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Voltage-dependent transient calcium currents in freshly dissociated capillary endothelial cells

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Dissociated capillary endothelial cells display a voltage-dependent Ca current activating around the resting potential. The initial transient component of the current corresponds to a Ca channel of the T type. Some cells also display a plateau component corresponding to a distinct dihydropyridine-sensitive Ca channel. Depolarization induced by high external K^+ elicits an increase in cytoplasmic Ca concentration. Confluent cells have been found to express the same Ca permeabilities.

Bovine endothelial cell; Calcium current; Patch-clamp; Fura-2

1. INTRODUCTION

Indirect pharmacological evidence [1–4] suggests that voltage-gated Ca entry occurs in endothelial cells. However, to date, extensive studies failed to demonstrate such an entry, whether using electrophysiological methods [5–7] or measurement of internal Ca concentration [8,9], or of Ca fluxes [10–11]. In these studies, endothelial cells were of aortic or venous origin, and had been maintained in culture for successive passages. Here we show voltage-dependent Ca currents (for review, see [11]) to be present in freshly dissociated capillary endothelial cells. In confluent cultures, this Ca entry is detectable at the level of the elementary events, a level at which it had not yet been looked for.

2. MATERIALS AND METHODS

2.1. *Cell culture and their histochemical identification*

Capillary endothelial cells and chromaffin cells were separated by differential plating [13] after enzymatic dissociation of bovine adrenal glands collected at the abattoir [14]. Cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 15% heat-inactivated horse serum and plated directly on a plastic Petri dish. Ac-LDL loading [15] was obtained by exposing the cells for 4 h at 37°C to 10 μg/ml di-acetyl-LDL (Biomedical Technologies, USA) added to DMEM.

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2.2. *Electrophysiological measurements*

For current recordings in the patch-clamp whole-cell configuration (EPC 7 amplifier, List, Darmstadt), the perfusion solution contained in mM: 130 choline Cl, 10 TEA Cl, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 Hepes/TrisOH, pH 7.4, 10 glucose; and the pipette solution in mM: 120 CsCl, 20 TEA Cl, 2 $MgCl_2$, 11 EGTA/CsOH, 1 $CaCl_2$, 10 Hepes/CsOH, pH 7.2. For cell-attached single calcium channel recordings, the pipette solution contained in mM: 110 $BaCl_2$, 10 Hepes/TrisOH, pH 7.4. To zero the membrane potential, 'symmetrical' K conditions were achieved by replacing external choline Cl by K gluconate. Current traces were recorded and stored on a Sony PCM digitizing recorder. Traces were filtered before sampling for computer analysis (Plessey 6220, PDP11/23 DEC compatible).

2.3. *Optical measurements*

Petri dishes with a glass bottom coated with polyornithine (500 μg/ml) were used. Cells were loaded with fura-2 [16] by incubation with 5 μM fura-2 acetoxymethyl ester (Molecular Probes), in saline with 0.1% BSA, at 37°C for 1–2 h. Fluorescence of single cells was monitored at 37°C with a dual excitation microspectrofluorimetry apparatus designed by one of us (J.L.R.). Fluorescence was measured through a 510 nm filter and the ratio R of the values measured at 350 nm vs 380 nm excitation was calculated after subtraction of the background at

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each wavelength. One point was obtained every 6 s. Calibrations were performed with 2 μM of ionomycin to equilibrate intra- and extracellular Ca. The cytosolic calcium was estimated using $[Ca^{2+}]_i = K_d \cdot \beta \cdot (R - R_{min})/(R_{max} - R)$, with K_d = 225 nM [17], $\beta = 5.0 \pm 1.7$, $R_{min} = 0.9 \pm 0.1$, $R_{max} = 5.6 \pm 1.1$ ($n = 5$).

3. RESULTS AND DISCUSSION

An enriched population of endothelial cells was obtained from bovine adrenal medulla (see section 2). These cells were stained by Ac-LDL, were immunoreactive to an antibody directed against the von Willebrand Factor (Amersham, RPN 1135) and immunohistochemical evidence for the presence of endothelin-I was obtained (R. Miller, personal communication). Their electrophysiological characterization was carried out in the 3–4 days preceding their growth to confluence. At that time they showed a typical cobblestone-like pattern. Their visual identification at earlier stages was made possible by their characteristic granulous surface under modulation contrast optics.

When impaled, dispersed endothelial cells had a resting potential of ≈ -50 mV in 5 mM K^+ physiological solution at 20°C, and no transient voltage-dependent Na^+ current could be detected. In contrast, inward-going Ca^{2+} currents could be evoked as soon as 3 h after plating (fig.1). In 5 mM Ca^{2+}, a low-threshold inward current was elicited when depolarizing the membrane potential above -50 mV from the holding potential of -80 mV, and it reached a maximum value of 35 ± 25 pA (SD, $n = 56$) between -30 and -10 mV. Abolished in Ca-free medium, it was blocked by 33% in 50 μM Cd^{2+} (not illustrated) and reversibly reduced by a low concentration of amiloride (100 μM, fig.1A). Inactivation by 50% occurred at -59 mV (fig.1B). These are characteristics of the T-type Ca^{2+} current [12]. Although present in all cells, this current was sometimes almost completely inactivated at resting potential (fig.1B). In most

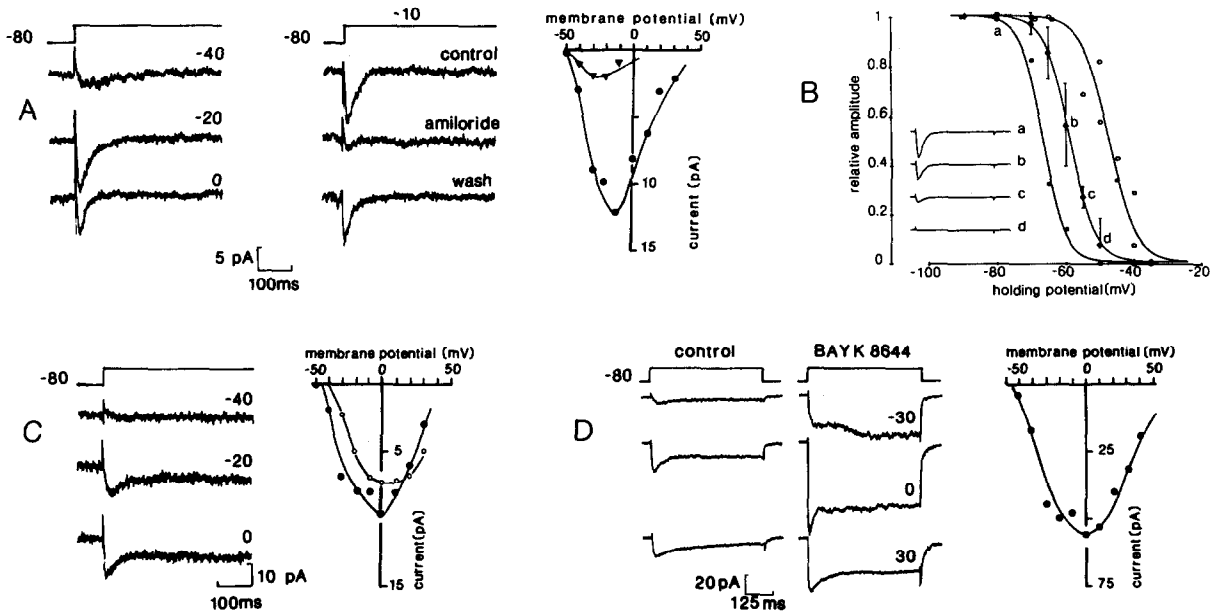

Fig.1. Voltage-dependent calcium currents recorded in whole-cell configuration. (A) Current was elicited by depolarizing steps from -80 mV to the potential indicated on each trace (left); reversible blockade by 100 μM amiloride is shown (center); at the right, current–voltage (I–V) relationships established for peak current: in control (●) and in the presence of 100 μM amiloride (▼). (B) Maximal current evoked by depolarization to -20 mV was reduced when membrane potential was held for 10 s at: a, -80 mV; b, -60 mV; c, -55 mV; d, -50 mV (inset). Inactivation curve shows the current amplitude (normalized with respect to maximal current) versus holding potential (11 cells; bars: ± SD; 2 extreme cases are also illustrated). (C) In some other cells (protocol as in A), the initial transient current was followed by a plateau current (left). Corresponding I–V curves (right) are superimposed (transient peak current (●) and plateau current (○)). (D) Cells with a plateau component (control, left) show a sensitivity to BAY K 8644 (10 μM, center). At the right, I–V curve of the plateau induced by BAY K 8644. Sampling at 2 kHz, and filter at 500 Hz.

cases, it displayed a transient time course, but in 16 out of a total of 72 whole-cell recordings, a sustained plateau was observed with a variable amplitude (11 ± 9 pA, n = 14, 5 mM Ca; note large SD). Noticeably both the transient and the sustained currents activated in the same range of potential (fig.1C) and one could wonder whether or not this plateau corresponded to some atypical T current. Application of BAY K 8644 (10 μM) on cells displaying a plateau current increased its amplitude (fig.1D), revealing a dihydropyridine sensitivity characteristic of an L-type current.

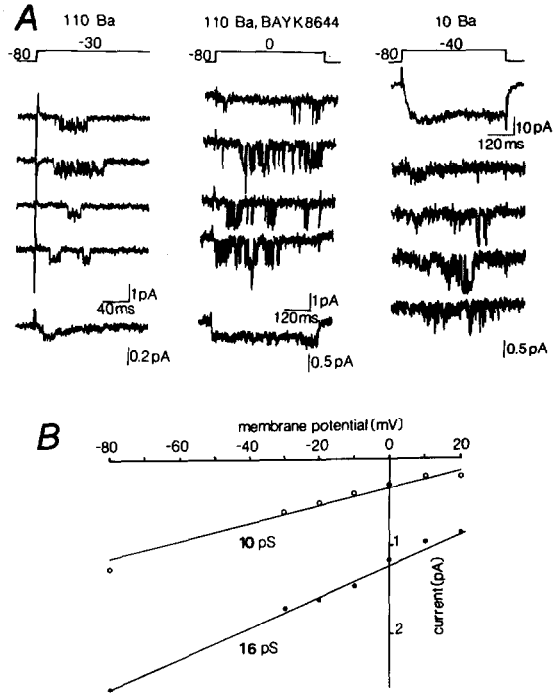

Fig.2. Two types of calcium channel activity can be recorded in the cell-attached configuration (in 110 mM $BaCl_2$). (A) Depolarizing steps from −80 to −30 mV opened small channels (left) and an averaged trace (n = 75) shows a transient activity (bottom left). These channels were blocked when holding the membrane potential at −30 mV. In addition, in the presence of 10 μM BAY K 8644 (center), a larger elementary event was observed and an averaged trace (n = 25) shows a sustained plateau (bottom center). In cells displaying a total current with a large plateau component (top right), an activity with two channel sizes could be recorded after excision of an outside-out patch (right), even though BAY K 8644 was absent. (B) Corresponding I–V curves for the two types of unitary events (cell-attached conditions, 110 mM $BaCl_2$). Straight lines are least-squares fits. Filter at 750 Hz, and sampling at 3.3 kHz.

Two types of underlying channels were thus expected. Actually, in most cells, elementary events of small amplitude were evoked above −30 mV (fig.2A, left), with a time course and inactivation characteristics of a T-type activity. Their conductance was 9.9 ± 0.5 pS (13 cells, fig.2B). In the presence of BAY K 8644, a sustained activity of large amplitude events was also displayed during the depolarizing steps (fig.2A, center). It was continued as a tail activity when membrane potential returned to the holding value. Altogether, these are characteristics of L-type channels, with a conductance of 16.2 ± 0.9 pS (7 cells, fig.2B). Channel openings of the two sizes were recorded in the absence of BAY K 8644 (fig.2A, right). We, therefore, suggest that the small events can be assigned to a T-type activity, and the large ones to an L-type activity giving rise to the plateau component with a BAY K 8644 sensitivity. Preliminary

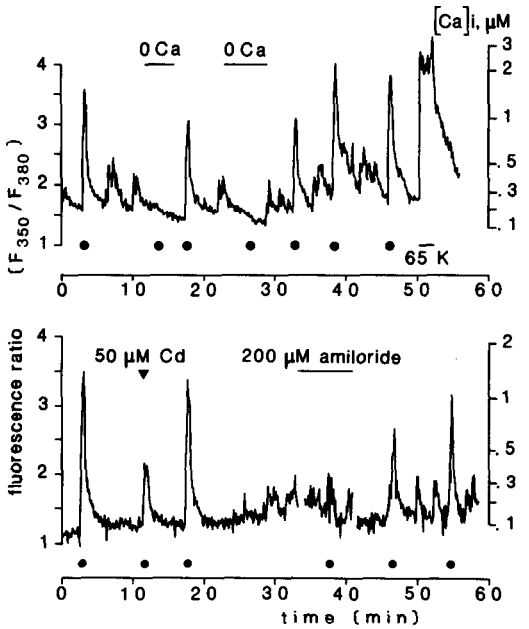

Fig.3. Depolarization-induced changes in cytosolic free Ca concentration. Extracellular K^+ was transiently increased during 30 s (dots) from 2 to 20 mM (upper panel) or from 5 to 30 mM (lower panel); except where indicated, Ca^{2+} was simultaneously increased from 5 to 10 mM. Upper panel: the resulting increase of intracellular Ca^{2+} was abolished in the absence of external Ca (10 mM Mg^{2+}); a larger and longer depolarization, in the presence of 65 mM K^+, did not induce a much larger Ca entry. Lower panel: this entry of external Ca was always reduced by 50 μM Cd; depending on the cells, it was also reversibly reduced by 200 μM amiloride.

observations indicate that confluent cells also express voltage-dependent Ca channels. Similarly, at least a T current could be recorded in endothelial cells freshly redissociated from a culture just grown to confluence.

Intracellular calcium measurement using fura-2 fluorescence showed that transient increases of external K^+ induced a concentration-dependent rise in internal Ca concentration, which was suppressed in Ca-free medium (fig.3, top). These K^+-evoked responses were reversibly reduced by 50 μM Cd; they were also partially or sometimes totally suppressed by low concentrations of amiloride (fig.3, bottom). This is consistent with a Ca entry at least partially mediated by a T-type Ca current. In this tissue, small Ca currents (mainly T-type under physiological conditions, as shown above) are thus able to elicit a rise of internal Ca up to the μM range. Stimulation of this voltage-dependent Ca entry via elevated K^+ – if it proved to be a common feature to all endothelial cells – would be an important factor to take into account not only in case of tissue injury, but also in all processes where a tetanic activity occurs in an excitable tissue.

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