Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4715–4719, May 1995
Physiology

Oxygen-sensitive calcium channels in vascular smooth muscle and their possible role in hypoxic arterial relaxation

(O_2 sensing/hypoxia/cytosolic Ca^{2+} oscillations)

A. FRANCO-OBREGÓN, J. UREÑA, AND J. LÓPEZ-BARNEO*

Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla, Facultad de Medicina, Avenida Sánchez Pizjuán, 4, E-41009, Seville, Spain

Communicated by Clay M. Armstrong, University of Pennsylvania Medical Center, Philadelphia, PA, February 15, 1995 (received for review December 10, 1994)

ABSTRACT We have investigated the modifications of cytosolic [Ca^{2+}] and the activity of Ca^{2+} channels in freshly dispersed arterial myocytes to test whether lowering O_2 tension (PO_2) directly influences Ca^{2+} homeostasis in these cells. Unclamped cells loaded with fura-2 AM exhibit oscillations of cytosolic Ca^{2+} whose frequency depends on extracellular Ca^{2+} influx. Switching from a PO_2 of 150 to 20 mmHg leads to a reversible attenuation of the Ca^{2+} oscillations. In voltage-clamped cells, hypoxia reversibly reduces the influx of Ca^{2+} through voltage-dependent channels, which can account for the inhibition of the Ca^{2+} oscillations. Low PO_2 selectively inhibits L-type Ca^{2+} channel activity, whereas the current mediated by T-type channels is unaltered by hypoxia. The effect of low PO_2 on the L-type channels is markedly voltage dependent, being more apparent with moderate depolarizations. These findings demonstrate the existence of O_2-sensitive, voltage-dependent, Ca^{2+} channels in vascular smooth muscle that may critically contribute to the local regulation of circulation.

Oxygen tension (PO_2) has been recognized for decades as an important factor in the local regulation of vascular tone *in vivo* (1), and it is known that hypoxia causes relaxation of systemic arteries *in vitro* (2–4). Nevertheless, the mechanisms underlying this physiological response to low PO_2 remain largely unknown. Based on studies done in organ bath preparations, it is believed that oxygen influences vascular resistance by directly interfering with the rise of cytosolic Ca^{2+} required for contraction of smooth muscle cells (4–7); nevertheless, very little is understood about oxygen-regulated processes in arterial myocytes. It has been suggested that dilatation of the coronary artery (8, 9), and perhaps other arteries, in response to extreme hypoxia might be mediated by myocyte hyperpolarization in response to the opening of ATP-regulated K^+ channels. However, it seems unlikely that this is the only underlying mechanism because the sensitivity to hypoxia in most arteries occurs over a physiologic range of PO_2 without a compromise of energy metabolism (3). Since in the past few years O_2-regulated ion channels have been demonstrated to participate in a number of cellular functions (10), we hypothesized that vascular tone could be also regulated by direct modulation of voltage-gated Ca^{2+} channels by PO_2. Here, we show the reversible inhibition of the macroscopic Ca^{2+} current of arterial myocytes in response to hypoxia, which can explain the suppression of the cytosolic [Ca^{2+}] oscillations in the same cells by low PO_2. These data demonstrate the existence of O_2-regulated Ca^{2+} channels in vascular smooth muscle and suggest that they might participate in hypoxic arterial relaxation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

METHODS

Experiments were performed on enzymatically dispersed smooth muscle cells from the celiac and femoral arteries of adult rabbits. In brief, arteries were removed, placed in cold (4°C) Hanks' balanced salt solution, and opened longitudinally. Their outer and inner surfaces were cleaned of the adventitia and endothelium, respectively. Pieces of artery (≈1 mm^2) were placed into 5 ml of Hanks' solution to which 7 mg of papain, 5 mg of collagenase (Sigma, type IA), and 3.5 mg of bovine serum albumin (Sigma, fraction V) had been added. The tissue was then stored in this enzyme solution for 1–6 hr at 6°C. In preparation for the dissociation of smooth muscle cells, the tissue and enzyme solution were placed at 37°C for 15–25 min with low stirring. Upon detecting the first free cells, the tissue was then transferred to fresh Hanks' solution containing bovine serum albumin (10 mg/50 ml) and mechanically dissociated through the fire-polished tip of a glass pipette. Cells were plated on pieces of poly(L-lysine)-coated glass coverslips. For experiments, a coverslip was placed in a recording chamber of ≈0.2 ml with continuous flow of solution. External solutions were equilibrated with either air (PO_2 ≈ 150 mmHg) or mixtures of N_2 and air to obtain the desired O_2 concentrations. PO_2 in the chamber was monitored with an O_2-sensing electrode (11). Cytosolic [Ca^{2+}] was estimated in unclamped myocytes loaded with fura-2 acetoxymethyl ester. Experiments were performed on an inverted microscope with standard optical components and equipped for epifluorescence and dual-wavelength photometry (12, 13). Calibration of the fluorescence signals in terms of [Ca^{2+}] was performed *in vitro* as described (14). Macroscopic calcium currents were recorded in isolation using the whole-cell configuration of the patch-clamp technique (15, 16) after blockade of the voltage-dependent K^+ channels. Although Na^+ channels are practically absent in arterial myocytes, some experiments were also performed with tetrodotoxin (0.2 μM) added to the external solution. The holding potential was either −80 or −70 mV. Ba^{2+} was used as charge carrier instead of Ca^{2+} to favor the flow of current through the Ca^{2+} channels, and ATP was added to the internal solution to prevent the wash-out of the channels. The composition of the recording solutions is given in the figure legends. Capacity current transients were well fitted by single exponential functions with average time constants of 120 ± 45 μs (n = 24) and 113 ± 30 μs (n = 10) (mean ± SD) for celiac and femoral myocytes, respectively. This indicates that despite the large size of the cells (≈100 μm in length and 6–8 μm in diameter), we had a reasonably fast voltage-clamp and, thus, we could directly monitor tail currents. Voltage-clamp speed was favored by ballistic charge of membrane capacitance and the use of low-resistance electrodes (between 1 and 3 MΩ) (16). Due to the relatively small size of the currents, series resistance was not systematically compensated. Analog current signals were low-pass filtered

*To whom reprint requests should be addressed.

(cutoff frequency between 3 and 10 kHz), digitized at a sample interval of 20 or 50 μs, and stored on computer for analysis. The experiments were conducted at room temperature (22–25°C).

RESULTS

The effect of hypoxia on single arterial smooth muscle cells was first studied by monitoring the modifications of cytosolic [Ca^{2+}] in response to changes in Po_2 with double-wavelength microfluorimetry. Fura-2-loaded myocytes stimulated with a purinergic agonist (ATP) generated rhythmical oscillations of cytosolic [Ca^{2+}]. In all myocytes exhibiting regular Ca^{2+} oscillations that were then subjected to the complete experimental protocol (n = 8), exposure to hypoxia (switching from a bath solution equilibrated with a Po_2 of 150 to one of 20 mmHg) elicited a marked reversible reduction in the amplitude and frequency of the Ca^{2+} spikes. Hypoxic treatment eventually resulted in the complete suppression of the Ca^{2+} oscillations. A representative example of this cellular response to low Po_2 is shown in Fig. 1*A*, which also includes the signal from an O_2-sensing electrode placed in the vicinity of the cell. In those myocytes that had a relatively high resting [Ca^{2+}] (above 50 nM), the inhibition of the oscillations by hypoxia was accompanied by a decrease in resting cytosolic Ca^{2+} levels. These observations indicate that low Po_2 may exert its relaxing action by decreasing intracellular [Ca^{2+}], which is the variable that determines contraction in vascular smooth muscle (17–20). Although largely due to Ca^{2+} release from intracellular stores (21, 22), Ca^{2+} oscillations in excitable (23) and nonexcitable (24) cells require, and are facilitated by, transmembrane Ca^{2+} influx. In accord with this idea, depolarization of myocytes with 60 mM external K^+ elicits an increase in the frequency of the Ca^{2+} oscillations preceding a maintained elevation of cytosolic [Ca^{2+}] (Fig. 1*B*). An opposite effect (abolishment of the Ca^{2+} spikes and decrease of resting cytosolic Ca^{2+}) was observed after briefly removing external Ca^{2+} (Fig. 1*C*) or blockade of voltage-gated Ca^{2+} channels with nifedipine (Fig. 1*D*).

The hypoxic suppression of Ca^{2+} oscillations could be caused by various O_2-dependent cellular processes. Refilling of stores previously depleted with caffeine was not prevented by hypoxia. Furthermore, hypoxia did not affect the release of Ca^{2+} from internal stores evoked by either caffeine (10 mM) or norepinephrine (3 μM). Therefore, one of the actions of low Po_2 might be to inhibit Ca^{2+} influx through voltage-dependent channels. This was directly tested by recording the current through Ca^{2+} channels in whole-cell patch-clamped myocytes. Current sweeps generated in response to step depolarizations to +10 mV in a celiac myocyte exposed to normoxic (control and recovery) and hypoxic external solutions are shown in Fig. 2*A*. The recordings demonstrate an ≈40% reversible reduction in current amplitude upon exposure to low Po_2. The relationship between current amplitude and Po_2 is illustrated in Fig. 2*B*, where the values of peak current (dots) elicited by depolarizing pulses delivered at different Po_2 levels are plotted. The graphs indicate that the reversible hypoxic inhibition of current amplitude occurs roughly with the time course of bath exchange and that it is particularly apparent at Po_2 levels below 70 or 80 mmHg. The time course of the inhibition of the calcium current by low Po_2 was similar to that of nifedipine block (not shown). For comparison, reversible hypoxic inhibition of Ca^{2+} channel activity in a femoral myocyte is illustrated in Fig. 2*C*. Similar qualitative results have been obtained in all cells studied so far (n = 33). It has been shown that vascular smooth muscle cells contain two major kinetically

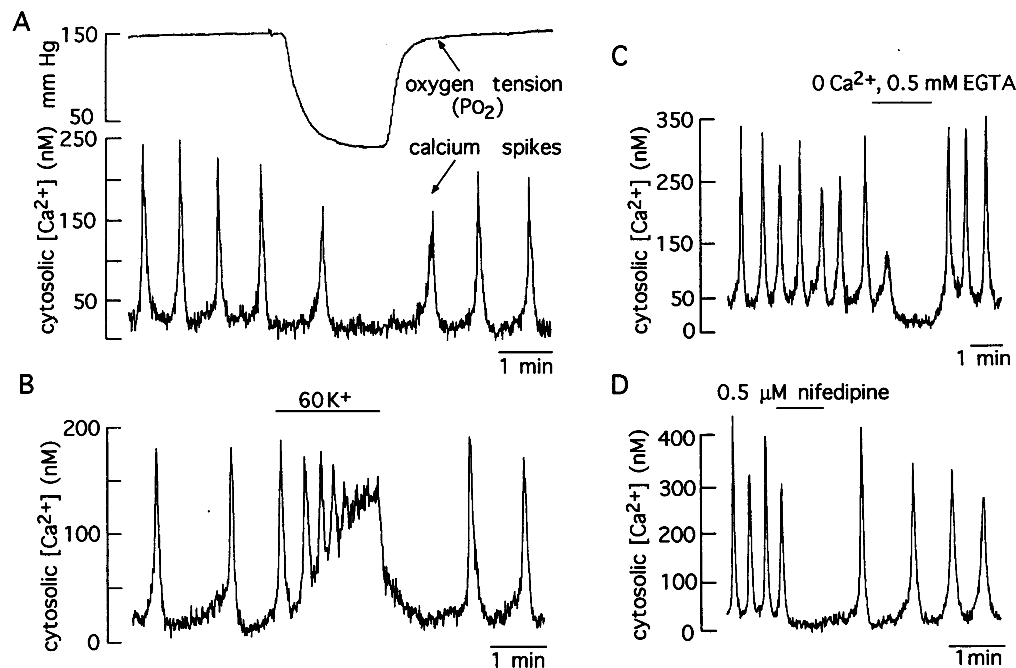

FIG. 1. Oscillations of cytosolic [Ca^{2+}] in arterial myocytes and effect of changes in ambient oxygen tension (Po_2). (*A*) Parallel recordings of Po_2 in the recording chamber and intracellular [Ca^{2+}] in a fura-2-loaded myocyte illustrating the reversible inhibition of Ca^{2+} oscillations in response to hypoxia. Note that the spikes are suppressed upon reaching extreme low Po_2 levels (≈20 mmHg). Average resting cytosolic [Ca^{2+}] was 61 ± 28 nM (mean ± SD, n = 22) and oscillations were triggered with a transient (≈30 s) exposure to 1 mM ATP (26). The average frequency and amplitude of the oscillations were 1.6 ± 0.6 spikes per min and 271 ± 172 nM (n = 8), respectively. (*B*) Reversible increase of the frequency of Ca^{2+} oscillations in response to membrane depolarization by 60 mM external K^+. Reinitiation of the oscillations after recovery of the basal Ca^{2+} levels was obtained by a brief pulse of 1 mM ATP. (*C* and *D*) Suppression of Ca^{2+} oscillations after brief removal and chelation of external Ca^{2+} (*C*) or blockade of voltage-gated Ca^{2+} channels with nifedipine (*D*). The application of the different test solutions is indicated by the horizontal bars. The standard external solution contained (in mM) 140 NaCl, 2.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes (pH 7.35–7.40). The 60 K^+ solution contained 82.7 mM NaCl and 60 mM KCl. Nifedipine (0.5 μM) was added to the external solution. The 0 Ca^{2+}, EGTA solution contained 4 mM $MgCl_2$ and 0.5 mM EGTA.

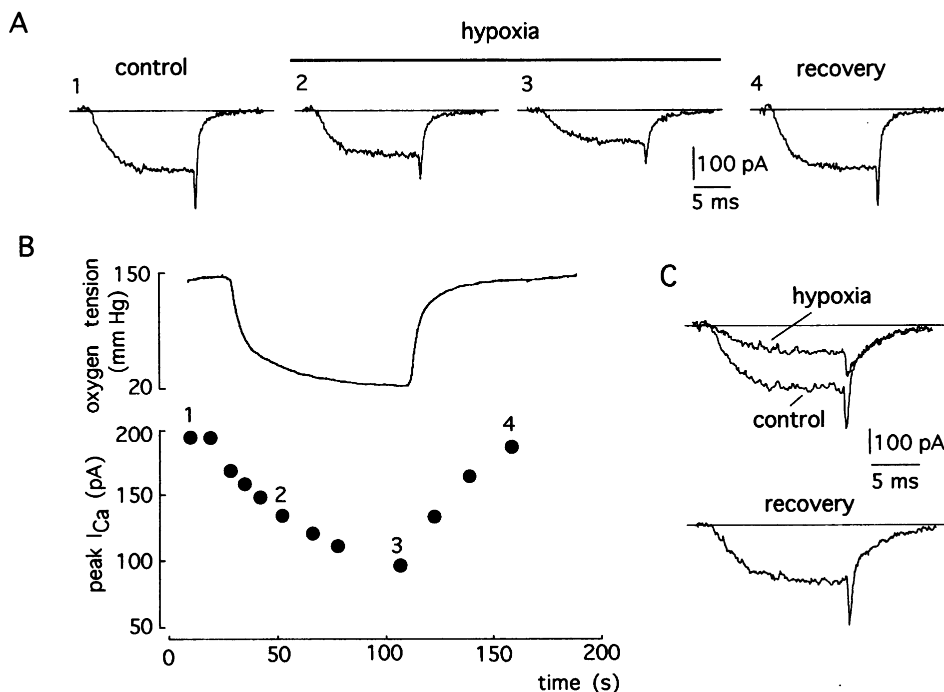

FIG. 2. Inhibition of voltage-gated Ca^{2+} channels by low P_{O_2}. (*A*) Macroscopic calcium currents recorded from a myocyte dispersed from the celiac artery during 15-ms step depolarizations to +10 mV from a holding potential of −80 mV. Exposure to hypoxia (switching from an external solution equilibrated with $P_{O_2} \approx 150$ mmHg to another with $P_{O_2} \approx 20$ mmHg; records 2 and 3) induces an inhibition in current amplitude. Reversibility is illustrated by the recovery trace. (*B*) Parallel time courses of the changes of P_{O_2} in the chamber and the reduction in current amplitude. The records shown in *A* are indicated by the corresponding number. Current amplitudes were measured immediately before the end of the depolarizing pulses. (*C*) Reversible reduction of calcium current amplitude by low P_{O_2} (20 mmHg) in a myocyte dispersed from the femoral artery. Depolarizing pulses to +10 mV (15 ms) were applied from a potential of −80 mV. Note that a slow component of the tail current was unaffected by low P_{O_2}. The recording solutions contained (in mM) the following: External [140 NaCl, 2.7 KCl, 10 $BaCl_2$, and 10 Hepes (pH 7.4)]. Internal (solution in the patch pipette and inside the cell) [100 CsCl, 25 CsF, 2 $MgCl_2$, 10 Hepes, 10 EGTA, 5 bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate, and 4 MgATP (pH 7.3)].

distinct populations of Ca^{2+} channels (L and T type) (25, 26) that, as in other cell types (16, 27), can be identified by their deactivation time courses as slow- (T) and fast- (L) deactivating channels. In many femoral and celiac myocytes we detect two components of the tails. The slowly deactivating component of the tail current, which is resistant to nifedipine (Fig. 3*A*) and inactivated by a small depolarizing prepulse (Fig. 3*B*), was unaffected by hypoxia (Fig. 3*C*) in all cells tested (see also Figs. 2*C* and 4*A*). These observations indicate that the effect of low P_{O_2} is selective for the dihydropyridine-sensitive, fast-deactivating (L-type), channels, whereas the slow-deactivating (T-type) Ca^{2+} channel population is unaffected by changes in P_{O_2}.

A remarkable characteristic of the regulatory action of oxygen on Ca^{2+} channel activity is its strong voltage dependence. Fig. 4*A* shows a family of calcium currents generated by depolarizations from −80 mV to the indicated membrane potentials. Current traces recorded in a low P_{O_2} solution (H) are compared with those obtained at normal P_{O_2} (C). Recovery from hypoxia was almost perfect and an example is shown at +10 mV (trace R). Low P_{O_2} produced an inhibition of current amplitude that was larger with moderate depolarization. With stronger depolarization the effect of hypoxia was almost negligible. In celiac myocytes, the average inhibition of current amplitude by hypoxia ($P_{O_2} \approx 20$ mmHg) was 43.17% ± 13.8% (mean ± SD, $n = 12$) of the control value at 0 mV, but only 2.08% ± 7% ($n = 8$) at +20 mV. In femoral myocytes, these values were 40% ± 27% ($n = 7$) at 0 mV and 10% ± 13% ($n = 6$) at +20 mV. The voltage dependence of the hypoxic inhibition of Ca^{2+} channel activity is also clearly evident in Fig. 4*B*, where we have plotted the average current–voltage relationship under normoxic (filled symbols) and hypoxic (open symbols) conditions obtained with measurements from four cells.

DISCUSSION

Our results indicate that exposure to low P_{O_2} leads to inhibition of Ca^{2+} influx through L-type Ca^{2+} channels in arterial myocytes. This is selective since T-type Ca^{2+} channels appear to be unaffected. The effect of O_2 tension on the Ca^{2+} channels is fast, is completely reversible, and occurs with P_{O_2} levels within the physiological range. This phenomenon explains the decrease of cytosolic Ca^{2+} upon exposure to low P_{O_2} and, thus, it is conceivable that it contributes to the hypoxic relaxation of systemic arteries. In agreement with previous work by other authors (28, 29), we have not detected so far any modulatory effect of P_{O_2} on the macroscopic K^+ currents of systemic myocytes. Pharmacological studies done in isolated perfused hearts have suggested that hypoxic vasodilatation of the coronary arteries could be a consequence of the decrease of intracellular ATP and the subsequent hyperpolarization caused by the opening of ATP-sensitive (K_{ATP}) K^+ channels (8, 9). Activation of K_{ATP} channels in response to hypoxia most likely requires maintained exposure to extreme low P_{O_2} (8, 30). Thus, although possibly important, this process may act on a slower, more protracted, time scale than the acute response of Ca^{2+} channels described here, occurring over a full range of P_{O_2} values and surely critical for an immediate arterial relaxation in response to hypoxia.

The Ca^{2+} channels selectively regulated by P_{O_2} are of the dihidropyridyne-sensitive, L-type, which are broadly distributed in vascular smooth muscle and are known to be activated by norepinephrine and other vasoactive agents (20, 26, 31). In mesenteric artery myocytes, the membrane potential–force

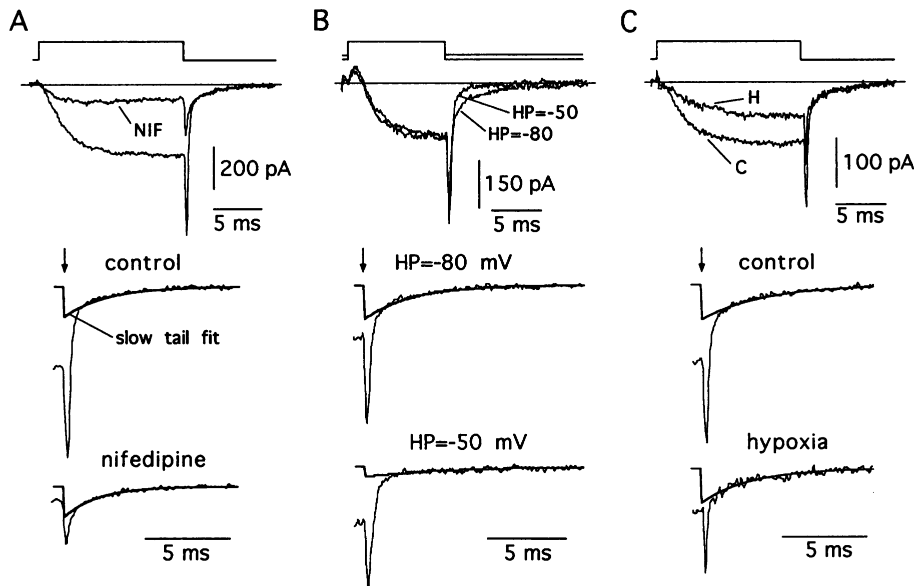

FIG. 3. Separation of fast- and slowly deactivating components in the Ca^{2+} tail currents and selective inhibition of the fast component by low P_{O_2}. (*A*) Superposition of current traces recorded in a celiac myocyte during 15-ms depolarizations to +10 mV from −80 mV in the control solution and after addition of 0.2 μM nifedipine (upper panel). The tail currents in the two experimental conditions are shown at a expanded time scale in the middle and bottom panels. As in other cell types (16, 27), the tail currents have fast and slow components that most likely represent the L-type (fast-deactivating) and T-type (slow-deactivating) Ca^{2+} channels described in arterial smooth muscle cells (25, 26). Nifedipine almost completely abolished the fast component of the tail but left unaltered the slow component. This is shown by the similar amplitude of single exponential functions that in the two experimental conditions were fitted to the slow component of the tail. The exponential functions were extrapolated to the onset of repolarization (indicated by downward arrows). (*B*) Superposition of current traces obtained from a femoral myocyte during 10-ms depolarizations to +10 mV from the indicated holding potentials (HP) (upper traces). The middle and bottom panels show the exponential functions fitted to the slow component of the tail at the two holding potentials. Note that a small maintained depolarization (from −80 to −50 mV) leads to >80% reduction of the slow component in the tail, indicating inactivation of the T-type Ca^{2+} channels. The fast component of the tail was almost unaltered. (*C*) Superposition of current traces in a celiac myocyte generated during 15-ms step depolarizations to +10 mV from −80 mV in a myocyte exposed to normoxic (C, control, $P_{O_2} \approx 150$ mmHg) and hypoxic (H, $P_{O_2} \approx 20$ mmHg) solutions (upper panel). As in *A*, the recordings in the middle and bottom panels show that the slow component of the tail currents was unaffected by low P_{O_2} and that the reduction of current amplitude is due to selective inhibition of the fast component of the tail current.

relation almost perfectly matches with the voltage dependence of Ca^{2+} channel open probability (p_{open}) (20). Thus, is has been suggested that this channel type is a major regulator of smooth muscle tension and hence of arterial tone (20, 28, 31).

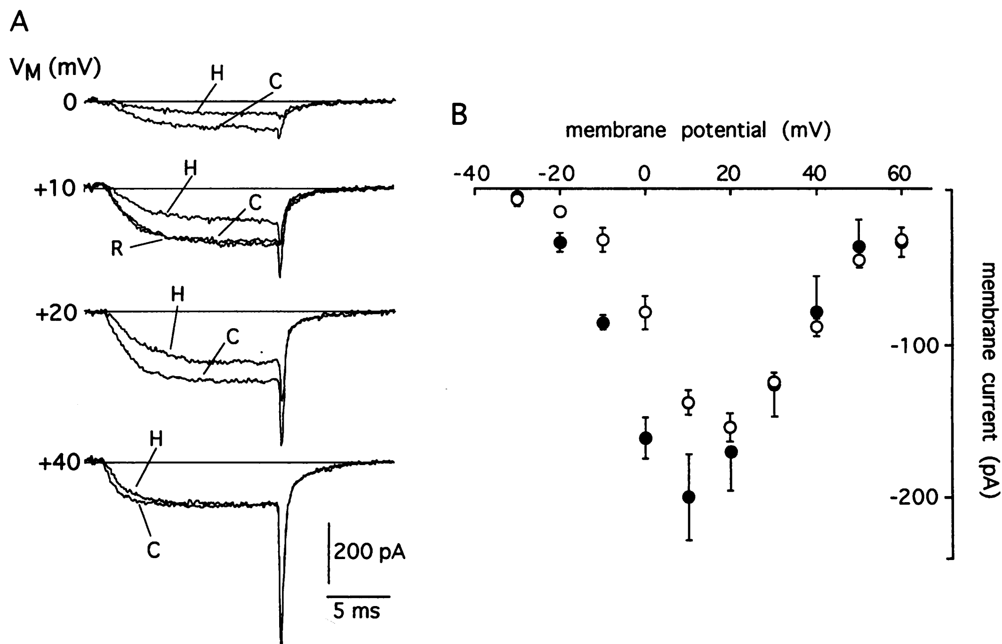

FIG. 4. Voltage dependence of the inhibitory effect of low P_{O_2} on the Ca^{2+} channels. (*A*) Current traces recorded during 15-ms step depolarizations from −80 mV to the indicated membrane potentials. Sweeps recorded from the same celiac myocyte in normoxic (C, $P_{O_2} \approx 150$ mmHg) and hypoxic (H, $P_{O_2} \approx 20$ mmHg) solutions are superimposed. Note that the effect of low P_{O_2} is larger with moderate depolarizations. (*B*) Average current–voltage relation in normoxic (filled symbols) and hypoxic (open symbols) solutions. Current amplitudes were measured in four celiac myocytes before the end of 15-ms depolarizing pulses. Vertical bars are the standard error of the mean.

Interestingly, the inhibition of the calcium current by low Po_2 is more pronounced at potentials between −30 and 0 mV (see Fig. 4), which is the range at which the p_{open}–voltage relationship of the channels is very steep (20, 31). Therefore, in partially depolarized myocytes low Po_2 would be expected to have a major influence on channel p_{open}. In good agreement with this idea, early studies had already shown that the sensitivity of systemic arteries to oxygen is more obvious when testing is carried out on precontracted samples using low to moderate concentrations of agonist (5).

In conclusion, we have found in arterial smooth muscle a type of Ca^{2+} channel modulation that may participate in hypoxic arterial dilatation. The properties of the oxygen-sensitive Ca^{2+} channels make them well suited for having a major physiological role in the fast adaptation of regional arterial resistance to the degree of blood oxygenation. The existence of oxygen-sensitive K^+ channels in various tissues has been previously described (10) but oxygen-sensitive Ca^{2+} channels are without precedent in the literature. Besides celiac and femoral myocytes (representative examples of visceral and skeletal muscle arteries), we have observed a similar regulatory action of Po_2 in muscle cells dispersed from rat and rabbit mesenteric arteries as well as from the main trunk of the rabbit pulmonary artery. This suggests that O_2-regulated Ca^{2+} channels are perhaps distributed throughout the circulatory system and in different species. This channel type might be involved in some cardiocirculatory disturbances such as hypertension.

This work was supported by grants from the Dirección General de Investigación Científica y Técnica (DGICYT) of the Spanish Ministry of Science and Education and the European Community (DGXII). A.F.-O. is an International Human Frontiers Science Program Postdoctoral Fellow.

1. Sparks, H. V. (1980) in *Vascular Smooth Muscle*, Handbook of Physiology, The Cardiovascular System, eds. Bohr, D. F., Somlyo, A. P. & Sparks, H. V. (Am. Physiol. Soc., Bethesda), Vol. 3, pp. 475–513.
2. Hellstrand, P., Johansson, B. & Norberg, K. (1977) *Acta Physiol. Scand.* **100,** 69–83.
3. Detar, R. (1980) *Am. J. Physiol.* **238,** H761–H769.
4. Marriot, J. F. & Marshall, J. (1990) *J. Physiol. (London)* **422,** 1–13.
5. Chang, A. E. & Detar, R. (1980) *Am. J. Physiol.* **238,** H716–H728.
6. van Breemen, C. & Saida, K. (1989) *Annu. Rev. Physiol.* **51,** 315–329.
7. Ebeigbe, A. B., Pickard, J. D. & Jennett, S. (1980) *Q. J. Exp. Physiol.* **65,** 273–292.
8. Daut, J., Maier-Rudolf, W., von Beckerath, N., Mehrke, G., Günther, K. & Goedel-Meinen, L. (1990) *Science* **247,** 1341–1344.
9. von Beckerath, N., Cyrys, S., Dischner, A. & Daut, J. (1991) *J. Physiol. (London)* **442,** 297–319.
10. López-Barneo, J. (1994) *Trends Neurosci.* **17,** 133–135.
11. Ganfornina, M. D. & López-Barneo, J. (1992) *J. Gen. Physiol.* **100,** 401–426.
12. Toledo-Aral, J., Ureña, J., Castellano, A. & López-Barneo, J. (1993) *J. Physiol. (London)* **472,** 327–340.
13. Almers, W. & Neher, E. (1985) *FEBS Lett.* **192,** 13–18.
14. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
15. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. (1981) *Pflügers Arch.* **391,** 85–100.
16. Castellano, A. & López-Barneo, J. (1991) *J. Gen. Physiol.* **97,** 303–320.
17. Blatter, L. A. & Wier, W. G. (1992) *Am. J. Physiol.* **263,** H576–H586.
18. Weissberg, P. L., Little, P. J. & Bobik, A. (1989) *Am. J. Physiol.* **256,** C951–C957.
19. Gustafsson, H. & Nilsson, H. (1993) *Acta Physiol. Scand.* **149,** 283–291.
20. Nelson, M. T., Standen, N. B., Brayden, J. E. & Worley, J. F. (1988) *Nature (London)* **336,** 382–385.
21. Tsien, R. W. & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6,** 715–760.
22. Amundson, J. & Clapham, D. (1993) *Curr. Opin. Neurobiol.* **3,** 375–382.
23. Friel, D. D. & Tsien, R. W. (1992) *Neuron* **8,** 1109–1125.
24. Girard, S. & Clapham, D. (1993) *Science* **260,** 229–232.
25. Benham, C. D., Hess, P. & Tsien, R. W. (1987) *Circ. Res.* **61,** 10–16.
26. McDonald, T. F., Pelzer, S., Trautwein, W. & Pelzer, D. J. (1994) *Physiol. Rev.* **74,** 365–507.
27. Matteson, D. R. & Armstrong, C. M. (1986) *J. Gen. Physiol.* **87,** 161–182.
28. Yuan, X., Goldman, W. F., Tod, M. L., Rubin, L. J. & Blaustein, M. P. (1993) *Am. J. Physiol.* **264,** L107–L115.
29. Post, J. M., Hume, J. R., Archer, S. L. & Weir, E. K. (1992) *Am. J. Physiol.* **262,** C882–C890.
30. Benndorf, K., Bollmann, G., Friedrich, M. & Hirche, H. (1992) *J. Physiol. (London)* **454,** 339–357.
31. Nelson, M. T., Patlak, J. B., Worley, J. F. & Standen, N. B. (1990) *Am. J. Physiol.* **259,** C3–C18.