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Voltage-dependent calcium entry in confluent bovine capillary endothelial cells

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Confluent bovine capillary endothelial cells display, when examined for voltage-dependent calcium entries using cell-attached channel recordings, two types of Ca^{2+} channels (4 and 23.5 pS in 110 mM Ba^{2+}) both sensitive to the dihydropyridine Ca agonist BAY K 8644. In contrast to isolated cells, confluent cells display no T-type, low threshold activity, and Ca currents were typically only elicited at very depolarized potentials. In these cells, voltage-dependent calcium entries will only be made operative by substances able to shift their activation towards the resting potential.

Confluent endothelial cell; Calcium channel; Bovine

1. INTRODUCTION

On isolated capillary endothelial cells recorded shortly after dissociation, we recently showed that membrane depolarization elicits Ca entry [1]. At least three types of voltage-dependent Ca channels were implicated. A T-type channel, elicited with a low threshold and sensitive to amiloride, and two high-threshold Ca channels (SB and L) distinguished by their conductances and sensitivity to dihydropyridines [2]. However, on the same type of preparation, even direct approaches, whether using electrophysiological methods [3–5] or measurements of internal Ca concentration or Ca fluxes [6,7], failed to detect a voltage-dependent Ca entry (for reviews see [8,9]). Since all biochemical and physiological work has been carried out on either organized tissue or confluent cells in culture, we thought it important to check whether the potentiality of such a Ca entry was maintained once this endothelial cell type had become confluent, or whether cell contact, which blocks mitotic processes, also down-regulates the voltage-dependent Ca permeability.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Bovine adrenal medulla, collected at the abattoir, were enzymatically dissociated by retrograde perfusion of collagenase through the vascular bed [10]. Two cell types were collected: chromaffin cells and capillary endothelial cells. The latter were selected by differential plating using their selective adherence [11]. DMEM culture medium (Dulbecco's modified essential medium) was supplemented with 15% heat-inactivated horse serum. Growth to confluence and one subsequent passage could be obtained after seeding directly on Corning dishes.

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2.2. *Electrophysiological measurements*

Endothelial cells observed in contrast optics were recorded at room temperature in cell-attached configuration of the patch-clamp technique (EPC 7 amplifier, List, Darmstadt, Germany). For recordings of single channels, the pipette solution contained in mM: 110 $BaCl_2$, 10 HEPES/TrisOH, pH 7.4. To zero the membrane potential, the following external solution was used (in mM): 130 potassium gluconate, 2 $MgCl_2$, 10 EGTA/KOH, 5 HEPES/KOH, pH 7.4. In these conditions, the actual values of the membrane potential are opposite to the potential applied to the pipette. Drugs were introduced in the bath using a fast perfusion system: perfusions were achieved by gravity feed, using a four-barrel pipette (200 μm tip diameter, each) system. Sampling and filtering conditions are given in the figure legends.

3. RESULTS AND DISCUSSION

Most of our records were obtained from cells at the edge of small groups of endothelial cells, at a time when they already displayed a characteristic polygonal shape and were surrounded by 4.4 cells on average. When full confluence was reached, each cell was surrounded by 6.1 other cells. When whole-cell recordings were carried out using a pipette solution with 130 mM K^+, these cells had a resting potential of -56 ± 6 mV ($n = 5$) in a 5 mM K^+ external solution. On hyperpolarization below the resting potential, a strong inward rectifier developed (not illustrated) which was likely to contribute to the definition of the resting potential.

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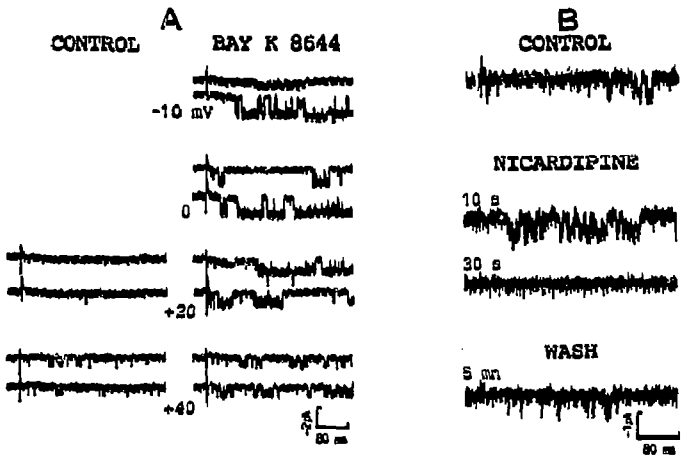

Fig. 1. Dihydropyridine sensitivity of the activity recorded on confluent endothelial cells. (A) Current traces recorded when changing the membrane potential from −80 mV to a depolarized potential (as given under each pair of traces) show an activity elicited above +40 mV in control conditions (on the left) and above −10 mV when perfusing 10^{-6} M BAY K 8644 on the same cell (on the right). Note the presence of two sized events on traces recorded at −10 mV. (B) During superfusion on the same cell with 10^{-5} M nicardipine (while repeatedly depolarizing to +40 mV), traces (from top to bottom) show a short-term facilitatory effect followed by total blockade of all activity 30 s later. Recovery was slow, and obtained after 5 min. Filter, 1 kHz; sampling, 5 kHz.

3.1. *Two types of Ca activities are evidenced in confluent cells*

In no case was a low threshold activity ever observed. Only very small events below −0.5 pA were elicited in the already depolarized domain of potential up from −10 mV. When further depolarizing the patch, above +40 mV, some larger inward elementary events (around −1 pA) could be recorded.

As illustrated by the traces of Fig. 1A, both these activities were sensitive to dihydropyridines. A superfusion of the Ca agonist (±) BAY K 8644 (above 10^{-8} M): (i) alters the *small amplitude events* by promoting more numerous long duration events (see Fig. 1A, top trace at the right); and (ii) shifts the activation curve of the *large size activity* towards more hyperpolarized potentials making it detectable at −10 mV, i.e. at a 50 mV more hyperpolarized potential than in control conditions. Conversely, the BAY K 8644-induced activity (Fig. 1B) was abolished reversibly by nicardipine, a DHP antagonist.

3.2. *Conductance and gating properties of the Ca channel activities*

With BAY K 8644 in the pipette, the *activity of small amplitude* (Fig. 2A) was easily characterized. The duration of the events extended, in fact, from a few ms (which corresponded to their minimal duration for discrimination from the noise level) to, at times a few hundred ms, the longest duration of the depolarizing step. Further, in a few patches (n = 5) where these were recorded in isolation, we estimated the conductance of the corresponding channel to be 2–4 pS (Fig. 2C). This channel shows no difference with the SB channel we have identified in isolated endothelial cells [2].

The *large size events* showed all the characteristics of an L-type channel. Firstly, their mean conductance between −40 and +40 mV was estimated to be 23.5 pS

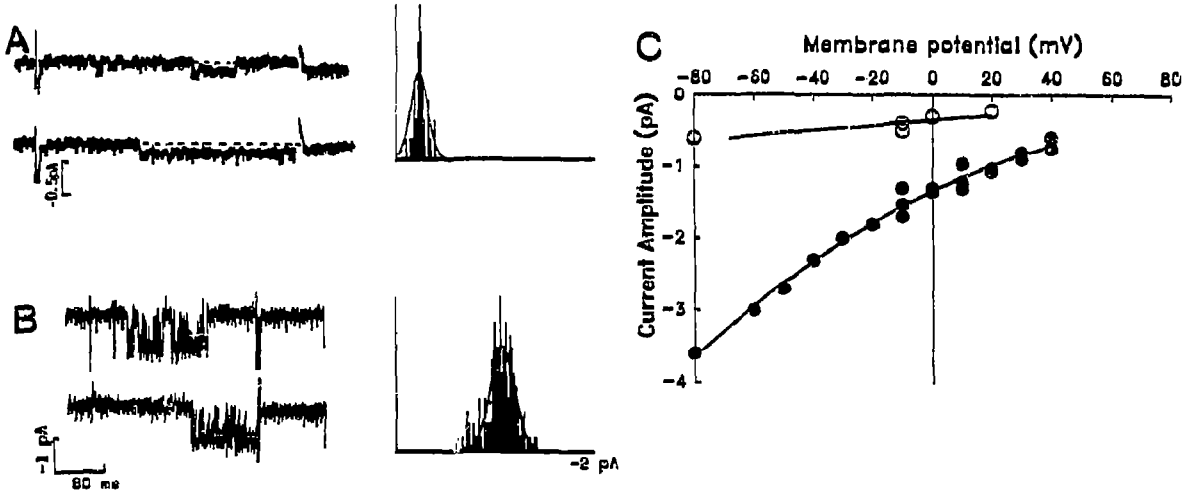

Fig. 2. Ca channel conductance in the presence of 10^{-6} M BAY K 8644. (A) During step-wise depolarization of a patch to +20 mV (from holding potential, −80 mV), current traces (left) displayed events mainly of −0.23 pA (as estimated from the amplitude histogram (right) where maximal number of events in ordinate is n = 5; filter, 750 Hz; sampling, 2 kHz). (B) In most patches, a similar depolarization evoked a larger activity of −1.1 pA (histogram to the right; maximal number of events, n = 12; filter, 1 kHz; sampling, 5 kHz). (C) Current-voltage relationships for the two types of activity, respectively (○, 3 cells and ●, 4 cells). Above −10 mV, current amplitude was measured during the depolarizing step; otherwise, it was measured after a depolarization to +20 mV on return to the holding potential (between −30 mV and −80 mV). Lines show eye-fitted curves. Linear regression over all points yielded elementary conductances of 3.7 and 23.5 pS for (○) and (●), respectively and in the latter case, when using data between −20 and +40 mV, estimated conductance was only 17 pS.

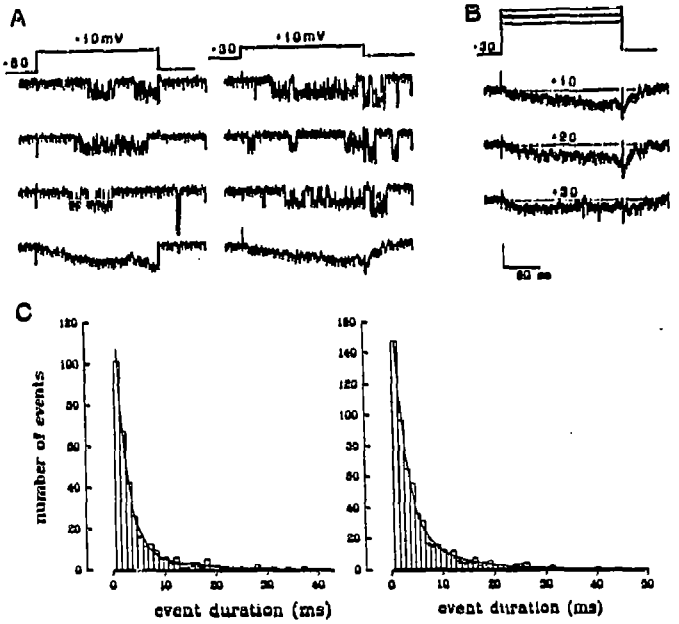

Fig. 3. Activation and gating characteristics of the L-type activity. A single patch. (A) An L-type activity was evoked in isolation at +10 mV from either −80 mV (left) or −30 mV (right). From top to bottom, are given the depolarization protocol, three current traces and the average of 55 and 63 current traces respectively. Note the delay before activation and also, after repolarization to −30 mV, a tail activity which is the sum of successive reopenings. (B) Activation kinetics at +10, +20 and +30 mV, successively, were estimated on averaged traces (from about 60 traces). Using a mono-exponential fit to describe the currents rising phase, time constants for activation were estimated to be 150, 80 and 25 ms, respectively. Vertical scales: 2 pA and 0.5 pA for the individual and averaged traces, respectively. (C) Histograms of the open time for the events elicited at +10 mV from −80 mV and −30 mV (left and right, respectively). Double exponential fits yielded time constants of 2.0 ms and 10.0 ms, and 1.9 and 6.8 ms, respectively (and relative amplitude of the slow component of 0.06 and 0.22). Filter, 1 kHz; sampling, 5 kHz.

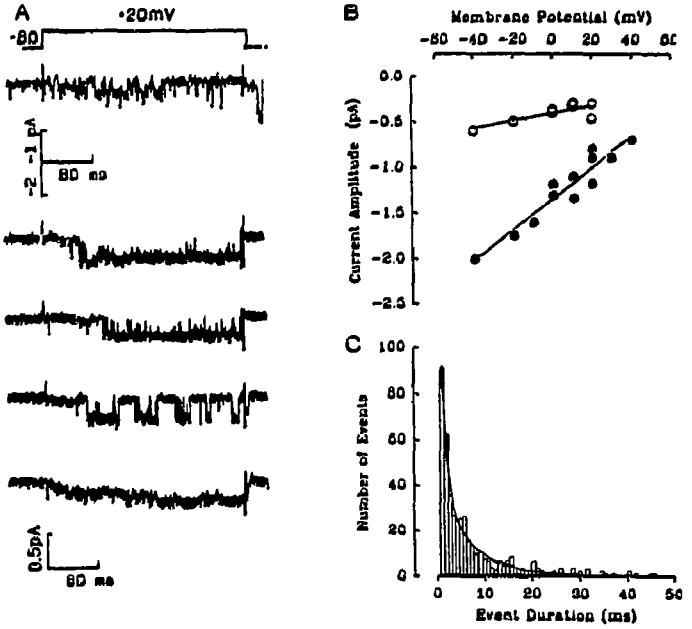

Fig. 4. A BAY K 8644-like activity may be encountered. (A) These traces show that, at +20 mV, a membrane potential usually devoid of activity in control conditions, small sized events (top trace) and an L-type activity (three bottom current traces, and average trace) may be elicited. Note the delay for first opening and the long duration openings. Filter and sampling: 500 Hz and 2.5 kHz, 1 kHz and 5 kHz, respectively. (B) In 5 such cells, estimated conductance was 2.5 pS (○) and 17 pS (●), respectively. (C) For the L-type activity, the histogram of event duration at +20 mV was best described using two exponentials with time constants of 0.95 ms and 6.0 ms, respectively, and in a ratio of 1: 0.25.

(Fig. 2C, filled circles). Secondly, the most conspicuous effect of BAY K 8644 was to favour openings of long duration. This effect was voltage-dependent, being further pronounced when the membrane potential was maintained at a more depolarized holding potential (Fig. 3C). This increase in activity on addition of the DHP Ca agonist develops characteristically as a slow process unless very depolarized membrane potentials are used. For instance in the case illustrated in Fig. 3A, the rise time was 150 ms with step depolarizations to +10 mV from a potential of either −80 mV or −30 mV; one can further see it to be accelerated by 5 times when the step depolarization was to +30 mV instead of +10 mV. Finally, as noticeable in Fig. 3A, a depolarized holding potential in the presence of BAY K 8644 promotes activity after the repolarisation (see bottom right trace in Fig. 3A, where the response is prolonged by a tail current on repolarization to −30 mV). Close examination of the individual traces shows that this current was built first by the current flow through those channels that were already open during the depolarization, but which had a very fast closing rate at more hyperpolarized potentials, plus additional reopenings within the 250 ms after repolarization at a membrane potential of −30 mV. The latter observation suggests that, on depolarization, the channel undergoes an inactivation subsequent to the opening, which gives way to successive closings and openings, for a repolarization above −60 mV, before going to the silent resting state. When repolarizing to −80 mV, the sequential transition from the inactivated state to the resting state through the open state is favored.

In a few cases, a two-size activity was observed in the range of potentials from 0 to +40 mV, where usually no activity was recorded in the absence of BAY K 8644. As typified by the case illustrated in Fig. 4, it showed all the characteristics (size and open times) of the BAY K 8644-induced activity just described above.

In conclusion, distinct pictures emerge for endothelial cells as to their permeability to calcium, depending on whether they are isolated (with T-, L-type and SB channels [2]) or have grown to confluence (with only the DHP-sensitive L and SB channels). As a result of contact inhibition; (i) the T-type channels are no longer recorded; (ii) when considering the DHP sensitive activ-

ity, we show it to be similar in both the isolated and confluent cells though in the latter case the threshold to elicit any activity is limited, in most cases, to depolarized membrane potentials above +40 mV. This makes it easy to understand why the voltage-dependent Ca entries would have escaped investigators until now. The question immediately arises of the possible physiological importance of the voltage-dependent permeabilities we have just described in confluent cells. It can be readily answered that in basal conditions they are likely to be of poor physiological significance since they develop at membrane potentials at least 80 mV above the resting potential. Along this line, we could not detect by microfluorometry any reliable increase of the cytoplasmic Ca concentration in confluent cells exposed to an elevated K concentration. Nevertheless we show here that a route for a voltage-dependent Ca entry does exist. It will be made operative by a substance able to shift the voltage-dependency of the channel gate towards the resting potential (see a similar proposal in [12]). Since some confluent endothelial cells show activation of L- and SB-type channels at −20 mV, we suggest that an endogenous, possibly intracellular component can provide a modulation of Ca entry via a shift in the activation characteristic of the voltage-dependent Ca channels. These Ca^{2+} entries will then add to the ones operating through the pathway of aspecific ionic channels [13,14].

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REFERENCES

[1] Bossu, J.L., Feltz, A., Rodeau, J.L. and Tanzi, F. (1989) FEBS Lett. 255, 377–380.
[2] Bossu, J.L., Elhamdani, A., Feltz, A., Tanzi, F., Aunis, D. and Thierse, D. (1992) Pflügers Arch. (in press).
[3] Colden-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T. and Kunze, D.L. (1987) Circulation Res. 61, 632–640.
[4] Johns, A., Lategan, T.W., Lodge, N.J., Ryan, U.S., Van Breemen, C. and Adams, D.J. (1987) Tissue Cell 19, 1–13.
[5] Takeda, K., Schini, V. and Stoeckel, H. (1987) Pflügers Arch. 410, 385–393.
[6] Bussolino, F., Aglietta, M., Sanavio, F., Stacchini, A., Lauri, D. and Camussi, G. (1985) J. Immunol. 135, 2748–2753.
[7] Whorton, A.R., Young, S.L., Data, J.L., Barchowsky, A. and Kent, R.S. (1982) Biochim. Biophys. Acta 712, 79–87.
[8] Bregestovski, P.D. and Ryan, U.S. (1989) J. Mol. Cell. Cardiol. 21, 103–108.
[9] Nilius, B. and Riemann, D. (1990) Gen. Physiol. Biophys. 9, 89–12.
[10] Bader, M.F., Ciesielska-Treska, J., Thierse, D., Hesketh, J.E. and Aunis, D. (1981) J. Neurochem. 37, 917–933.
[11] Banerjee, D.K., Ornberg, R.L., Youdim, M.B.H., Heldman, E. and Pollard, H.B. (1985) Proc. Natl. Acad. Sci. USA 82, 4702–4706.
[12] Nelson, M.T., Standen, N.B., Brayden, J.E. and Worley III, J.F. (1988) Nature 336, 382–385.
[13] Lansman, J.B., Hallam, T.J. and Rink, T.J. (1987) Nature 325, 811–815.
[14] Lückhoff, A. and Busse, R. (1990) Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 94–99.