A patch-clamp study of the effects of cicletanine on whole-cell calcium current in ventricular myocytes.

M Gisbert, P Mery, R Fischmeister

A PATCH-CLAMP STUDY OF THE EFFECTS OF CICLETANINE ON WHOLE-CELL CALCIUM CURRENT IN VENTRICULAR MYOCYTES

GISBERT M.-P., MERY P.-F., FISCHMEISTER R.*

Laboratory of Cardiac Cellular Physiology, INSERM U-241, Paris-Sud University, F-91405 Orsay, France.

Summary: *The effects of extracellular application of cicletanine on the voltage-sensitive calcium current (I_{Ca}) was studied in isolated cells from frog ventricle. Myocytes were isolated by enzymatic dissociation and I_{Ca} was measured using the whole-cell configuration of the patch-clamp technique modified to permit intracellular perfusion with various substances. Cicletanine (10 to 100 μM) had no effect on control I_{Ca}. However, when I_{Ca} was enhanced by superfusion of the cell with saturating doses of β-adrenergic agonist (isoprenaline, 2 μM) or by intracellular perfusion with maximal doses of cAMP (20 μM), cicletanine exerted a dual effect on I_{Ca}. At 10 μM, cicletanine generally induced a transient or sustained stimulation of I_{Ca} (5 to 40%), while 100 μM of the drug generally reduced I_{Ca}. The effects of cicletanine were reversible and not voltage-dependent. These results suggest that cicletanine affects I_{Ca} by acting on a mechanism occurring after cAMP synthesis, by enhancing cAMP concentration (e.g. through an inhibition of cAMP phosphodiesterase) or facilitating cAMP-dependent phosphorylation of the Ca channels.*

Introduction

The new diuretic and antihypertensive drug, cicletanine, was shown to reduce contraction induced by membrane depolarization (KCl) or $CaCl_2$ in rabbit aorta, mesenteric artery and rat portal vein (1). Cicletanine was also shown to antagonize the contractions caused by noradrenaline and phenylephrine on isolated rat aorta (2). Since Ca^{2+} ions play a determinant role in vascular contractility, it has been suggested that cicletanine may interact directly and/or indirectly with membrane Ca^{2+} transport (3, 4). Ca influx through Ca channels may be one of several mechanisms by which cicletanine could partially counteract the increase in cytosolic free Ca^{2+} concentration induced by various agonists and membrane depolarization (3). In some respects, the contractile and diuretic effects of cicletanine resemble those of the atrial natriuretic factor (ANF). As the authors recently reported, ANF has strong inhibitory effects on Ca channels in frog ventricular myocytes (5, 6). Thus, it was of interest to study the effects of cicletanine on Ca current in cardiac cells, where Ca current has been relatively well characterized.

* *Author to whom correspondence should be addressed at:* Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241, Université de Paris-Sud, Bât. 443, F-91405 Orsay, France.

Materials and methods

The methods used for cell dissociation, whole-cell patch-clamp recording, superfusion and internal perfusion of the cells, and data analysis have been extensively described in previous papers (6–9) and were used without major modification in the present study.

Briefly, for routine monitoring of calcium current (I_{Ca}), the frog *(Rana esculenta)* ventricular cell was depolarized every 8 s from −80 mV holding potential to 0 mV for 200 ms. To accurately measure I_{Ca} with no contamination of other ionic currents, the cells were bathed in K-free, 20 mM Cs Ringer solution containing: 88.4 mM NaCl, 20 mM CsCl, 22.9 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate and 0.3 μM tetrodotoxin (Sankyo, Japan). The standard internal solution in the patch-electrode (1–3 megohms resistance) contained: 120 mM CsCl, 5 mM K_2EGTA, 4 mM $MgCl_2$, 5 mM Na_2CP, 3 mM Na_2ATP, 0.4 mM Na_2GTP, adjusted to pH 7.1 with KOH. Solutions were applied to the exterior of the cell by placing the cell at the opening of 250 μm inner diameter capillary tubing (flow rate of 10 μl/min) (7). All external solutions were gassed with 95% O_2 and 5% CO_2 (pH = 7.4) and experiments were carried out at room temperature (19.5–22.5 °C). Solutions were applied to the interior of the cell via the patch electrode which could be perfused and thus permitted modification of the internal solution (8, 9). Under these conditions, the current traces were digitized at 10 KHz (12-bit A/D converter) by a Compaq 286 Desk-Pro computer and I_{Ca} was measured on-line, using programs written in the Pascal language, as the difference between the peak inward current and the current at the end of the 200-ms pulse (7).

Drugs. (±)-Isoprenaline and cyclic AMP were obtained from Sigma Chemical Co. (USA). (±)3-(4-Chlorophenyl) - 1,3-dihydro - 7-hydroxy - 6-methylfuro(3,4-*c*)pyridine (cicletanine hydrochloride) was provided by IHB Laboratories (Le Plessis Robinson, France). Stock solutions (0.2 M) of cicletanine were made from crystalline powder dissolved in DMSO. Control solutions contained identical amounts of DMSO as the cicletanine-containing solutions.

Results

Frog ventricular cells possess only one type of calcium current (10, 11) which corresponds to the high-threshold L-type Ca channels (12), so that the I_{Ca} current elicited by a voltage-clamp depolarization from −80 mV to 0 mV corresponds to a single population of Ca channels. The effects of 1 to 100 μM concentration of (±)-cicletanine hydrochloride were tested on I_{Ca} measured every 8 s by a depolarizing pulse from −80 mV holding potential to 0 mV for 200 ms. Cicletanine had negligible effects on the control I_{Ca}. I_{Ca} was 98.24 ± 9.5% of control value (mean ± s.e.m., n = 10) when the cell was superfused with 10 μM cicletanine and 101.13 ± 10.3% (n = 11) with 100 μM cicletanine. However, when I_{Ca} was increased by exposing the cell to 2 μM isoprenaline (ISO), cicletanine had more pronounced effects (Fig. 1). At 1 μM concentration, cicletanine was without significant effect on I_{Ca}, but at 10 μM cicletanine strongly enhanced ISO-elevated I_{Ca}. A further increase in the dose of cicletanine to 100 μM was accompanied by a strong inhibition of I_{Ca} following a transient initial stimulatory period. The holding current (at −80 mV) and the quasi-steady-state current at the end of the depolarizing pulse to 0 mV were not modified by cicletanine, reflecting the absence of an effect of the drug on the electrogenic Na,K pump. The effects of cicletanine on I_{Ca} were essentially reversible (Fig. 1).

The current-voltage *(I–V)* relationship for I_{Ca} was not significantly modified by cicletanine (Fig. 2). The control *I–V* curve was symmetrical with a maximal I_{Ca} amplitude observed around 0 mV membrane

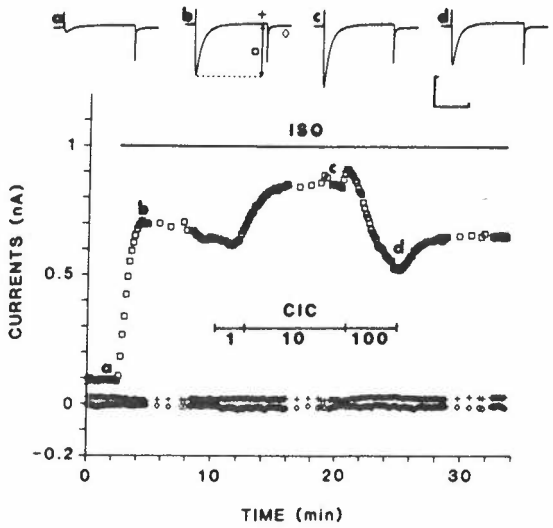

Fig. 1 Effects of cumulative doses of cicletanine (CIC) on membrane currents. The four current traces (top) were recorded at the times indicated by the corresponding letters on the bottom graph: (a) control; (b) 2 μM isoprenaline (ISO); (c) ISO + 10 μM cicletanine (CIC); (d) ISO + 100 μM CIC. Current traces were recorded on and replayed from VCR magnetic tape (bandwidth DC to 20 KHz, 16 bit resolution) and digitized at 10 KHz. Calibration bars: horizontal 100 ms; vertical 400 pA. Each set of symbols (bottom) corresponds to measurements obtained by a depolarizing pulse made every 8 s from −80 mV holding potential to 0 mV for 200 ms. Net amplitude of I_{Ca} (squares) was determined by the difference between peak inward current and the current at the end of the depolarizing pulse to 0 mV (i.e. quasi-steady-state current). Holding current at −80 mV (diamonds) and the current at the end of the pulse (crosses) were obtained as indicated on the (b) current trace shown on the top. The cell was externally perfused with 2 μM ISO in the absence or presence of increasing doses of CIC (1, 10 and 100 μM) during the periods indicated.

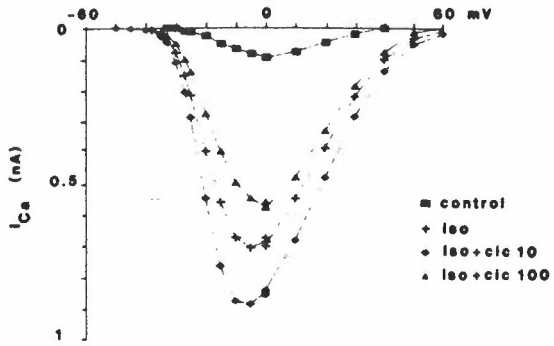

Fig. 2 Current–voltage *(I–V)* relationships of net I_{Ca}. The cell was depolarized from −80 mV holding potential to various potentials for 200 ms, and the net I_{Ca} was determined as indicated in Fig. 1. Squares, control conditions; crosses, the cell was superfused with 2 μM isoprenaline (ISO); diamonds, ISO + 10 μM cicletanine (CIC); triangles, ISO + 100 μM CIC.

potential; 2 μM ISO increased I_{Ca} about 6-fold (6.84 ± 1.49, $n = 7$) and slightly shifted the maximal current potential by ~5 mV in the negative direction, most probably due to an incomplete series resistance compensation (7). A 4-min exposure to 10 μM cicletanine induced a further increase in I_{Ca} amplitude by ~25% at each membrane potential, while a 4-min application of 100 μM cicletanine reduced I_{Ca} elevated by ISO by ~20%. These changes in I_{Ca} amplitude induced by cicletanine were not accompanied by any apparent modification in the kinetics of activation and inactivation of I_{Ca} (Fig. 1, top).

Inactivation and reactivation properties of I_{Ca} were studied in the presence and absence of cicletanine using double-pulse protocols. Inactivation (Fig. 3) was determined by examining the effect of 200-ms prepulses to various potentials on the response to a subsequent test pulse to 0 mV. Prepulses between −60 mV and 0 mV progressively decreased I_{Ca} with complete inactivation being reached between −10 and +10 mV. With prepulses above +20 mV, the test I_{Ca} progressively increased. The inactivation curve was not significantly modified by either ISO or cicletanine in the negative range of potentials. However, for membrane potentials above +20 mV, ISO increased the amount of inactivation (7, 11); I_{Ca} inactivation by prepulses to +100 mV was increased from 50% in the control to 65% in ISO. The addition of 10 μM cicletanine to the ISO solution was without further effect on the inactivation curve. However, a larger dose of the drug (100 μM) further increased the

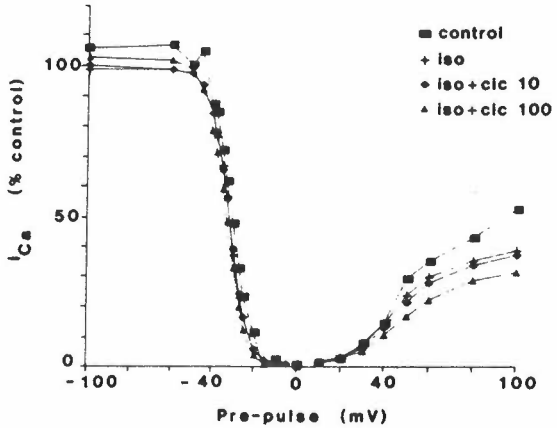

Fig. 3 Inactivation curves of I_{Ca}. Inactivation was measured with a double-pulse protocol (see ref. 7 and text for more details). Symbols refer to the same cell and experimental conditions as in Fig. 2.

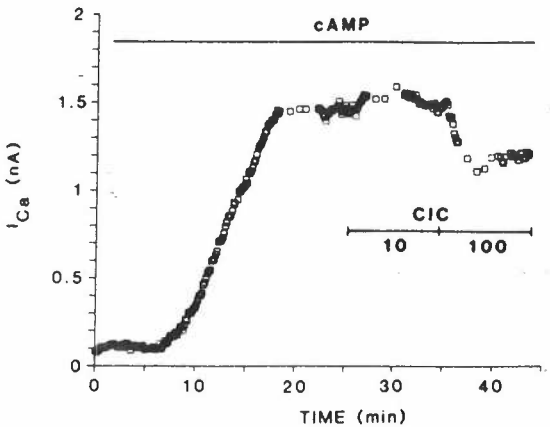

Fig. 4 Effects of cicletanine on I_{Ca} enhanced by intracellular perfusion with cAMP. At time zero, the cell was perfused with control intracellular solution (see Materials and methods). After about 2 min, the solution in the patch electrode was switched to one containing 20 μM cAMP and the new intracellular perfusion continued throughout the whole experiment. The cell was exposed to 10 and 100 μM cicletanine (CIC) during the periods indicated.

degree of inactivation induced by strong positive potentials. Such changes in the inactivation curve could result from an enhancement in the Ca-mediated inactivation process (13), possibly due to an increased intracellular Ca^{2+} concentration near the channels induced by high doses of cicletanine.

Recovery from inactivation was determined by measuring I_{Ca} elicited by a 200-ms test pulse to 0 mV that followed a 200-ms prepulse to 0 mV and a subsequent variable recovery interval at −80 mV. As already reported (7, 11) reactivation of I_{Ca} occurred faster in control conditions than when I_{Ca} was increased by ISO. However, cicletanine (10 or 100 μM) added to the ISO solution did not induce any additional modification in the reactivation of I_{Ca} (not shown).

The observation that cicletanine affected I_{Ca} only when it had been elevated by β-adrenergic stimulation with ISO raises questions about the mechanism(s) of action of the drug. β-Adrenergic agonists stimulate the activity of adenylate cyclase and increase the level of intracellular cyclic AMP (cAMP). The increase in cAMP is responsible for the β-adrenergic stimulation of I_{Ca}, by increasing the degree of phosphorylation of the Ca channels, since a similar action is seen when cAMP is perfused into the cell (7). In an attempt to clarify whether the action of cicletanine occurs before or after cAMP synthesis, the authors studied the effects of cicletanine on calcium current which was elevated by direct application of intracellular cAMP. In the experiment shown in Fig. 4, after the level of I_{Ca} had stabilized for a few minutes after disruption of the membrane patch, the solution in the patch-pipette was switched to one containing 20 μM cAMP. Five minutes after the beginning of the perfusion with the new intracellular solution, the level of I_{Ca} started to increase, reflecting the diffusion of cAMP into the cell. When I_{Ca} stabilized at a value ≈12-fold larger than the control, the cell was externally exposed to 10 and 100 μM cicletanine; 10 μM cicletanine induced a small and transient stimulation of I_{Ca} (5 to 20%), while 100 μM induced a marked diminution.

Although the average stimulatory effects of 10 μM cicletanine were smaller on cAMP-elevated I_{Ca} than on ISO-elevated I_{Ca}, the differences were not statistically significant. This would suggest that cicletanine acts on a mechanism which occurs after cAMP synthesis in the cascade of events leading to β-adrenergic agonist stimulation of I_{Ca}. A possible candidate for the stimulatory action of cicletanine may be an inhibition of cAMP hydrolysis. Such an inhibition would lead to local increases in cAMP levels stimulating the phosphorylation of Ca channels. An inhibition of cAMP phosphodiesterases would be expected to have different effects depending on: (i) the degree of stimulation of cAMP synthesis with β-adrenergic agonists; or (ii) the concentration of cAMP reached near the membrane when the cell is perfused with a cAMP-containing solution (14). Such variations in the increase of local cAMP concentrations would most probably reflect different stimulations of I_{Ca}, relative to control levels, by ISO or cAMP. Thus, if inhibition of cAMP phosphodiesterase is one mechanism of action of cicletanine, cells in which ISO- or cAMP-induced stimulations of I_{Ca} were the smallest would be expected to exhibit the largest relative stimulations by 10 μM cicletanine (and the smallest inhibitions by 100 μM). Figure 5 represents a summary of all the experiments conducted with externally applied ISO (0.1 or 2 μM) or internally applied cAMP (20 μM) when the cells were then exposed to 10 and/or 100 μM cicletanine. The relative variations of I_{Ca} induced by cicletanine are plotted as a function of the relative stimulatory effect of ISO or cAMP on I_{Ca}. Figure 5 represents only steady-state effects of cicletanine, measured 3 to 5 min after exposure to the drugs. The analysis of the response to cicletanine was complicated by the presence of frequent initial transient stimulatory effects of the drug on I_{Ca}, probably due to two opposite effects taking place at the same concentration of cicletanine. This, as well as significant cell-to-cell variations in I_{Ca} amplitude (10, 11), may be partly responsible for the large scatter of the data. However, a slight negative correlation seemed to exist between the response to cicletanine and the relative stimulation of I_{Ca} by ISO or cAMP.

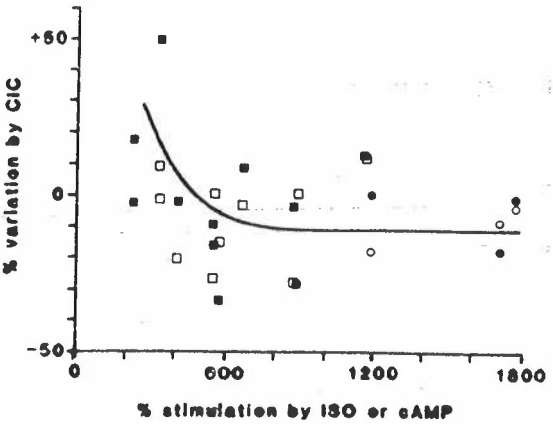

Fig. 5 Plot of the relative variations of I_{Ca} induced by cicletanine (CIC) (10 μM, filled symbols; 100 μM, open symbols) vs. the relative stimulations induced by isoprenaline (squares) or cAMP (circles). The line represents a fit by eye to the data obtained with 10 μM CIC (i.e. filled symbols). The data represent only steady-state effects of cicletanine, observed after 3 to 5 min exposure to the drug.

Discussion

While cicletanine was without any significant effect on basal I_{Ca} levels, this antihypertensive drug exerted two opposite effects on Ca channels when their activity had been elevated by either the externally applied β-adrenergic agonist isoprenaline or intracellularly perfused cAMP. At 10 μM concentrations, cicletanine induced a transient or sustained stimulation of I_{Ca}, while at 100 μM the drug generally reduced I_{Ca}. However, a large scatter in the response of the cells to cicletanine was observed and the overall effect of the drug on I_{Ca} was relatively small (< 10% on average).

The dual effect of cicletanine may be due to: (i) the presence of non-specific effects at high concen-

trations; or (ii) the opposite actions on I_{Ca} of the two enantiomers (+) and (−). The second hypothesis could be verified by analysing the effects on Ca channels of each of the enantiomers in comparison with the action of the racemic form studied here.

An increase in I_{Ca} by cicletanine seen in some of the present experiments would lead to a more pronounced influx of Ca^{2+} ions which would result in a positive inotropic effect on the heart. In vascular smooth muscle, such an effect on sarcolemmal Ca transport would lead to an increase in the vascular contraction. This is somewhat suprising in view of the fact that cicletanine has been demonstrated to possess strong antihypertensive properties (1–3). Clearly, a description of the effects of cicletanine on vascular smooth muscle Ca channels would be required in order to resolve this paradox. However, a possible reason for a difference in the action of cicletanine on Ca channels in heart and smooth muscle cells could be due to the recent evidence that the cAMP-mediated regulation of Ca channels is probably absent in smooth muscle cells (15–17). Since it has been observed here that a stimulation of Ca channels activity by cAMP-dependent mechanisms was a prerequisite for the response of cardiac Ca channels to cicletanine, such a response to the drug may be absent in Ca channels from vascular smooth muscle cells.

The enhancement of I_{Ca} by 10 μM cicletanine, seen in some of the present experiments, when the level of cAMP inside the cell was supposedly maximal, most likely reflects either an additional local increase in cAMP concentration (e.g. due to an inhibition of cAMP phosphodiesterase) or a facilitation of cAMP-dependent protein kinase which phosphorylates various proteins, including Ca channels. It should be noted that both of these actions would induce a relaxation of vascular smooth muscle. If cAMP hydrolysis were fast enough to locally reduce the concentration of cAMP available for the phosphorylation of Ca channels, then an inhibition of the cAMP-phosphodiesterase may increase local concentrations of the nucleotide and, thus, enhance I_{Ca} (14). Although the authors do not have strong evidence in favour of such an inhibition of phosphodiesterase activity induced by cicletanine, the present data is consistent with such a hypothesis.

The effects of cicletanine on cardiac Ca channels are clearly different from the strong and sustained inhibitory action of ANF (5, 6). Whether these two substances share some common mechanisms at the cellular level in vascular smooth muscle cells remains to be explored.

Acknowledgements

The authors wish to thank Michel Pucéat for preparing the cells, Patrick Lechêne for excellent computer assistance and Dr. Alvin Shrier for reading the manuscript. This work was supported by a grant from the Henri Beaufour Institute.

References

(1) Baranes J., Hellegouarch A., Clostre F., Braquet P. *New diuretic drug studies using isolated depolarized veins.* Naunyn-Schmied. Arch. Pharmacol., **324** (suppl.), R51, 1983.

(2) Auguet M. et al. *Antihypertensive effect of cicletanine on SHR-stroke-prone rats: Possible interaction with alpha-adrenoceptors.* In: 10th World Congress of Cardiology, Washington, 14–19 September, 1986.

(3) Chabrier P. et al. *Cicletanine.* In: Scriabine A., ed. "Cardiovascular drug reviews." Raven Press, New York, 1988, in press.

(4) Galli C. et al. *Evidence for an antagonistic effect of R(−)cicletanine on the increases in cytosolic free Ca^{2+} contents induced by the Ca^{2+} ionophore A23187 in macrophages and vascular smooth muscle cells.* Pharmacol. Res. Comm., 1987, in press.

(5) Fischmeister R., Gisbert M.-P. *Effects of atrial natriuretic factor on calcium current in single cells of frog heart.* J. Physiol. (London), **390**, 65P, 1987.

(6) Gisbert M.-P., Fischmeister R. *The atrial natriuretic factor regulates the calcium current in frog isolated cardiac cells.* Circ. Res., 1988, in press.

(7) Fischmeister R., Hartzell H.C. *Mechanism of action of acetylcholine on calcium current in single cells from frog ventricle.* J. Physiol. (London), **376**, 183, 1986.

(8) Fischmeister R., Hartzell H.C. *Cyclic guanosine 3′,5′-monophosphate regulates the calcium current in single cells from frog ventricle.* J. Physiol. (London), **387**, 453, 1987.

(9) Hartzell H.C., Fischmeister R. *Cyclic GMP and cyclic AMP produce opposite effects on Ca current in single heart cells.* Nature, **323**, 273, 1986.

(10) Fischmeister R., Argibay J.A., Hartzell H.C. *Modifications of cardiac calcium current in cells with inherent differences in current density.* Biophys. J., **51**, 29a, 1987.

(11) Argibay J.A., Fischmeister R., Hartzell H.C. *Inactivation, reactivation and pacing dependence of calcium current in frog cardiocytes: Correlation with current density.* J. Physiol. (London), 1988, in press.

(12) Nilius B, Hess P., Lansman J.B., Tsien R.W. *A novel type of cardiac calcium channel in ventricular cells.* Nature, **316**, 443, 1985.

(13) Mentrard, D., Vassort G., Fischmeister R. *Calcium-mediated inactivation of calcium current in cesium-loaded frog atrial cells.* J. Gen. Physiol., **83**, 105, 1984.

(14) Simmons M.A., Hartzell H.C. *Roles of phosphodiesterases in regulation of calcium current in isolated cardiac myocytes.* Mol. Pharmacol., 1988, in press.

(15) Ohya Y., Kitamura K., Kuriyama H. *Modulation of ionic currents in smooth muscle balls of the rabbit intestine by intracellularly perfused ATP and cyclic AMP.* Pflügers Arch., **408**, 465, 1987.

(16) Droogmans G., Declerck I., Casteels R. *Effect of adrenergic agonists on Ca^{2+}-channel currents in single vascular smooth muscle cells.* Pflügers Arch., **409**, 7, 1987.

(17) Galizzi J.-P., Qar J., Fosset M., Van Renterghem C., Lazdunski M. *Regulation of calcium channels in aortic smooth muscle cells by protein kinase C activators (diacylglycerol and phorbol esters) and by peptides (vasopressin bombesin) that stimulate phosphoinositide breakdown.* J. Biol. Chem., **262**, 6947, 1987.