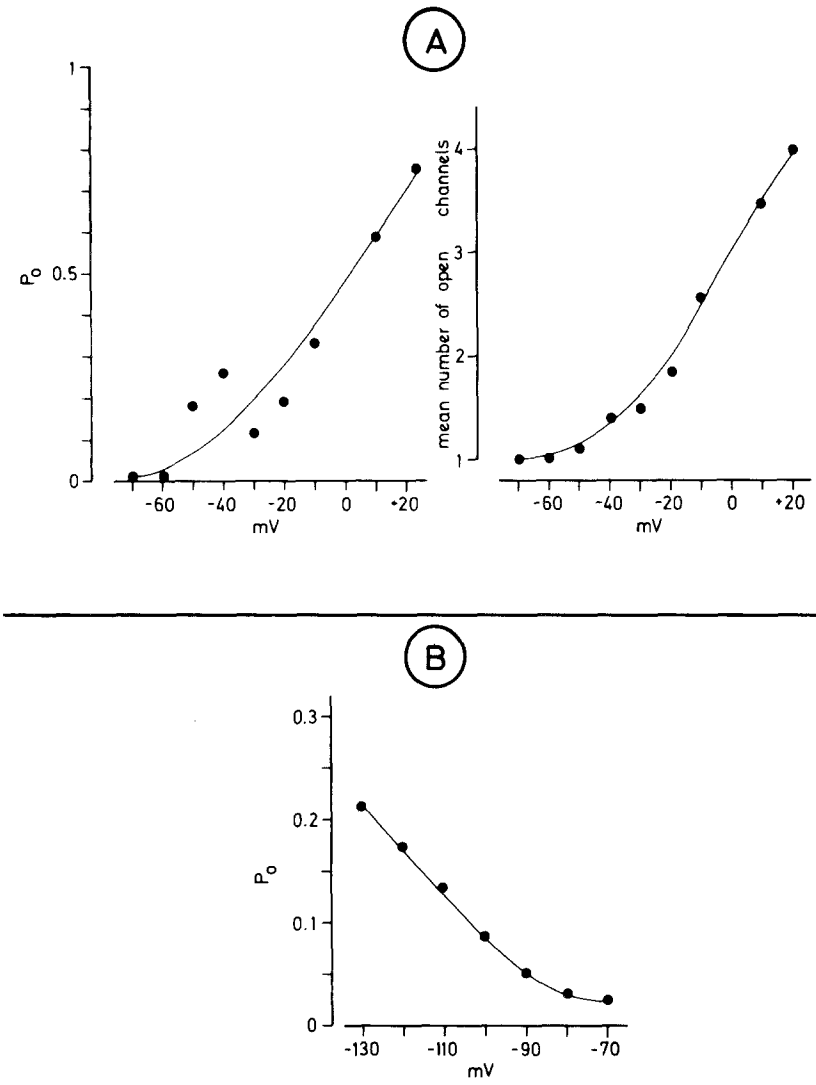

Fig. 3. Voltage-dependent activation of a 160-pS channel (*A*) and of a 12-pS channel (*B*). Open probability P_o and the number of open channels N (right graph in *A*), were calculated from $I = P_o \cdot N \cdot i$ where "I" represents, average current and "i" the unitary current in each sample of 250 msec (mean values of all the samples continuously collected with a constant rate of 0.33 Hz during a given experimental condition). The curves relating P_o and the mean number of open channels to membrane potential were drawn by eye

patches, their cationic selectivity could not yet be studied and remains to be elucidated. Small conductance channels cannot discriminate between K^+ and other cations. This was found in three inside-out patches exposed to an asymmetrical cationic concentration. The external side faced 140 mM K^+ but the cytoplasmic side was exposed to a low (5.4 mM) K^+, high (120 mM) Na^+ medium. The I/V relations determined experimentally between −130 and −70 mV intersected after extrapolation, the voltage axis between +16 and +18 mV (mean: +17 ± 1 mV), and thus yielded a reversal potential (E_{rev}) which deviates by 64 mV from the value of +81.7 mV calculated from the Nernst equation for E_K. By applying the Goldman constant field equation (Goldman, 1943), E_{rev} was calculated to be close to the experimentally observed values if P_K was taken to be 1.8-fold larger than P_{Na}. Thus, P_K dominates over other cationic permeabilities only slightly, making the small conductance channel nonselective for K^+.

Both channels show strong resemblance in their opening and closing kinetics (Fig. 2*A* and 2*B*). The open-time distributions consist of two components, an initial fast one governed by time constants between 1 and 5 msec and a much slower phase. Biexponential open-time histograms were consistently found in all patches and suggest two open states in both channels. As expected from the burst-like activity, the closed-time histogram was the sum of two or, in small conductance nonselective channels, of three exponentials. Whether three closed states may be actually attained cannot, however, be definitely decided. The ultralong third component of the histogram could also reflect gaps between individual channels instead of the closing of one channel when several functioning channels are present in a patch.

Figure 3 demonstrates that the activation of both channels is voltage-dependent. In the case of the large conductance channel (Fig. 3*A*), a shift of the membrane potential to more positive values

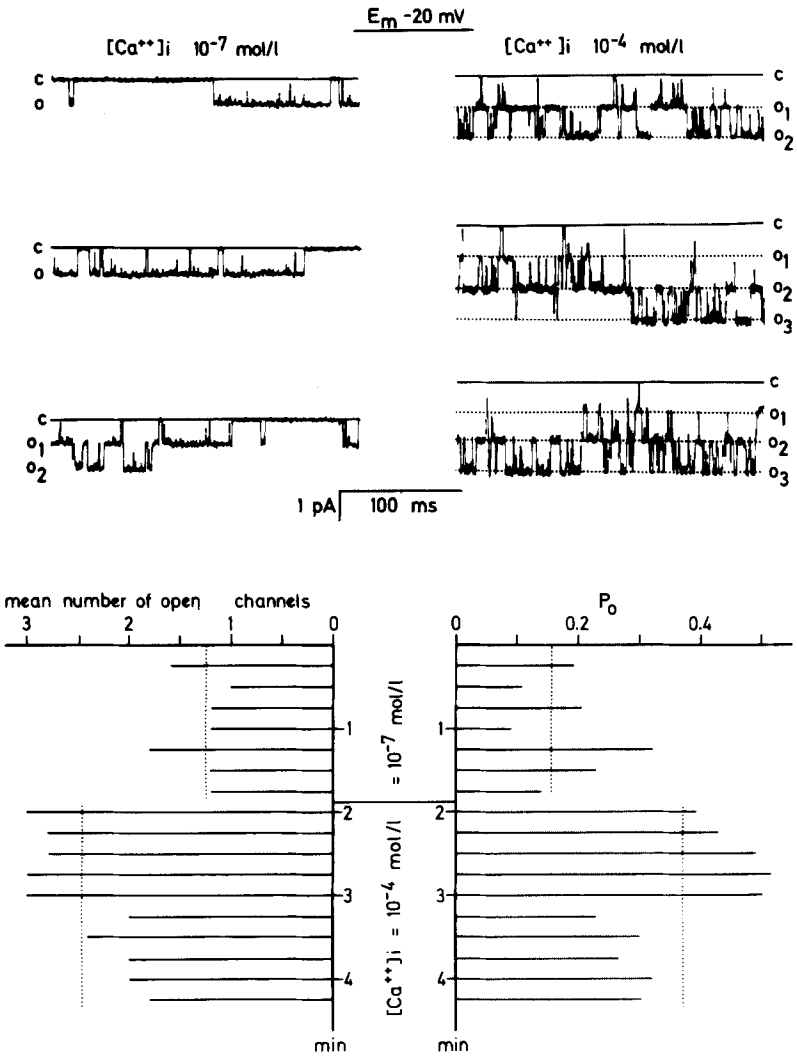

Fig. 4. Ca^{2+}-dependent activation of a 160-pS channel. Upper part: Successive current recordings before (left) and after (right) elevating the Ca^{2+} concentration at the cytoplasmic side of the inside-out patch from 10^{-7} to 10^{-4} M. Solid lines indicate zero current (c) and the dashed lines represent the several current levels (o_1 – o_3). Lower part: Mean number of open channels (left) and open probability P_o (right) before and after the change in Ca^{2+} concentration. Each bar represents a 15-sec value calculated from five samples which had been collected with a constant rate of 0.33 Hz. The dotted lines symbolize the overall mean values for 10^{-7} M Ca^{2+} and 10^{-4} M Ca^{2+}, respectively

strongly increases the probability of the channel being in the open state. This, consequently, increases the mean number of open channels in multi-channel patches. At the same internal Ca^{2+} concentration (10^{-7} M), Wong, Lecar and Adler (1982) found a similar $P_o(V)$ in 208-pS K^+ channels from anterior pituitary cells. Facilitation of the open state at more positive potentials also was manifested in a prolongation of the mean open time $\bar{t}_o$. $\bar{t}_o$ proved to be almost as voltage sensitive as P_o and increased from 2.0 msec at −70 mV to 10.1 msec at −10 mV. By contrast, the small conductance channel responded to depolarization with a decline of P_o which was accompanied by a decrease in $\bar{t}_o$. On changing the membrane potential from −100 to −60 mV for example, $\bar{t}_o$ went down from 8.2 to 3.8 msec. In most of the patches, P_o approached zero between −70 and −50 mV.

Large conductance channels were found to be Ca^{2+}-activatable. Elevating the internal Ca^{2+} concentration from 10^{-7} to 10^{-4} M abruptly increased the open probability. In an experiment performed at −20 mV and depicted in Fig. 4, P_o rose from 0.16 to 0.38. Simultaneously, there appeared an approximately two-fold increase in the mean number of open channels. A time-dependent analysis (*see* lower part in Fig. 4) revealed that, after an initial maximum, P_o and the mean number of open channels subsequently tended to decrease, approaching a somewhat smaller level. This apparently paradoxical reaction strongly resembles a Ca^{2+}-induced blockade in Ca^{2+}-activatable K^+ channels which develops in the presence of relatively high Ca^{2+} concentrations (10^{-4} M) (Eckert & Lux, 1977; Vergara & Latorre, 1983). The unitary current size changed only slightly, if at all.

The same Ca^{2+} concentration change evoked a different response in small conductance channels (Table). As analyzed at −100 mV, P_o remained unaffected. A lack of response of P_o was also observed

Table. The influence of the cytoplasmic Ca^{2+} concentration on 12-pS nonselective channels[a]

	$Ca_i^{2+} = 10^{-7}$ M			$Ca_i^{2+} = 10^{-4}$ M		
	P_o	MOT (msec)	Current size (pA)	P_o	MOT (msec)	Current size (pA)
patch 26	0.079	5.7	1.17	0.070	5.6	1.02
patch 27	0.056	5.4	1.25	0.023	14.6	1.33
patch 32	0.017	4.9	1.09	0.044	6.1	1.25
patch 45	0.099	4.4	1.09	0.057	5.6	1.02
patch 50	0.099	4.3	1.25	0.075	8.3	1.17
Mean ± SEM	0.070 ± 0.015	4.9 ± 0.27 msec	1.17 ± 0.04 pA	0.054 ± 0.009	8.0 ± 1.71 msec	1.16 ± 0.06 pA

[a] P_o means open probability, MOT is the mean open time. The unitary current size in each inside-out patch was obtained from the peak of the amplitude distribution. All patches were kept at −100 mV.

during the first seconds even in cases when the internal Ca^{2+} concentration was slowly increased so that the final level, 10^{-4} M, was attained only within 80 to 90 sec. Nevertheless, in four out of the five patches studied, $\bar{t}_o$ increased from 4.9 ± 0.3 msec at 10^{-7} M Ca^{2+} to 8.0 ± 1.7 msec at 10^{-4} M Ca^{2+}. The mean closed time varied insignificantly.

Discussion

The present experiments demonstrate for the first time the existence of K^+-permeable channels in aortic endothelial cells. Two types were found which differ in the unitary conductance, in the voltage- and Ca^{2+}-dependence of activation and, very probably, in their cationic selectivity. Both the 150-pS channel and the 12-pS, nonselective cationic channel can attain multiple open and closed states.

Large K^+ channels with a conductance of approximately 200 pS, first detected by Marty (1981) in chromaffin cells, are widespread in biological membranes and exist in excitable cells as well as in several epithelial tissues known to be unable to generate action potentials (for review *see* Petersen and Maruyama, 1984). Ca^{2+}-dependent activation is a characteristic property of large K^+ channels proven in chromaffin cells (Marty, 1981), cultured rat muscle cells (Pallotta, Magleby & Barrett, 1981), pituitary cells (Wong et al., 1982), sympathetic neurons (Adams et al., 1982) and in T-tubules of skeletal muscle (Latorre, Vergara & Hidalgo, 1982). They are selective for K^+. In *Aplysia*, the ratio P_K/P_{Na} was found to be 1:0.009 (Gorman, Woolum & Cornwall, 1982). A similar ratio was obtained in rat skeletal muscle (Blatz & Magleby, 1984). The present experiments demonstrate the endothelial membrane as another location of Ca^{2+}-activatable large conductance channels. This property together with a voltage-dependence of activation which is typical for large K^+ channels suggests that the endothelial 150-pS channel may also be selective for K^+.

Endothelial Ca^{2+}-activatable 150-pS channels may attain at least two open and two closed configurations. The transition from one state to another appears as a function of voltage. Membrane depolarization strongly favors the open state. As inferred from studies of Ca^{2+}-dependent K^+ outward currents in *Helix* (Lux & Hofmeier, 1982*a,b*), voltage and Ca^{2+} ions could work together in order to bring the channel into the conductive state.

150-pS channels were observed in only two out of the 55 patches studied. Their activity proved to be independent of the patch configuration since patch excision neither led to a run-down nor induced channel activity in silent cell-attached patches. This largely rules out a link with cellular metabolism, specifically with ATP which blocks 65 to 80 pS K^+ channels in heart muscle (Noma, 1983; Trube & Hescheler, 1984) and other cells. Another reason for their rarity might relate to cellular developmental factors associated with the culturing of aortic endothelial cells. Analogous to secreting epithelia, the transmembrane cation transport across the luminal and abluminal membrane of the endothelium might be asymmetrical. As evidenced in brain capillary endothelium (Betz, Firth & Goldstein, 1980) and consistent with epithelia, ATPase-mediated Na^+/K^+ exchange for example, is mainly located in the abluminal membrane of endothelial cells (Mircheff, 1983). Consequently, the distribution of 150-pS channels might also be asymmetrical. As the luminal and the abluminal surface differ in the structure of their glycocalices, one might also expect that cultured cells could retain this feature. This, in turn, might enable the cells to be oriented preferentially with a particular side in attaching to the bottom of culture dishes. The hypothetical assumption that the cell surface with the higher channel density usually has the greater tendency for ad-

hesion would explain the apparent rarity of the 150-pS channels described here since this area of the cell membrane would not have been accessible to the patch pipette. On the other hand, the density of 150-pS channels might be a function of cellular age. As shown in cultured human monocytes, a 240-pS K^+ channel was extremely rare in freshly isolated cells but became increasingly frequent during the subsequent culturing over several days (Gallin, 1985).

Because of such uncertainties, the physiological significance of the 150-pS channel is difficult to judge. Assuming a cytoplasmic Ca^{2+} concentration near 10^{-7} M, the $P_o(V)$ found in the present experiment suggests a minimal open probability close to the resting potential of the endothelial cell. If the 150-pS channel is a K^+ channel, its Ca^{2+}-dependent activation makes this channel a potential regulation system by virtue of its ability to hyperpolarize the endothelial cell. Although the Ca^{2+} movements across endothelial cell membranes are far away from being well understood, similar principles to those described in other cell types may be supposed. Accordingly, one response associated with hyperpolarization could be a decline in Ca^{2+} influx. Then, activation of the 150-pS channel could counteract external stimuli thereby diminishing or abolishing their promoting action of the Ca^{2+} influx. As Ca^{2+} ions may be important for the synthesis of several endothelial vasoactive autacoids (Peach et al., 1985; Lückhoff & Busse, 1986), 150-pS K^+-permeable channels could indirectly control many cellular functions.

The 12-pS channel resembles nonselective cationic channels found in cardiac (Colquhoun et al., 1981) and neuroblastoma (Yellen, 1982) cells which are likewise unable to discriminate between K^+ and Na^+. This channel may provide a physiological pathway for inward movements of Na^+ in endothelial cells thereby causing a drop in membrane potential. But it is necessary to note, in considering its functional relevance, that the open probability of the 12-pS channel was found to approach the minimal level near −60 mV, a potential close to the resting potential of endothelial cells. The 12-pS channel also has multiple open and closed states. But, the activation of this channel is favored by hyperpolarization, which appears to be a discriminating kinetic feature. Even nonselective cationic endothelial channels in cardiac and neuroblastoma cells are not reported to possess this remarkable property. Whether such a channel might be a specific constituent of aortic endothelium surface membrane awaits further clarification.

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