Voltage-gated Calcium Channel Currents in Human Coronary Myocytes

Regulation by Cyclic GMP and Nitric Oxide

Jean-François Quignard,* Jean Marc Frapier,‡ Marie-Cécile Harricane,* Bernard Albat,‡ Joël Nargeot,* and Sylvain Richard*
**Centre de Recherches de Biochimie Macromoléculaire, CNRS, UPR 9008, INSERM U 249, Université de Montpellier I, 34033 Montpellier Cedex, France; and ‡Service de Chirurgie Thoracique et Cardio-vasculaire, Hôpital Arnaud de Villeneuve, 34033 Montpellier, France*

Abstract

Voltage-gated Ca^{2+} channels contribute to the maintenance of contractile tone in vascular myocytes and are potential targets for vasodilating agents. There is no information available about their nature and regulation in human coronary arteries. We used the whole-cell voltage-clamp technique to characterize Ca^{2+}-channel currents immediately after enzymatic dissociation and after primary culture of coronary myocytes taken from heart transplant patients. We recorded a dihydropyridine-sensitive L-type current in both freshly isolated and primary cultured cells. A T-type current was recorded only in culture. The L- (but not the T-) type current was inhibited by permeable analogues of cGMP in a dose-dependent manner. This effect was mimicked by the nitric oxide–generating agents *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine which increased intracellular cGMP. Methylene blue, known to inhibit guanylate cyclase, antagonized the effect of SNAP. Inhibitions by SNAP and cGMP were not additive and seemed to occur through a common pathway. We conclude that (*a*) L-type Ca^{2+} channels are the major pathway for voltage-gated Ca^{2+} entry in human coronary myocytes; (*b*) their inhibition by agents stimulating nitric oxide and/or intracellular cGMP production is expected to contribute to vasorelaxation and may be involved in the therapeutic effect of nitrovasodilators; and (*c*) the expression of T-type Ca^{2+} channels in culture may be triggered by cell proliferation. (*J. Clin. Invest.* 1997. 99:185–193.) Key words: human coronary • transmembrane signaling • Ca^{2+} channels • contractile tone • proliferation

Introduction

Intracellular free calcium (Ca^{2+}) is the major determinant of vascular tone (1, 2). Since contraction depends crucially upon extracellular Ca^{2+}, transmembrane voltage-gated Ca^{2+} channels are perhaps the most important physiological regulator of Ca^{2+} entry into vascular smooth muscle cells (2–7). By sensing membrane depolarization, these channels open and thereby flux Ca^{2+}. Opening may additionally be modulated by hormones, transmitters, and second messengers (4, 6, 7). Ca^{2+} channels are key targets in the control of vascular tone by antihypertensive and vasodilating drugs (2–7). This is the case for those ligands referred to as Ca^{2+} antagonists [e.g., dihydropyridines (DHPs)][1] that promote a nonconducting state of the L-type Ca^{2+} channel, and for agents such as K^+ channel activators that hyperpolarize the membrane potential, thus deactivating Ca^{2+} channels. The final effect is vasorelaxation of the arterial muscle.

Two types of Ca^{2+} channels with distinct electrophysiological and pharmacological properties have been identified in mammalian coronary myocytes. The main type, described in bovine, rabbit, porcine, and guinea pig myocytes (8–14), is the DHP-sensitive L-type current involved in excitation–contraction coupling. The second, referred to as T-type, has been described only in guinea pig (14, 15). Its functional role and pharmacology are still not well-defined owing to the lack of specific ligands. Although Ca^{2+} channel antagonists have found worldwide acceptance in the therapy of coronary heart disease, suggesting the presence of L-type Ca^{2+} channels (7, 16, 17), the precise nature and regulation of the Ca^{2+} channels present in human coronary myocytes has not been studied yet. For example, the existence of T-type Ca^{2+} channels in these myocytes is still not known. Over the last years, there has been an explosive increase in our knowledge of the electrophysiology of human cardiac cells, revealing significant differences in cellular behavior with animal models (18). In contrast, there is a large deficit of information concerning the human coronary artery. In this work, using the whole-cell patch-clamp method, we have characterized Ca^{2+}-channel currents in human coronary myocytes. We have also investigated the regulation of these

Address correspondence to Sylvain Richard, Centre de Recherches de Biochimie Macromoléculaire, CNRS, UPR 9008, INSERM U 249, Université de Montpellier I, Route de Mende, BP 5051, 34033 Montpellier Cedex, France. Phone: 33-467-61-33-55; FAX: 33-467-52-15-59; E-mail: Richard@xerxes.crbm.cnrs-mop.fr

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1. *Abbreviations used in this paper:* db-cGMP, dibutyryl cGMP; DHPs, dihydropyridines; HP, holding potential; HVA, high voltage–activated; I_{Ba}, Ba^{2+} current; $I_{Ba,L}$, L-type Ba^{2+} current; $I_{Ba,T}$, T-type Ba^{2+} current; I_{Ca}, Ca^{2+} current; $I_{Ca,L}$, L-type Ca^{2+} current; LVA, low voltage–activated; NO, nitric oxide; SIN-1, 3-morpholinosydnonimine; SM, smooth muscle; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TTX, tetrodotoxin.

currents by two classes of potent vasodilating agents: Ca^{2+}-channel antagonists and the endothelium-derived relaxing factor nitric oxide (NO).

Methods

Myocyte isolation and primary cultures. Coronary myocytes were obtained from 13 transplanted hearts of patients (males; aged 50±9 yr) with end-stage heart failure (NYHA classes III and IV) caused by dilated or ischemic disease (ejection fraction: 20±5%). Samples of the left descending artery were removed and placed in a sterile physiological solution (A) containing (in mmol/liter): NaCl, 132; KCl, 5; $MgCl_2$, 1.5; dextrose, 11; Hepes, 13; $CaCl_2$, 1; penicillin (60 μg/ml); and 1% bovine serum albumin (pH adjusted to 7.4 with NaOH). The media, dissected from the vessel, were subjected to enzymatic digestion using solution A (without Ca^{2+}) supplemented with 0.2 mg/ml papain (Sigma Chemical Co., St. Louis, MO) plus 0.1 mM dithiothreitol (19), 0.3 mg/ml type II collagenase (Worthington Biochemical Corp., Freehold, NJ), and 0.2 mg/ml elastase (Boehringer Mannheim, Indianapolis, IN). After 25 min of incubation at 37°C, the dissociation was achieved by gentle mechanical trituration. Well-relaxed long spindle-shaped cells (Fig. 1 *A*) were obtained (yield ~ 70%). These cells, which contracted upon depolarization by KCl (data not shown), were used for electrophysiology immediately or, alternatively, plated at low density (between 2×10^4 and 2×10^5 cells per dish) for culture. Culture maintenance medium [MEM and Ham's F-10 (1:1 vol/vol); supplemented with 10% fetal calf serum, 1 mM glutamine, 50 μM ascorbic acid, 15 μM proline, 100 U/ml penicillin, and 100 μg/ml streptomycin; Eurobio, Les Ulis, France] was changed every 2 d. Cultured cells proliferated rapidly. Their size increased by four- to eightfold reflecting an hypertrophic process. Cell capacitance increased from 39±14 pF (n = 64) to 190±80 pF (n = 194). Confluence was obtained 15–20 d after plating. Electrophysiological studies of cultured cells were started 4 d after plating.

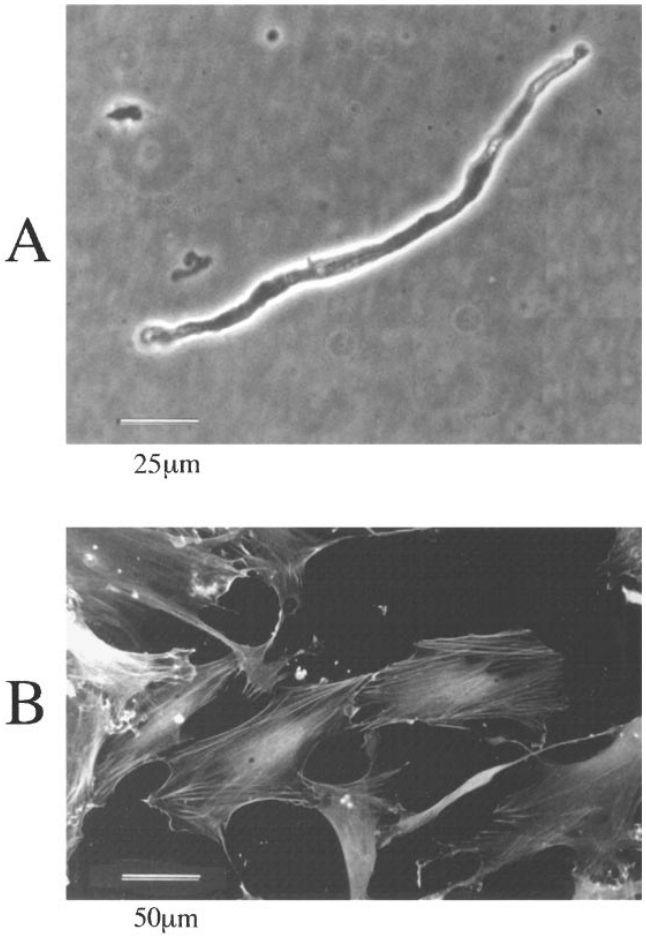

Figure 1. Freshly isolated and cultured human coronary myocytes. Photographs of (*A*) a single freshly isolated and (*B*) cultured myocytes with immunodetection of α-SM actin (5 d after plating; see Methods).

Immunofluorescence. The cells on coverslips were fixed in 3% paraformaldehyde-PBS, at pH 7.4, and permeabilized with methanol at −18°C for 6 min to detect α-smooth muscle (SM) actin. A polyclonal antibody (gift of V. Hanin, CRBM, Montpellier, France) was directed against the first specific amino acids of α-SM actin. Fluorescent goat anti–rabbit FITC-conjugated antibodies (Immunotech, Marseilles, France) were used (20). The cultured cells were uniformly positive for the expression of α-SM actin forming large and dense bundles (Fig. 1 *B*) which confirmed the muscular origin of all cultured cells (21).

Electrophysiological recordings. The whole-cell patch-clamp method (Bio-Logic RK-300 patch-clamp amplifier; Bio-Logic, Claix, France) was used (20–22°C) in conventional conditions to isolate Ca^{2+}-channel currents (19, 22, 23). The recording pipettes were filled with (mM): CsOH, 130; EGTA, 10; Hepes, 25; Mg-ATP, 3; Na-GTP, 0.5; glucose, 10; succinic acid, 5; and aspartic acid, 5. The bathing solution contained (mM): CsOH, 120; $BaOH_2$ (or $CaOH_2$), 20; Hepes, 10; 4-aminopyridine, 5; and glucose, 10. For both solutions, pH was adjusted to 7.3 with CH_3SO_3H (310–330 mosM/liter). Voltage errors resulting from residual, uncompensated series resistance ($\leq$ 1 MΩ) were $<$ 2 mV (currents $<$ 200 pA). Experimental parameters were controlled with an IBM PC. Capacitive transient and linear leakage currents were often subtracted using the P/-4 protocol. Data acquisition and analyses were performed using the pCLAMP software (v. 6.0; Axon Instruments, Inc., Foster City, CA). Current amplitude was measured as the difference between the maximal inward current and the holding current. Results are expressed as mean±SD. Statistical comparisons between groups of values were performed using Student's t test ($P <$ 0.05 considered significant).

Drugs. SNAP (±-*S*-nitroso-*N*-acetylpenicillamine) and SIN-1 (3-morpholinosydnonimine hydrochloride) (BIOMOL Research Labs Inc., Plymouth Meeting, PA) were prepared directly with the test solution just before use. Stock solutions (10 mM) of dibutyryl (db)- and 8-bromo cGMP (Sigma Chemical Co.) and tetrodotoxin (TTX) were diluted with the test solution to the final concentration. Nicardipine, PN200-110 (Sandoz Ltd., Basel, Switzerland), Bay-K 8644 (Bayer Tour, Puteaux, France), and diltiazem (Sigma Chemical Co.) were dissolved in 50% ethanol as concentrated stock solutions (10 mM) stored first at −20°C before the final dilution. Control and test solutions were applied to the extracellular side of cells using a multiple microcapillary (200 μM inner tubing, flow rate 0.5 ml·min^{-1}) gravity-driven perfusion system placed nearby to the cell ($<$ 1 mm). Rapid solution changes could be made within seconds.

Intracellular cGMP measurements. Cultured myocytes were grown until subconfluence was reached (12–15 d). cGMP was measured after exposure to the NO donor SNAP as described before (24). Briefly, myocytes were rinsed out three times and preincubated for 20 min with Krebs bicarbonate buffer equilibrated with 95% O_2/5% CO_2. Myocytes were then incubated for 5 min in this medium in the presence (or absence) of 10 μM SNAP and 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. The reaction was stopped by replacing the medium with 0.5 ml of ethanol/formic acid (95%/5%, vol/vol). Cells were scraped and suspensions were centrifuged for 5 min at 10,000 *g*. After lyophylization, cGMP was assayed after acetylation by radioimmunoassay (24).

Results

L-type Ca^{2+}-channel currents in freshly isolated myocytes. Ca^{2+}-channel currents were successfully recorded in freshly isolated human coronary myocytes using either physiological

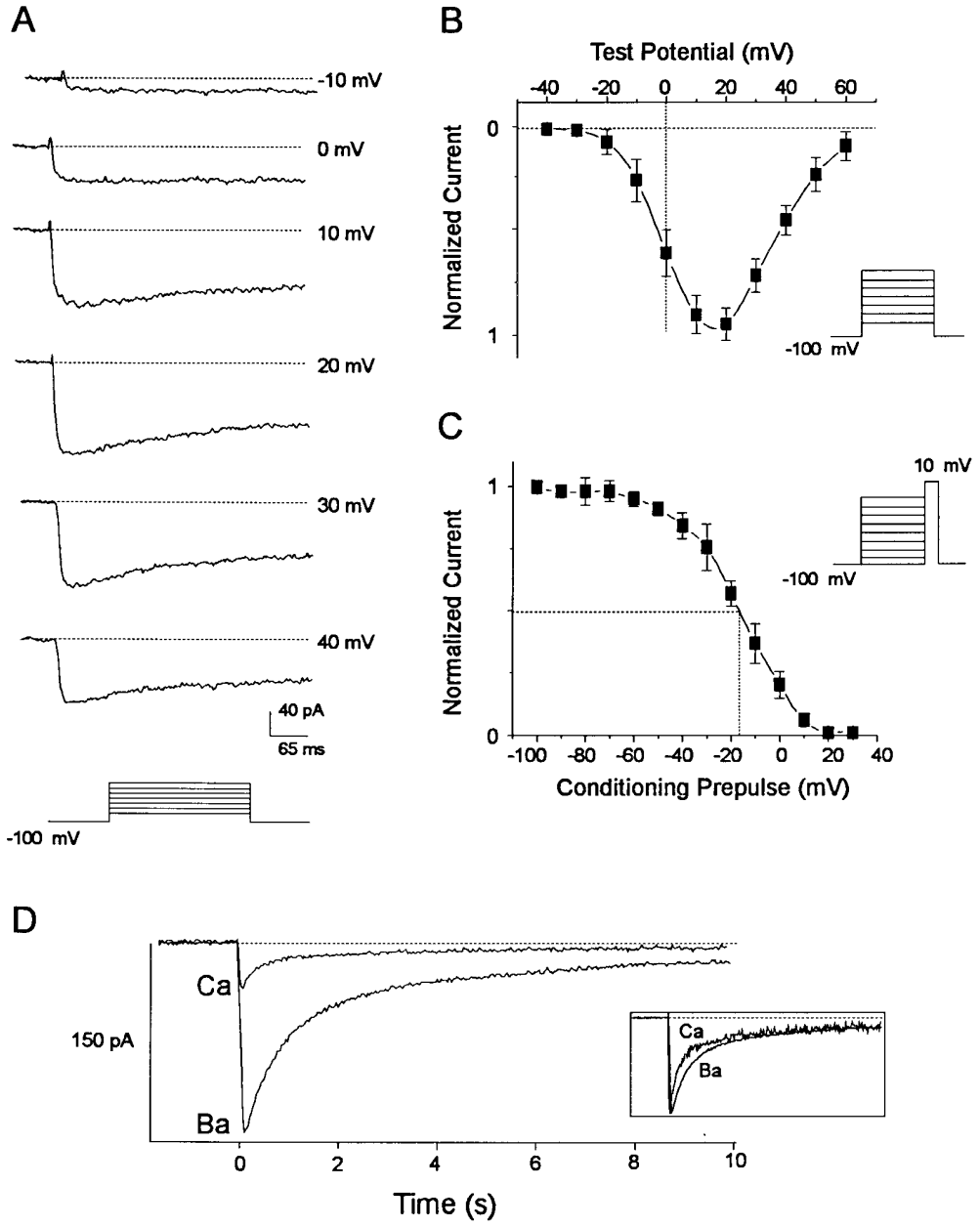

Figure 2. Electrophysiological properties of Ca^{2+}-channel currents in freshly isolated human coronary myocytes. (*A*) Typical wave form of I_{Ba} evoked for various depolarizations from a HP of −100 mV in the same cell. (*B*) Mean normalized (±SD; 21 cells) I-V relationship of I_{Ba} for potentials comprised between −40 and +60 mV. (*C*) Mean (±SD; seven cells) normalized steady state inactivation curve of I_{Ba}. I_{Ba} was evoked at +10 mV after conditioning prepulses at various voltages, measured for each prepulse, normalized, averaged, and plotted as a function of the conditioning voltages. The curve could be fitted assuming a Boltzmann distribution ($I/I_{max} = 1/[1 + exp^{V - V0.5)/k}]$) where $V_{0.5}$ is the voltage at which there is 50% inactivation (−16±1 mV), k (13±1) is the slope factor, and I is the relative amplitude of the recorded current. (*D*) Comparison of I_{Ba} and I_{Ca} in a same cell. The Ca^{2+}-channel currents were recorded using first Ba^{2+} and then Ca^{2+} using an EGTA-free pipette solution. (*Inset*) I_{Ba} and I_{Ca} have been scaled for comparison of kinetics.

Ca^{2+} (I_{Ca}) or Ba^{2+} (I_{Ba}) ions as charges carrier. These currents were abolished in Ca^{2+}- or Ba^{2+}-free solutions and were insensitive to the specific Na^+-channel inhibitor TTX at concentrations up to 10 μM (data not shown) confirming their nature. Fig. 2 *A* shows typical recordings of I_{Ba} evoked for various test potentials between −10 mV and +40 mV from the holding potential (HP) of −100 mV. I_{Ba} was evidenced in all myocytes investigated (n = 64). On average, I_{Ba} activated at depolarizations positive to −30 mV and peaked near +20 mV as shown by the current–voltage (I-V) relationship (Fig. 2 *B*). At +10 mV, current amplitude was 120±70 pA (n = 64). The averaged current density, estimated by measuring cell capacitance (39±14 pF; n = 64) which reflects cell membrane surface, was 3.2±1.8 pA/pF (n = 64). I_{Ba} was fully activable for all HPs < −60 mV as determined from the steady state inactivation relationship by use of a double-pulse protocol (Fig. 2 *C*). The mean $V_{0.5}$ (determined from the Boltzmann equation: see legend to Fig. 2 *C*) was −16±1 mV (n = 7) which indicated that Ca^{2+} channels are still available for opening in cells with low membrane resting potential.

I_{Ba} was sustained with nearly no decay during short test depolarizations at all voltages (Fig. 2 *A*). Long depolarizing steps (several seconds) were required in order to reveal inactivation (Fig. 2 *D*). When Ba^{2+} and Ca^{2+} were rapidly exchanged on an equimolar basis to determine current kinetics when the physiological ion Ca^{2+} is the charge carrier, there was a marked decrease of current amplitude. The ratio I_{Ca}/I_{Ba} for currents evoked at +10 mV was 0.26±0.05 (n = 10) which indicated that Ca^{2+} channels were much more permeable to Ba^{2+} than to Ca^{2+}. In contrast, current decay was not much changed and remained slow. This slow inactivation of I_{Ca} was not related to the presence of the Ca^{2+} buffer EGTA, which can prevent Ca^{2+}-dependent inactivation of I_{Ca} (6), in the patch pipette. Indeed, when EGTA was omitted from the patch-pipette, the

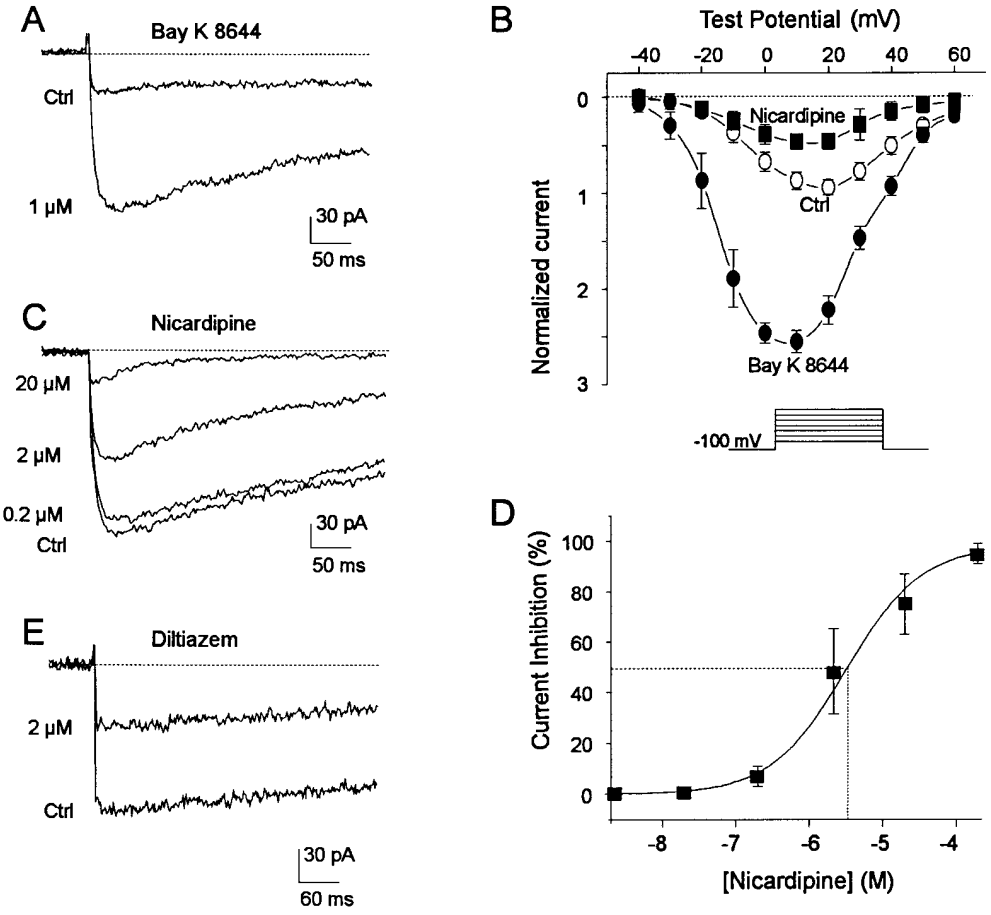

Figure 3. Effects of specific L-type Ca^{2+}-channel ligands on I_{Ba} in freshly isolated human coronary myocytes. (*A*) Effect of Bay-K 8644. I_{Ba}, evoked at +10 mV from HP −100 mV, was recorded before (*Ctrl*) and after 2 min of exposure to Bay-K 8644 (1 μM). (*B*) Mean (±SD) normalized I-V relationships of I_{Ba} in presence of Bay-K 8644 (*filled circles*; eight cells) and nicardipine (*filled boxes*; five cells). Control, *open circles*; 13 cells. (*C*) Concentration-dependent effects of nicardipine on I_{Ba} evoked as described in *A*. The cell was sequentially exposed to increasing concentrations (as indicated) of the drug until steady state inhibition was reached for each concentration (3 min). (*D*) Averaged normalized (±SD; five cells) dose–response relationship of the inhibition of I_{Ba} by nicardipine. For each cell, experimental data points were obtained using stepwise perfusion of concentrations as shown in *C*. The dose–response curve was fitted with Hill equation in the form: $I = I_0 + A/\{1 + (X/IC_{50})\}^{nH}$; where X is the drug concentration; I_0 is the maximal inhibition (percentage); A is the amplitude; and nH is the Hill coefficient. The IC_{50} determined is 2±0.9 μM (nH = 0.7±0.2). (*E*) Effect of diltiazem. I_{Ba}, evoked as described in *A*, was recorded before (*Ctrl*) and after 3 min of exposure to 2 μM diltiazem.

decay of I_{Ca} remained slow and was still similar to that of I_{Ba} (Fig. 2 *D*). Therefore, we concluded that inactivation of I_{Ca} is not significantly Ca^{2+} dependent. Because I_{Ba} was much larger than I_{Ca}, Ca^{2+}-channel currents were studied routinely using Ba^{2+} ions as the permeating ion.

We next evaluated the effect of various agonists and antagonists of the L-type Ca^{2+} channel. The DHP agonist Bay-K 8644 (1 μM) induced a 3.4±1.0 (n = 9) potentiation of peak I_{Ba} (Fig. 3 *A*). Both the threshold and the maximum of the I-V curve were shifted by ∼ −10 mV (Fig. 3 *B*) which is characteristic of the effect of Bay-K 8644 (6). In contrast, I_{Ba} was inhibited by two potent vasodilating agents, the DHP antagonists nicardipine (Fig. 3, *B* and *C*) and PN200-110 (82±5% at 2 μM; n = 3). Fig. 3 *C* shows the cumulative effects of increasing concentrations of nicardipine. As determined from the averaged dose-dependent curve, the half-maximal inhibition (IC_{50}) of I_{Ba} by nicardipine occurred at ∼ 2 μM (Fig. 3 *D*). I_{Ba} was also inhibited by the benzothiazepine diltiazem (Fig. 3 *E*). IC_{50} occurred at ∼ 2 μM (45±10%; n = 3). As expected from their voltage-dependent high-affinity block (3–7), inhibition of I_{Ba} by DHP antagonists was much enhanced by membrane depolarization. The modulation of the blocking effect of I_{Ba} by membrane potential is illustrated for PN200-110 (Fig. 4). For example, the percentage of inhibition of I_{Ba} by 0.1 μM PN200-110 increased significantly from 16±3% at HP −100 mV to 53±10% at HP −40 mV (n = 5; $P < 0.04$) (Fig. 4 *B*). Taken together, these results confirm that the high voltage–activated (HVA) Ca^{2+}-channel current recorded in freshly isolated human coronary myocytes is related to the L-type family of Ca^{2+} channels described in the cardiovascular system (3–14). Therefore, it is now referred to as $I_{Ba,L}$.

T-type Ca^{2+}-channel currents in primary cultured myocytes. No low voltage–activated (LVA) Ca^{2+}-channel current was present in any of the freshly isolated human coronary myocytes (n = 64; see also Fig. 2 *B*). In contrast, a transient LVA I_{Ba} was recorded in cultured cells in addition to a sustained HVA I_{Ba}. Fig. 5 *A* shows the typical wave form of a large transient LVA I_{Ba} recorded for a test depolarization as low as −30 mV. It is worth noting that the HVA I_{Ba} recorded in cultured cells (Fig. 5 *B*) had properties identical to those shown above for $I_{Ba,L}$ in freshly isolated cells as indicated by electrophysiological parameters such as threshold for activation (−30 mV), maximal peak amplitude (near +20 mV), steady state inactivation (mean $V_{0.5}$ −15±2 mV; n = 14), relative permeability ratio I_{Ca}/I_{Ba} (0.23±0.03 at +10 mV; n = 6), and pharmacological sensitivity to Bay-K 8644 (3.1±0.3 increase at 1 μM; n = 6) and nicardipine (IC_{50} = 3.5±1.0 μM at HP −100 mV; n = 6).

The LVA I_{Ba} was insensitive to the Na^+-channel blocker TTX (10 μM) and to Bay-K 8644 (1 μM; data not shown). When $I_{Ba,L}$ and the LVA I_{Ba} coexisted in the same cell, they were easily distinguishable by their activation threshold and kinetics of decay. The LVA I_{Ba} began to activate for test pulses

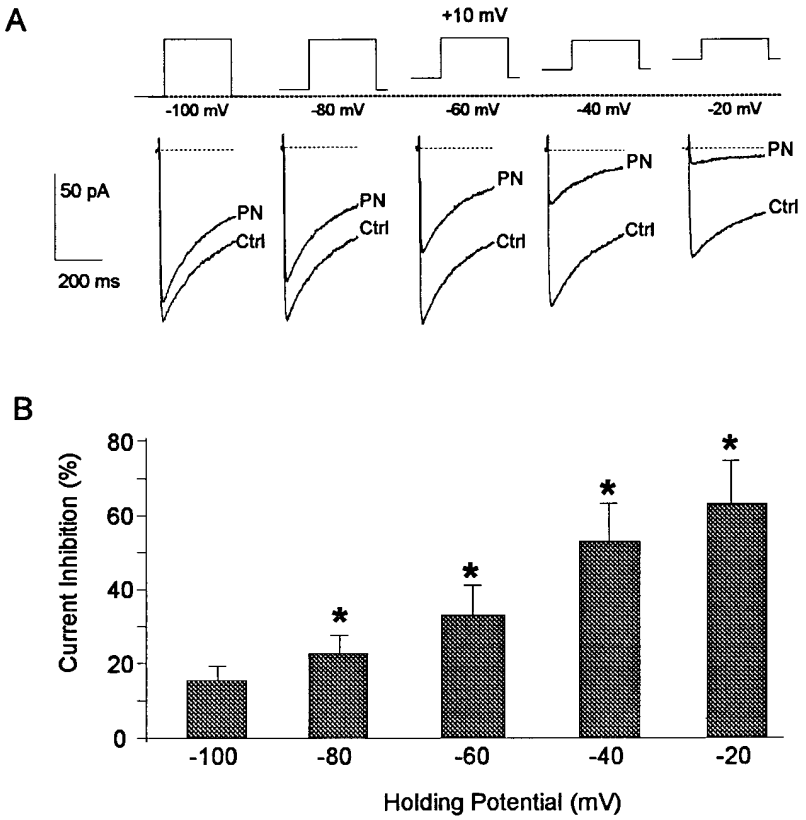

Figure 4. Voltage dependence of the effect of PN200-110 on $I_{Ba,L}$. (*A*) Effect of 0.1 μM PN200-110 on $I_{Ba,L}$ evoked at +10 mV from various HPs in the same cell. The cell was held for 2 min at each HP tested in the absence (*Ctrl*) and presence of PN200-110 (*PN*). (*B*) Bar graphs show mean inhibitions (±SD; five cells) of 0.1 μM PN200-110 obtained for each HP tested as explained in *A*. Current inhibition increased significantly ($P < 0.04$) with membrane depolarization at each represented voltage. Significance was assessed by use of a binomial test based on the sign of the difference between paired samples.

around −40 mV, i.e., 20 mV more negative than $I_{Ba,L}$ (Fig. 5 *C*). Furthermore, this current could be separated from $I_{Ba,L}$ by changing the HP from −100 mV to a more depolarized level (Fig. 5 *C*) as predicted from the steady state inactivation curve (Fig. 5 *D*). The mean $V_{0.5}$ were very different (Fig. 5 *D*). The relative permeability ratio I_{Ca}/I_{Ba} of the LVA I_{Ba} evoked at −30 mV was 0.96±0.05 ($n = 5$), i.e., also quite different from that of $I_{Ba,L}$ (see above). Therefore, the electrophysiological

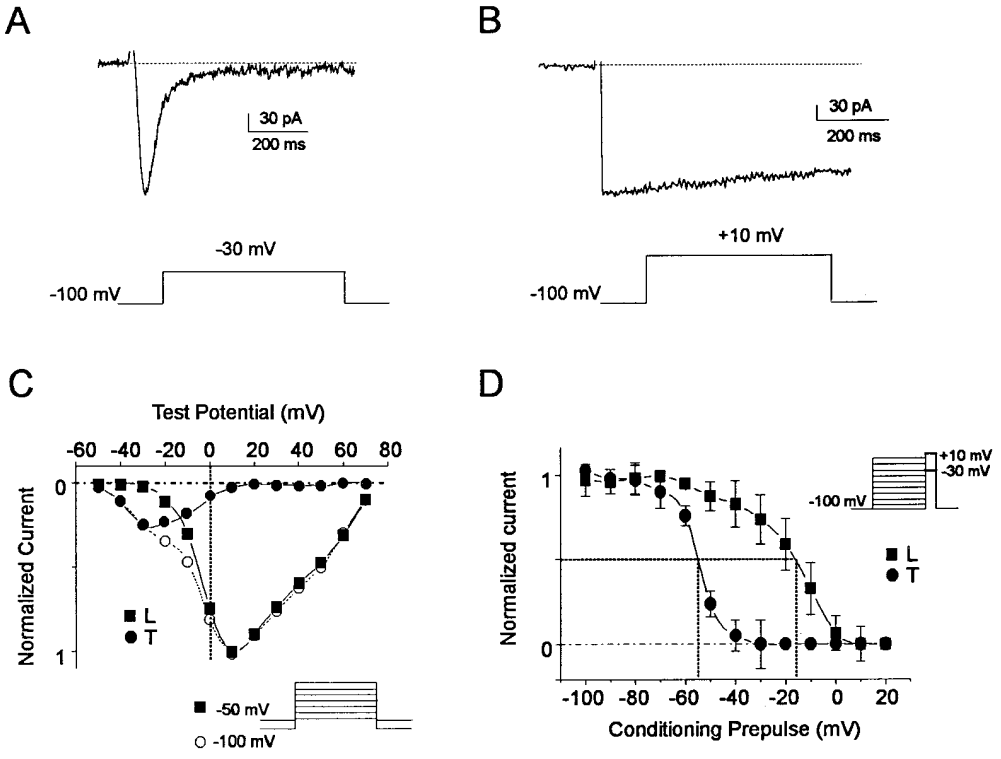

Figure 5. Two types of I_{Ba} in primary cultured human coronary myocytes. (*A*) Wave form of an LVA T-type I_{Ba} ($I_{Ba,T}$) evoked at −30 mV from an HP of −100 mV. (*B*) Wave form of a sustained HVA I_{Ba} ($I_{Ba,L}$) evoked at +10 mV from an HP of −100 mV in another cell. (*C*) Voltage-dependent separation of $I_{Ba,T}$ and $I_{Ba,L}$ in a cell where the two currents coexisted. This was obtained by changing the HP from −100 mV to −50 mV. Shown are complete I-V relationships of the global current $I_{Ba,L} + I_{Ba,T}$ (*open circles*) recorded from HP −100 mV, the depolarization-resistant $I_{Ba,L}$ (*filled boxes*) recorded from HP −50 mV and $I_{Ba,T}$ (*filled circles*) obtained by subtracting the two curves (*open circles − filled boxes*). (*D*) Mean (±SD) normalized steady state inactivation curves of $I_{Ba,T}$ (*filled circles*; three cells) and $I_{Ba,L}$ (*filled boxes*; 14 cells). $I_{Ba,T}$ was evoked at −30 mV and $I_{Ba,L}$ at +10 mV after 5-s conditioning prepulses. The curves were drawn as explained in Fig. 2 *C*. The mean $V_{0.5}$ determined from the Boltzmann equation (see legend to Fig. 2) were −55±1 mV ($k = 4±1$) for $I_{Ba,T}$ and −15±2 mV ($k = 10±2$) for $I_{Ba,L}$.

and the pharmacological properties of the LVA I_{Ba} recorded in cultured human coronary myocytes were consistent with those of Ca^{2+}-channel currents classically referred to as T-type ($I_{Ba,T}$) currents in vascular and nonvascular cells (3, 4, 6, 7, 14, 15, 19, 22, 23). In contrast to $I_{Ba,L}$ present in nearly all cultured cells investigated (72 out of 77), $I_{Ba,T}$ was found in only 25% of these cells and always in association with $I_{Ba,L}$. The absence of $I_{Ba,T}$ in freshly isolated cells was unrelated to the enzymatic treatment because $I_{Ba,T}$ was still observed in cultured cells exposed to the same enzymatic procedure (data not shown). It is also worth noting that we detected no significant difference in the nature, properties, and regulation (see below) of both $I_{Ba,L}$ and $I_{Ba,T}$ between cells obtained from patients with dilated cardiomyopathy and cells obtained from patients with an ischemic disease.

Regulation by cGMP and NO donors. The intracellular messenger cGMP has been shown to regulate Ca^{2+} currents in some vascular smooth muscle cells from animals (4). We investigated whether or not cGMP regulate Ca^{2+} currents in human coronary myocytes using permeable analogues. Fig. 6 *A* illustrates the inhibitory effect of 10 μM db-cGMP on $I_{Ba,L}$. The averaged inhibition occurred at all test potentials (Fig. 6 *B*). The inhibition was partially reversible upon washout of the drug (data not shown). Similar effects were observed on $I_{Ba,L}$ of cultured myocytes. The IC_{50} determined from the dose–effect curves was 0.8±0.2 μM (Fig. 6 *C*). Similar inhibitory effects were observed using 10 μM 8-bromo cGMP (42±8% decrease; $n = 4$). In contrast, at 100 μM, which is maximally effective for $I_{Ba,L}$, db-cGMP had no effect on $I_{Ba,T}$ in all cells tested ($n = 6$).

NO is a major endothelium-derived relaxing factor (4, 7, 25–30). It is a potent vasodilating agent which contributes to local regulation of vascular smooth muscle tone by modulation of several cellular processes. The principal physiological actions of NO are mediated by activation of a cytosolic guanylate cyclase and subsequent formation of cGMP (4, 7, 31, 32). A possible action of NO on voltage-gated Ca^{2+} channels has also been suggested previously (4, 5, 33, 34). Therefore, we have studied the effect of NO both on intracellular cGMP and Ca^{2+}-channel currents of human coronary myocytes using two different NO donors: SNAP and SIN-1, which spontaneously release NO under physiological conditions and are potent vasodilator in vitro and in vivo (26, 35, 36). When applied at 10 μM, SNAP induced (*a*) a rise in intracellular cGMP concentration of coronary myocytes (Fig. 7 *A*) and (*b*) a substantial decrease of $I_{Ba,L}$ either on freshly isolated myocytes (Fig. 7 *B*; mean inhibition 33±12%; $n = 6$) or on cultured cells (30±15%; $n = 5$). The decrease was observed at all test potentials (Fig. 7 *C*) and was reversible upon washout of the drug (Fig. 7 *D*). An average of 60±12% ($n = 5$) inhibition was reversed after 5 min of washout, suggesting that most of the decrease of $I_{Ba,L}$ was related to the presence of the NO donor and does not reflect spontaneous rundown of Ca^{2+} channel activity during the experiment. The effect of SNAP, which was not significantly different from that of 10 μM db-cGMP, could be prevented by the presence of methylene blue (Fig. 7 *E*) known to inhibit nonspecifically guanylate cyclase activity (31, 32, 37). Moreover, a saturating concentration db-cGMP (10 μM) occluded the effect of 10 μM SNAP in all cells tested ($n = 4$), suggesting that the effects of the two agents are not additive and are likely to occur through a common pathway. It should be noted that a similar inhibition of $I_{Ba,L}$ was obtained using SIN-1 (29±12% inhibition at 10 μM, $n = 4$; see Fig. 7 *E*). It is also worth noting that, in contrast to their effect on $I_{Ba,L}$, SIN-1 and SNAP (at 10 μM) had no effect on $I_{Ba,T}$ in any of the cultured cells tested ($n = 7$).

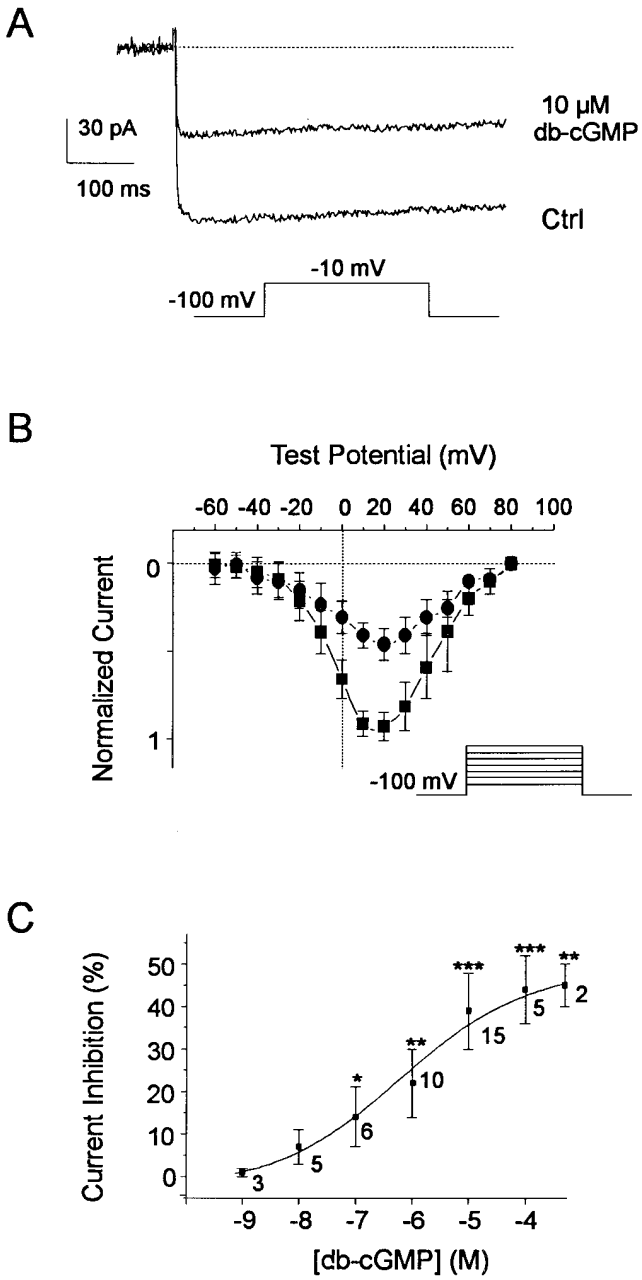

Figure 6. Inhibition of $I_{Ba,L}$ by db-cGMP. (*A*) Maximal inhibition (after 3 min of exposure) of $I_{Ba,L}$ by 10 μM db-cGMP in a freshly isolated human coronary myocyte. $I_{Ba,L}$ was evoked as indicated. (*B*) Mean (±SD; nine cells) normalized I-V relationships of $I_{Ba,L}$ recorded in the absence (*filled boxes*) and presence of db-cGMP (*filled circles*). (*C*) Averaged (±SD) dose–response curve of the inhibition of I_{Ba} by db-cGMP. The number of cells is quoted for each point. The IC_{50} determined after fitting the curve according to Hill equation (see legend Fig. 3) is 1.2±0.5 μM (nH = 0.5±0.1).

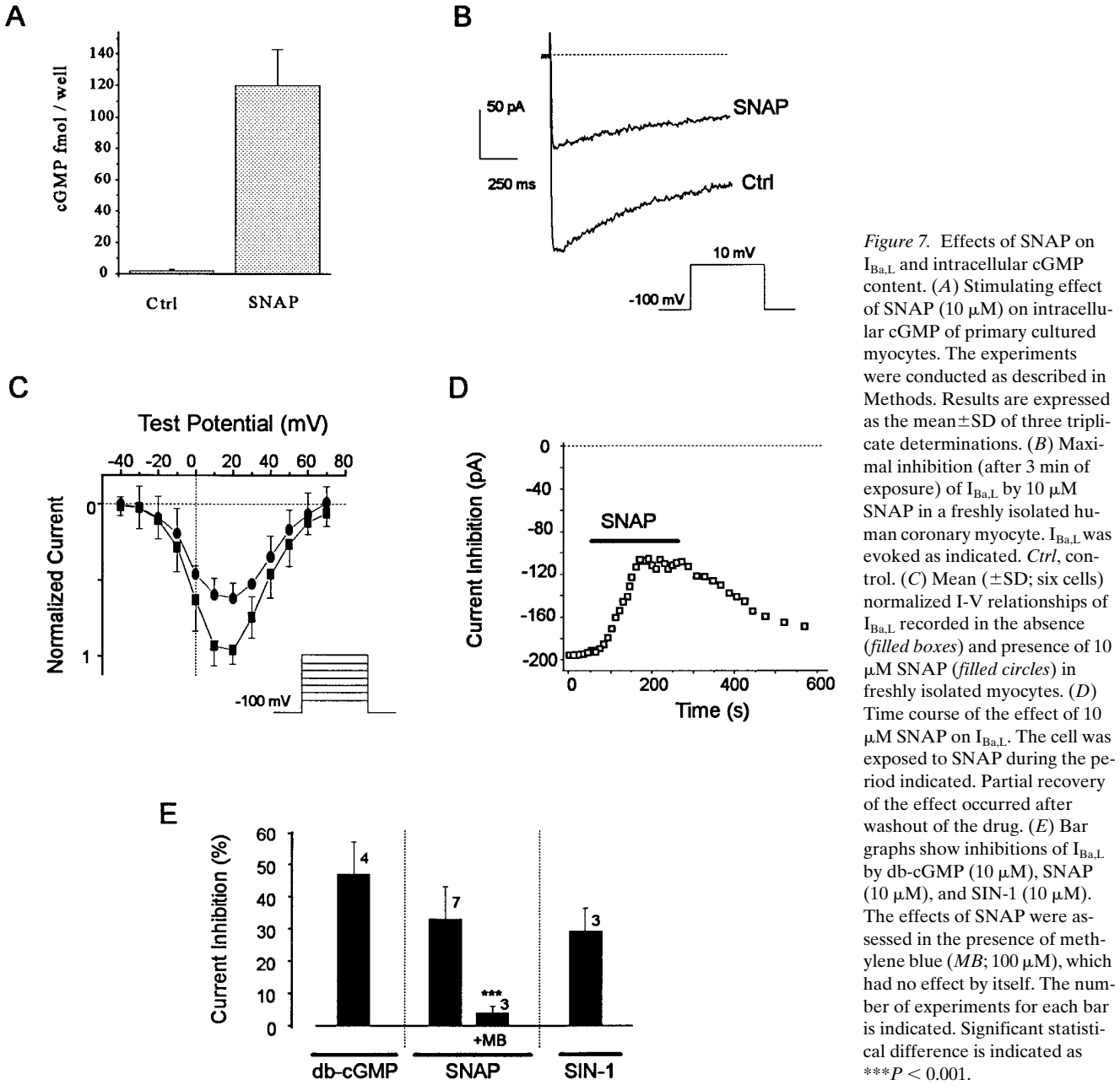

Figure 7. Effects of SNAP on $I_{Ba,L}$ and intracellular cGMP content. (*A*) Stimulating effect of SNAP (10 μM) on intracellular cGMP of primary cultured myocytes. The experiments were conducted as described in Methods. Results are expressed as the mean±SD of three triplicate determinations. (*B*) Maximal inhibition (after 3 min of exposure) of $I_{Ba,L}$ by 10 μM SNAP in a freshly isolated human coronary myocyte. $I_{Ba,L}$ was evoked as indicated. *Ctrl*, control. (*C*) Mean (±SD; six cells) normalized I-V relationships of $I_{Ba,L}$ recorded in the absence (*filled boxes*) and presence of 10 μM SNAP (*filled circles*) in freshly isolated myocytes. (*D*) Time course of the effect of 10 μM SNAP on $I_{Ba,L}$. The cell was exposed to SNAP during the period indicated. Partial recovery of the effect occurred after washout of the drug. (*E*) Bar graphs show inhibitions of $I_{Ba,L}$ by db-cGMP (10 μM), SNAP (10 μM), and SIN-1 (10 μM). The effects of SNAP were assessed in the presence of methylene blue (*MB*; 100 μM), which had no effect by itself. The number of experiments for each bar is indicated. Significant statistical difference is indicated as $***P < 0.001$.

Discussion

To our knowledge, this paper provides the first characterization of voltage-gated I_{Ca} in human coronary myocytes. Since species- and tissue-dependent variations are possible, it was of great interest to determine the nature and regulatory profile of Ca^{2+} channels in humans. There are four principal findings.

First, a DHP-sensitive L-type Ca^{2+} current ($I_{Ca,L}$) is the major pathway for voltage-gated Ca^{2+} entry in human coronary myocytes. This is consistent with findings in various animal species (7–14). The human $I_{Ca,L}$ resembles the $I_{Ca,L}$ described in detail in rabbit cells (9). It activates and peaks at similar voltages (~ -20 mV and +20 mV, respectively) when compared at equivalent concentrations of divalent cation (or after correction). The potential at which half of the channels are inactivated (~ -20 mV), the relative permeability to Ca^{2+} versus Ba^{2+} of those channels ($I_{Ca}/I_{Ba} < 0.3$), and the slow kinetics of $I_{Ca,L}$ are also similar (9). The lack of changes in kinetics of current decay when extracellular Ba^{2+} is replaced by Ca^{2+} indicates that, as in rabbit coronary cells where the time constants of inactivation of $I_{Ca,L}$ and $I_{Ba,L}$ differ by less than a factor of two (9), inactivation of the human coronary Ca^{2+} channels is not markedly Ca^{2+} dependent. The sustained Ca^{2+} entry resulting from slow inactivation of $I_{Ca,L}$, together with the fact that Ca^{2+} channels in arteries respond primarily to graded changes in membrane potential after agonist activation by hormones and neurotransmitters [rather than to action potentials as in cardiac cells (7, 38, 39)], is consistent with the slow development of vascular tone in physiological conditions.

Although many studies refer to DHP-sensitive L-type Ca^{2+} channels without making distinction between cardiac and vascular channels, we observed quite important differences. For

example, the ratio I_{Ca}/I_{Ba} is much higher (> 0.6) in cardiomyocytes (6, 40, 41). The cardiac $I_{Ca,L}$ also exhibit much faster decay kinetics (18, 40–42) with the time constants of inactivation of $I_{Ca,L}$ and $I_{Ba,L}$ differing by $>$ 10-fold (9). The presence of a marked Ca^{2+}-dependent inactivation (6, 40), may, at least in part, explain the difference. Different kinetics may reflect structural differences between L-type Ca^{2+} channels. The cardiac and vascular pore forming subunits (α_1C), which carry the DHP-binding site, are splice variants from the same gene resulting in 93% homology at the protein level (43). Another degree of diversity may be introduced by differences in the auxiliary subunits that make up the complete channel protein (4, 6, 7). For example, there are several isoforms of the β subunit considered as an endogenous modulator of I_{Ca} amplitude and kinetics. The possibility for opposite regulations of the coronary (decrease: present results) and the cardiac (increase: reference 44) human L-type I_{Ca} by NO confirms that they have quite distinct physiology. Concerted antinomic regulations are of great interest for the cardiovascular physiology (increased cardiac contraction; relaxation of the artery).

Second, the selective inhibitory effect of DHP antagonists for the coronary over the cardiac L-type Ca^{2+} channel in vivo, which determines their clinical profile (4, 6, 16, 17), can be accounted for mainly by the modulation of DHP blockade by membrane potential. Our direct measurements of Ca^{2+}-channel activity demonstrate that the sensitivity of the human coronary L-type I_{Ca} to DHP antagonists (and diltiazem as well) is similar to that of its counterpart in cardiac cells investigated at similar negative HP (6, 45). This property, which reflects modulation of DHP binding by voltage, is consistent with the general picture that the resting membrane potential (more depolarized by 20–30 mV in the coronary artery; 6, 7, 18, 46) is an important determinant of the vascular selectivity of DHPs. However, it may not be a unique factor. Differences in channel structure have also been suggested (47, 48).

Third, T-type Ca^{2+} currents are absent in freshly isolated human coronary myocytes. This is in agreement with findings in rabbit, bovine, and porcine studies (8–13) but contrasts with studies in guinea pig (14, 15). The difference may be related to size (resistance) of the vessels or may be purely species dependent. It is worth noting that the T-type I_{Ca} is also well-represented in guinea pig, but not in human cardiomyocytes (6, 18, 42). The T-type Ca^{2+} channel does not seem to be a major pathway for voltage-gated Ca^{2+} entry in the human coronary artery and is thus probably not involved in regulation of the contraction. Therefore, the L-type channel is probably the target for the therapeutically active vasodilating agents. In contrast, the presence of T-type I_{Ca} in cultured myocytes is of major interest because it may be related to dedifferentiation and proliferative activity of the cells in vitro as recently demonstrated for adult rat aortic myocytes (19, 22, 23). Indeed, proliferative disorders play a major role in coronary artery disease (2). Although there is here matter for interesting speculations concerning the possible role(s) of T-type I_{Ca} in coronary physiology, it remains to be seen whether or not T-type I_{Ca} can also be expressed in vivo.

The fourth finding of this study in human coronary myocytes provides clear evidence that NO- and cGMP-generating agents can decrease Ca^{2+} influx through voltage-gated Ca^{2+} channels. This partial ($< 50\%$) inhibition is selective, i.e., cGMP and NO inhibit only $I_{Ca,L}$ (T-type Ca^{2+} current is not targeted). The regulation by cGMP resembles that described in rabbit pulmonary artery and in portal vein and rat ventricular cells (4, 49–52). Our results suggest that the effect of NO on Ca^{2+}-channel currents of human coronary myocytes is mediated through a rise in intracellular cGMP. Therefore, one would expect that factors stimulating either NO production (e.g., neurotransmitters and circulating agents such as acetylcholine, arginine vasopressin, bradykinin; products of platelet aggregation such as ADP, ATP, serotonin, thrombin, and PAF; shear stress; hyperemia) or directly intracellular cGMP formation (e.g., atrial natriuretic peptide) inhibit human coronary L-type Ca^{2+} channels.

The endothelium and autonomic nerves provide the major factors regulating intracellular Ca^{2+} and, thereby, determine the contractile tone in arteries. Among endothelium-derived factors, the vasorelaxing messenger NO exerts multiple effects including extrusion of intracellular Ca^{2+}, decreased Ca^{2+} sensitivity of the contractile proteins, increased cAMP via inhibition of phosphodiesterase, and membrane hyperpolarization due to activation of K^+ channels (4, 30, 34, 37, 53–55). The endothelium-derived relaxing factor NO can cause hyperpolarization in many arteries, including guinea pig coronary artery (37). This effect, which involves direct or PKG-mediated activation of K^+ channels (53, 54), ends up into relaxation by deactivating (and closing) voltage-gated Ca^{2+} channels. However, NO-induced relaxation can also occur without detectable hyperpolarization (37). The direct inhibition of $I_{Ca,L}$ evidenced here in human coronary myocytes is consistent with this hyperpolarization-independent effect of NO. Partial inhibition ($< 50\%$) by saturating concentrations of cGMP (see also 49) and by NO suggests that Ca^{2+} channel activity is tonically inhibited by the endothelium. This system would provide the coronary artery with a highly sophisticated system for a fine and localized modulation of its basal contractile tone. Pharmacologically, this regulation could be, in part, involved in the vasodilating effects of nitrovasodilators used clinically.

In conclusion, to our knowledge, this is the first report on the characterization of Ca^{2+} currents in human coronary myocytes. Overall, our study establishes a basis for future studies concerning their regulation by many endogenous and exogenous agents, including drugs, targeting contractility and proliferation. Both contractile and proliferative abnormalities play a major part in coronary artery diseases (hypertension, atherosclerosis, myointimal proliferation after postangioplasty restenosis). Primary cultured coronary cells may be a useful in vitro system to understand also the possible role of Ca^{2+} channels in the mechanisms regulating cell replication (e.g., T-type: 19, 22) and the molecular basis of phenotypic modulation of Ca^{2+} channels which may occur during coronary diseases.

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References

1. Filo, R.S., D.F. Bohr, and J.C. Rüegg. 1965. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science (Wash. DC).* 147: 1581–1583.

2. Somlyo, A.P., and A.V. Somlyo. 1994. Signal transduction and regulation

in smooth muscle. *Nature (Lond.).* 372:231–236.

3. Bolton, T.B., and P. Pacaud. 1985. Voltage-dependent calcium channels of smooth muscle. *Proc. Natl. Acad. Sci. USA.* 81:6388–6392.

4. Gurney, A.M., and L.H. Clapp. 1994. Calcium channels and vasodilation. *Adv. Mol. Cell. Biol.* 8:21–41.

5. Cohen, R.A. 1993. Pathways controlling healthy and diseased arterial smooth muscle. *Am. J. Cardiol.* 42:39C–47C.

6. McDonald, T.F., S. Pelzer, W. Trautwein, and D. Pelzer. 1994. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.* 74:365–507.

7. Kuriyama, H., K. Kitamura, and H. Nabata. 1995. Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissues. *Pharmacol. Rev.* 47:387–514.

8. Klöckner, U., and G. Isenberg. 1985. Whole cell calcium channel currents of smooth muscle cells isolated from bovine coronary arteries: effects of nifedipine and Bay-K 8644. *Eur. J. Physiol.* 403:R22.

9. Matsuda, J.J., K.A. Volk, and E.F. Shibata. 1990. Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. *J. Physiol.* 427:657–680.

10. Ishikawa, T., J.R. Hume, and K.D. Keef. 1993. Modulation of K^+ and Ca^{2+} channels by histamine H1-receptor stimulation in rabbit coronary artery cells. *J. Physiol.* 468:379–400.

11. Lamb, F.S., K.A. Volk, and E.F. Shibata. 1994. Calcium-activated chloride current in rabbit coronary myocytes. *Circ. Res.* 75:742–750.

12. Goto, K., Y. Kasuya, N. Matsuki, Y. Tokuwa, H. Kurihara, T. Ishikawa, S. Kimura, M. Yanagisawa, and T. Masaki. 1989. Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA.* 86:3915–3918.

13. Klöckner, U., and G. Isenberg. 1991. Myocytes isolated from porcine coronary arteries: reduction of currents through L-type Ca-channels by verapamil-type Ca antagonists. *J. Physiol. Pharmacol.* 42:163–179.

14. Ganitkevich, V.Y., and G. Isenberg. 1990. Contribution of two types of calcium channels to membrane conductance of single myocytes from guinea-pig coronary artery. *J. Physiol.* 426:19–42.

15. Ganitkevich, V.Y., and G. Isenberg. 1991. Stimulation-induced potentiation of T-type calcium channel currents in myocytes from guinea-pig coronary artery. *J. Physiol.* 443:703–725.

16. Godfraind, T., and R.C. Miller. 1983. Pharmacology of coronary arteries. *In* Factors Influencing the Course of Myocardial Ischemia. Chapter 7. DeBakey and Gotto, editors. Elsevier Science Publishers, BV. 101–119.

17. Fleckenstein, A. 1990. History and prospects in calcium antagonist research. *J. Mol. Cell. Cardiol.* 22:241–251.

18. Coraboeuf, E., and J. Nargeot. 1993. Electrophysiology of human cardiac cells. *Cardiovasc. Res.* 27:1713–1725.

19. Neveu, D., J.F. Quignard, A. Fernandez, S. Richard, and J. Nargeot. 1994. Differential β-adrenergic regulation and phenotypic modulation of voltage-gated calcium currents in rat aortic myocytes. *J. Physiol.* 479:171–182.

20. Hanin, V., H. Naharisoa, V. Sarrade, and B. Calas. 1989. Production of oligoclonal antibodies directed to the N-terminal of smooth alpha actin using peptidyl-polyacrylic resins as direct immunogens. *Pept. Res.* 2:367–372.

21. Chamley, J.H., G.R. Campbell, and J.D. McConnell. 1977. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. *Cell Tissue Res.* 177:503–522.

22. Richard, S., D. Neveu, G. Carnac, P. Bodin, P. Travo, and J. Nargeot. 1992. Differential expression of voltage-gated Ca^{2+}-currents in cultivated aortic myocytes. *Biochim. Biophys. Acta.* 1160:95–104.

23. Akaike, N., H. Kanaide, T. Kuga, M. Nakamura, J. Sadoshima, and J. Tomoike. 1989. Low-voltage-activated calcium current in rat aorta smooth muscle cells in primary culture. *J. Physiol.* 416:141–160.

24. Marin, P., J.F. Quignard, M. Lafon-cazal, and J. Bockaert. 1993. Non-classical glutamate receptors, blocked by both NMDA and non NMDA antagonists, stimulate nitric oxide production in neurons. *Neuropharmacology.* 32:29–36.

25. Furchgott, R.F., and J.V. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond.).* 288:373–376.

26. Ignarro, L.J., R.E. Byrns, G.M. Buga, and K.S. Wood. 1987. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacological and chemical properties identical to those of NO radical. *Circ. Res.* 61: 866–879.

27. Lüscher, T.F., and P.M. Vanhoutte. 1990. The Endothelium: Modulator of Cardiovascular Function. CRC Press, Inc., Boca Raton, FL. 1–228.

28. Lüscher, T.F., V. Richard, and F.C. Tanner. 1991. Endothelium-derived vasoactive factors and their role in the coronary circulation. *Trends Cardiovasc. Med.* 1:179–185.

29. Cohen, R.A., and P.M. Vanhoutte. 1995. Endothelium-dependent hyperpolarization. *Circulation.* 92:3337–3349.

30. Umans, J.G. 1995. Nitric oxide in the regulation of blood flow and arterial pressure. *Annu. Rev. Physiol.* 57:771–790.

31. Feelisch, M., and E.A. Noack. 1987. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* 139:19–30.

32. Gruetter, C.A., D.Y. Gruetter, J.E. Lyon, P.J. Kadowitz, and L.J. Ignarro. 1981. Relationship between cyclic guanosine 3′:5′-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. *J. Pharmacol. Exp. Ther.* 219:181–186.

33. Collins, P., T.M. Griffith, A.H. Henderson, and M.J. Lewis. 1986. Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: a cyclic guanosine-monophosphate-mediated effect. *J. Physiol.* 381:427–437.

34. Kai, H., H. Kanaide, T. Matsumoto, and M. Nakamure. 1987. 8-bromo 3′:5′-cyclic monophosphate decreases intracellular free calcium concentrations in cultured vascular smooth muscle cells from rat aorta. *FEBS Lett.* 221:284–288.

35. Kowaluk, E.A., R. Poliszczuk, and H.L. Fung. 1987. Tolerance to relaxation in rat aorta: comparison of an *S*-nitrosothiol with nitroglycerin. *Eur. J. Pharmacol.* 144:379–383.

36. Henry, P.J., J.D. Horowitz, and W.J. Louis. 1989. Nitroglycerin-induced tolerance affects multiple sites in the organic nitrate bioconversion cascade. *J. Pharmacol. Exp. Ther.* 248:762–768.

37. Tare, M., H.C. Parkington, H.A. Coleman, T.O. Neild, and G.J. Dusting. 1990. Hyperpolarisation and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature (Lond.).* 346:69–71.

38. Droogmans, G., L. Reymaekers, and R. Casteels. 1977. Electro- and pharmaco-mechanical coupling in the smooth muscle cells of rabbit ear arteries. *J. Physiol.* 426:19–42.

39. Hirst, G.D.S., and F.R. Edwards. 1989. Sympathetic neuroeffector transmission in arteries and arterioles. *Physiol. Rev.* 69:546–604.

40. Lee, K.S., E. Marban, and R.W. Tsien. 1985. Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *J. Physiol.* 364:395–411.

41. Tiaho, F., C. Piot, J. Nargeot, and S. Richard. 1994. Regulation of the frequency-dependent facilitation of L-type Ca^{2+} currents in rat ventricular myocytes. *J. Physiol.* 477:237–252.

42. Piot, C., S. Lemaire, B. Albat, J. Séguin, J. Nargeot, and S. Richard. 1996. High frequency-induced upregulation of human cardiac calcium currents. *Circulation.* 93:120–128.

43. Koch, W., P.T. Ellinor, and A. Schwartz. 1990. cDNA cloning of a dihydropyridine-sensitive calcium channel from rat aorta. Evidence for the existence of alternatively spliced forms. *J. Biol. Chem.* 29:17786–17791.

44. Kirstein, M., M. Rivet-Bastide, S. Hatem, A. Benardeau, J.J. Mercadier, and R. Fischmeister. 1995. Nitric oxide regulates the calcium current in isolated human atrial myocytes. *J. Clin. Invest.* 95:794–802.

45. Diochot, S., S. Richard, M. Baldy-Moulinier, J. Nargeot, and J. Valmier. 1995. Dihydropyridines, phenylalkylamines and benzothiazepines block N-, P/Q- and R-type calcium currents. *Pflügers Arch.* 431:10–19.

46. Nakashima, M., J.V. Mombouli, A.A. Taylor, and P.M. Vanhoutte. 1993. Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J. Clin. Invest.* 92:2867–2871.

47. Welling, A., Y.W. Kwan, E. Bosse, V. Flockerzi, F. Hofmann, and R.S. Kass. 1993. Subunit-dependent modulation of recombinant L-type calcium channels (molecular basis for dihydropyridine tissue selectivity). *Circ. Res.* 73: 974–980.

48. Soldatov, N.M., A. Bouron, and H. Reuter. 1995. Different voltage-dependent inhibition by dihydropyridines of human Ca^{2+} channel splice variants. *J. Biol. Chem.* 270:10540–10543.

49. Clapp, L.H., and A.M. Gurney. 1991. Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. *Pflügers Arch.* 418:462–470.

50. Ishikawa, T., J.R. Hume, and K.D. Keef. 1993. Regulation of Ca^{2+} channels by cAMP and cGMP in vascular smooth muscle cells. *Circ. Res.* 73:1128–1137.

51. Mery, P.F., S.M. Lohmann, U. Walter, and R. Fischmeister. 1991. Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* 88:1197–1201.

52. Sumii, K., and N. Sperelakis. 1995. cGMP-dependent protein kinase regulation of the L-type Ca^{2+} current in rat ventricular myocytes. *Circ. Res.* 77: 803–812.

53. Lincoln, T.M., B. Pryzwanski, T.L. Cornwell, T.A. Wyatt, and L.A. MacMillan. 1993. Cyclic-GMP-dependent protein kinase in smooth muscle and neutrophils. *In* Advances in Second Messenger and Phosphoprotein Research. Vol. 28, Chapter 15. Raven Press, Ltd., New York. 121–132.

54. Taniguchi, J., K.I. Furukawa, and M. Shigekawa. 1993. Maxi K^+ channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscle cells. *Pflügers Arch.* 423:167–172.

55. Bolotina, V.M., S. Najibi, J.J. Palacino, P.J. Pagano, and R.A. Cohen. 1994. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature (Lond.).* 368:850–853.