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MYOCYTES ISOLATED FROM PORCINE CORONARY ARTERIES: REDUCTION OF CURRENTS THROUGH L-TYPE Ca-CHANNELS BY VERAPAMIL-TYPE Ca-ANTAGONISTS

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Myocytes were enzymatically isolated from large epicardial arteries of the pig. In the cell attached configuration, we studied currents through L-type Ca-channels. At 22°C, open channel conductance was 9 pS with 110 mM Ca^{2+} and 24 pS with 110 mM Ba^{2+} as charge carrier. According to the life time of the open state, 2 'modes' of gating are distinguished; mode 1 contributed time constants shorter than 1 ms, mode 2 those longer than 6 ms to the open time distribution. Mode 2 openings appeared spontaneously, more frequently with Ba^{2+} than with Ca^{2+} as charge carrier. The Ca-agonist Bay K 8644 (0.5 μM) facilitated the appearance of mode 2. Bath application of the phenylalkylamine D 600 (1 μM) did not change the gating modes, but it reduced the channel openness by increasing the percentage of blank records.

With whole cell recordings, we studied reduction of I_{Ca} by 1 μM D 600 at 3.6 mM $[Ca^{2+}]$ and 35°C. At a holding potential of —45 mV, D 600 induced an 'initial block' of 35% (10% at —65 mV). Upon repetitive 1 Hz pulsing (170 ms to 0 mV) an additional, 'use-dependent' block developed with time. More negative holding potentials attenuated reduction of I_{Ca} by D 600, hyperpolarizations to —100 mV had an 'unblocking' effect.

In regard to reduction of I_{Ca}, we compared the partially uncharged D 600 (membrane permeable) with the completely charged compound D 890 (membrane impermeable). When applied with the bath, 1 or 10 μM D 600 reduced I_{Ca} dose-dependently whereas D 890 was ineffective. When D 890 was applied via the patch electrode to the cytosol, it reduced I_{Ca}. We discuss that D 600 enters the cell in the uncharged lipid soluble form and reaches form the inside its receptor associated with the Ca-channel.

Key words: *Ca-channel; Ca-antagonists; vascular smooth muscle cell; open channel block.*

INTRODUCTION

The entry of Ca into vascular smooth muscle cells is largely through voltage-operated Ca-channels of the L-type (1, 2). This Ca-entry is one of the determinants of Ca-load of the cell, both in the stores (the sarcoplas-

mic reticulum) and the myoplasm. Thus, Ca-influx through Ca-channels is involved in control of contraction and, thereby, the diameter of coronary arteries (3). Ca-channels in coronary myocytes are affected by 'Ca-antagonists', as dihydropyridines (e. g. nifedipine) or phenylalkylamines as verapamil, D600 (gallopamil) and D890. Therefore, Ca-antagonists are used in the treatment of a variety of cardiovascular disorders including angina (4). But, only recently it has been possible to measure directly the action of Ca-antagonists on Ca-channels (5, 6, 1, 7, 8, 9).

Vascular Ca-channels, like Ca-channels in other tissues (10, 11), exist in at least three functional states: resting, open and inactivated. The open state can be easily reached from the resting state by membrane depolarization, but it cannot be readily reached from inactivated state which is refractory. The resting and inactivated states are non-conducting forms of the channel. Studies on verapamil-derivatives on Ca-channel currents in cardiac muscle suggested that these drugs bind preferentially to the open state (11). At potentials as negative as —80 mV (e. g. resting potential of heart ventricular cells), Ca-channels reside primarily in the resting state which prevents the drug-channel interaction. This does not hold true for porcine coronary myocytes that have an approximate resting potential of —45 mV. At this potential, a significant fraction of the channels will be in the open state available for the block by D600. Block of Ca-influx at resting potential was suggested to explain the sensitivity of resting tone to verapamil (12).

In this article we study the voltage- and use-dependence reduction of I_{Ca} by D600 and address the question whether the hypothesis of the open channel block is applicable to the coronary myocytes. In addition, we compare the effects of D600 and D890 and obtain evidence for the hypothesis that the drug interacts with the Ca-channel from the cytosolic site, similar as in heart ventricular L-type Ca-channels (13). By means of single-channel analysis we demonstrate that D600 does not change the fast gating-kinetics of this channel (for heart ventricular cells: 11).

METHODS

The vascular myocytes were isolated from large porcine epicardial coronary arteries. The principles of enzymatic cell isolation resembles the one described recently for the urinary bladder (14). Hearts were obtained in the local slaughterhouse. In the laboratory, the arteries were taken out. The muscularis was separated from the adventitia and the intima. At 37°C, chunks of muscular tissue (diameter about 2 mm) were stirred for 6 periods of 5 min each in 25 ml of a Ca-free solution which was composed of 90 mM NaCl, 1.2 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM glucose, 20 mM taurine, 5 mM HEPES, adjusted to pH 7.1 with NaOH. Thereafter, the chunks were incubated in the same Ca-free solution but complemented by 3 g per 1 collagenase (Sigma, St. Louis, C0130)

and 0.5 g per 1 protease (Sigma, St. Louis, P4630). The enzyme treatment delivered only broken material within the first incubation period (30 min) but numerous elongated cells after 60 and 90 min. The cells were harvested in the supernatant and suspended for storage at 4° C in a 'KB-medium' (15) composed of 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 2 mM Na_2ATP, 5 mM Na-pyruvate, 5 mM 5-OH-β-butyrate, 5 mM creatine, 20 mM taurine, fatty acid free albumin 1 g per 1, adjusted with KOH to pH 7.4.

Whole-cell recordings of membrane potential and current were performed at 35° C with a List-EPC7 amplifier (16). For the experiment, a drop of KB-medium containing the cells was pipetted into the experimental chamber (volume 100 µl). When the cells had settled down to the glass bottom, they were continuously superfused with a physiological salt solution (PSS) composed of 150 mM NaCl, 5.4 mM KCl, 3.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES/NaOH (pH 7.4). Patch-electrodes had fire-polished tips to give an inner diameter of about 1 µm and a resistance of about 3 MOhm. The electrodes were filled with a Cs-medium (17) composed of 130 mM CsCl, 5 mM Na-pyruvate, 5 mM Cs-oxalacetate, 5 mM Cs-succinate, 5 mM EGTA, 10 mM HEPES/CsOH (pH 7.4). D 600 or D 890 were added to this solution to give final concentrations between 1 and 100 µM.

Currents through single Ca-channels were recorded in the cell-attached configuration at 22° C. The patch electrodes contained a solution of 110 mM $BaCl_2$ or 110 mM $CaCl_2$, 5.4 mM KCl, 10 mM HEPES/Tris (pH 7.4). The myocyte's membrane potential was adjusted to zero by means of a bath solution composed of 100 mM K-glutamate, 30 mM KCl, 5 mM K-succinate, 5 mM K-oxalacetate, 5 mM creatine, 1 mM EGTA, 10 mM HEPES/KOH (pH 7.4).

A PDP 11/73 computer generated the pulse protocols. For single channel analysis, the records were low pass filtered at 1 kHz and digitized at 5 kHz; records with no channel openings were averaged for subtraction of the capacitive artifact and the linear leakage current. A threshold was set to half of the mean amplitude of the unitary current. Whole cell currents were sampled at 5 kHz and stored for further analysis. Computer playback via a graphical terminal and a line printer generated the figures. The amplitude of whole-cell I_{Ca} was defined as the most negative net current surge.

RESULTS

Elementary currents through L-type Ca-channels

(*Fig. 1*) shows currents through single Ca-channels recorded in the cell attached configuration. The holding potential was −50 mV, and 400 ms long pulses to 0 mV were superimposed at a rate of 0.5 Hz. When the currents were carried by Ca^{2+}-ions, depolarization evoked currents of approx. −0.5 pA amplitude and 0.2 −20 ms duration. At the holding potential of −50 mV, no single channel currents were seen.

One may think that a holding potential of −50 mV is insufficient for complete removal of inactivation, since whole cell I_{Ca} has a $V_{0.5}$ of −40 mV (V_{05} = potential of 50 % inactivation, see 18, 17). In the present case, the high pipette Ca^{2+} concentration (110 mM) screens external surface charges thereby shifting the steady-state inactivation curve of I_{Ca} by

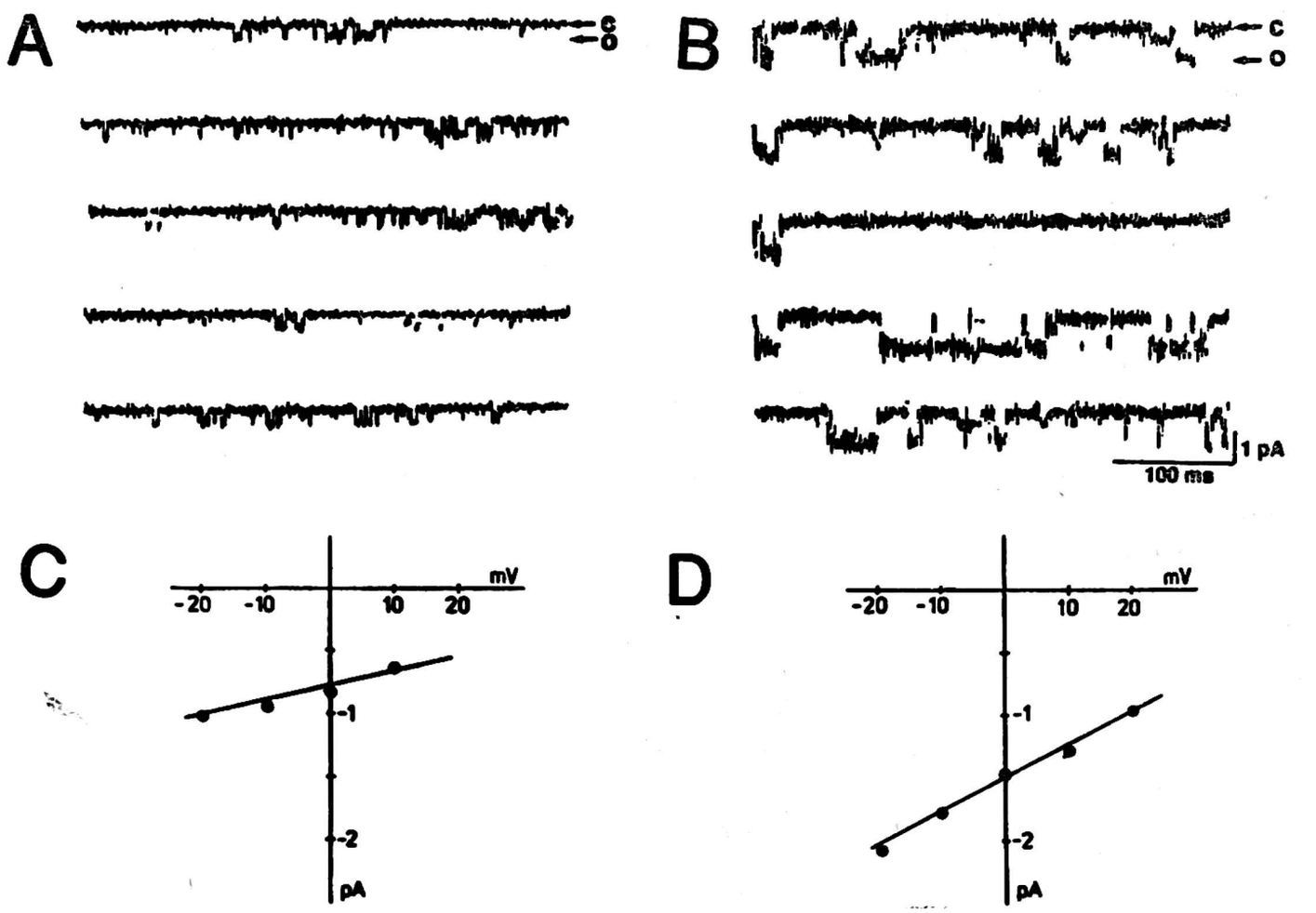

Fig. 1. Elementary currents through single L-type Ca-channels of a coronary myocyte Original records. 400 ms long depolarization from —50 to 0 mV (0.5 Hz). Pipette filled with either 110 mM $CaCl_2$ (**A**) or 110 mM $BaCl_2$ (**B**). Membrane potential 'zeroed' by superfusion of a high-K medium (22°C, 0.5 μM Bay K 8644). Arrow „c": zero current, channel closed. Arrow „o": channel open. **C, D**: Open channel current plotted versus patch potential. Open channel conductance for 110 mM Ca^{2+} is 9±1 pS (C, n = 4) and 24±2 pS (n = 10) for 110 mM Ba^{2+} (D).

about +30 mV (19). In case of 110 mM Ba^{2+} the shift amounts to about +20 mV. Thus, the holding potential of —50 mV is effectively —40 to —30 mV more negative than $V_{0.5}$ of steady state inactivation.

With 110 mM Ca^{2+}, we could measure single channel currents in a potential range between — 25 and +10 mV; at more negative potentials, the probability of channel openings was too small, and at more positive potentials, currents through maxi-K-channels were activated that masked the Ca-channel currents of small amplitude. From the individual records, the amplitude of the single channel current was evaluated with amplitude histograms (compare *Fig. 4A*). When the amplitudes were plotted versus the potential of the test-step, the plot resulted in approximately linear i-v relations (*Fig. 1C*). We estimated from the slope of these plots a single channel conductance of 9 pS±1 pS (mean±S.D., n = 4). This conductance resembles values published for the L-type Ca-channel in other cells (1, 20, 21).

(*Fig. 1B*) shows single channel currents carried by Ba^{2+}-ions. The amplitude of elementary Ba-currents is more than twice as large as the amplitude of Ca-currents. Correspondingly, the single channel conductance for Ba^{2+}-ions is 24 ± 2 pS ($n = 6$). The absolute conductance values, the conductivity ratio $g_{Ba}:g_{Ca}$—2.6:1, as well as the voltage dependence of currents strongly supports the idea that underlying channel is a Ca-channel of the L-type (1). Since larger current amplitude facilitates the data analysis, the following single channel data were recorded with Ba^{2+}-ions as charge carrier.

The kinetics of the currents through L-type Ca-channels

Data of Fig. 1 was taken in the presence of 0.5 μM Bay K 8644. This 'Ca-agonist', in coronary myocytes, increases the probability of the channel being in the open state (open probability P_o) by facilitation of openings with a long lifetime. That is, both in presence and in absence of Bay K 8644, the lifetimes of the open channel fall into two groups. The first group ('mode 1') contains openings with a mean lifetime of about 0.5 ms, the second one ('mode 2') those with a mean lifetime of about 7 ms. Usually, openings of different modes mix only rarely, i. e. we observe that the gating jumps from one to the other mode in such a way that openings of the same mode cluster together. This property resembles the '3-mode gating-model' of L-type Ca-channels that was originally described for heart ventricular cells (10). We like to apply this model also to the porcine coronary myocytes. According to this model mode 0 defines a mode where depolarization fails to open the channel (blanks), mode 1 a gating with short openings and mode 2 the gating with longer openings. To our experience, L-type Ca-channels of coronary myocytes enter the mode 2 also in the absence of Bay K 8644. It is worth noting that we obtained records with mode 2 gating under conditions where the chamber had never been exposed to Bay K 8644 before.

For Fig. 2, the traces with different gating-modes have been selcted and grouped together (10). Panel A shows the original tracings and panel B the corresponding analysis. The distribution of the open times can be fitted with a single exponential with a time constant (mean open time) of 0.5 ms (mode 1) or 7 ms (mode 2), respectively. Usually, the records are not grouped according to the different modes before analysis. Then, the openings with short and long life time superimpose, and the distribution of the open times is bi-exponential with time constants of about 0.6 and 7 ms. Openings of single L-type Ca-channels have been described to occur in bursts that cluster together (11). Since our analysis of closed state was

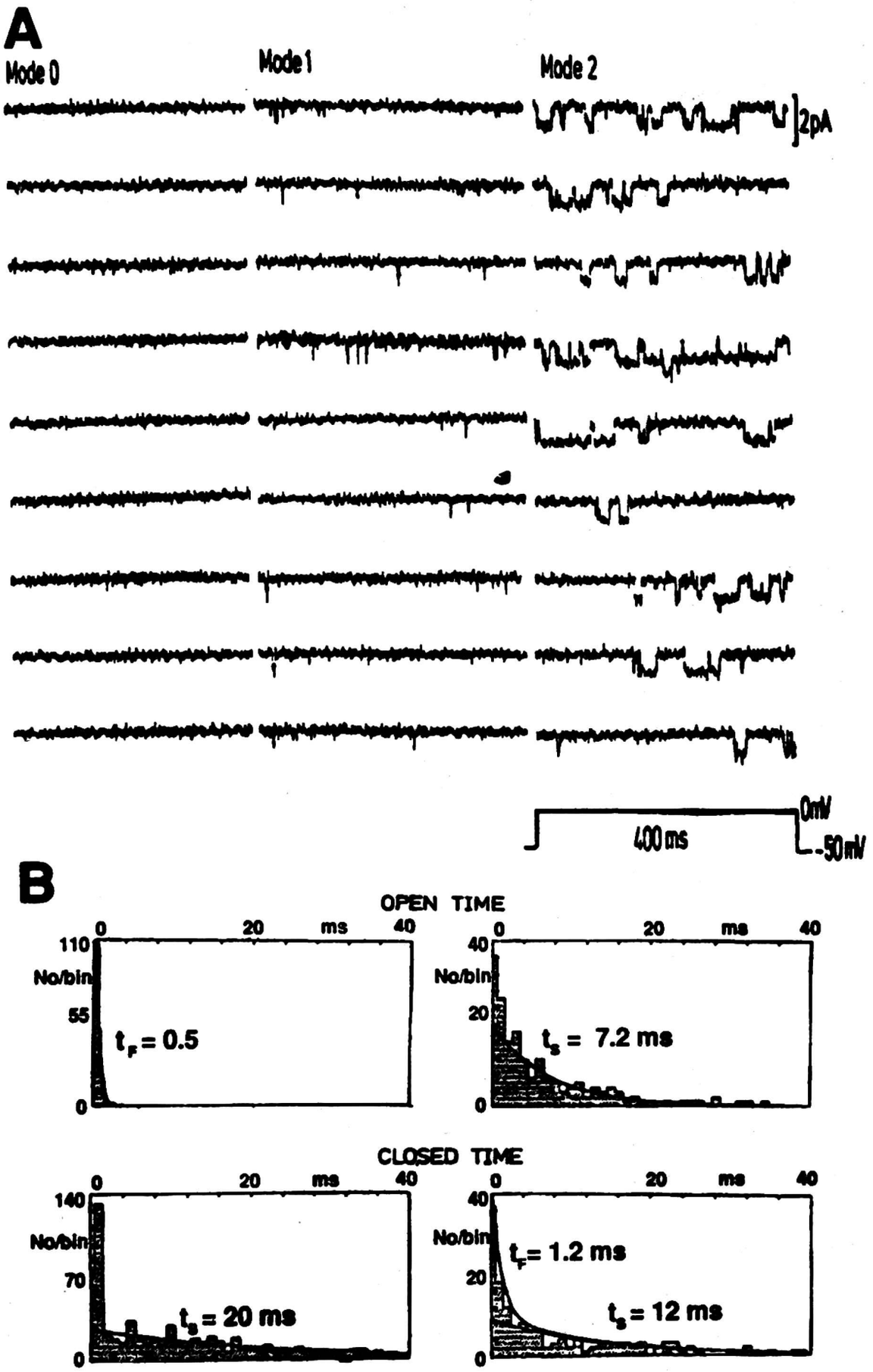

Fig. 2. 3 different modes of Ca-channel gating behaviour. Measurements in absence of Bay K 8644, 110 mM Ba^{2+} as charge carrier. A: Modes from selected sweeps. Mode 0: characterized by blanks. Mode 1: openings appear as spiky downward deflections. Mode 2: openings are long and well resolved. B: Open and closed time histograms for mode 1 (left) and mode 2 (right).

restricted to periods shorter than 40 ms, long 'interburst-intervals' are excluded from the analysis. In mode 1, the closed time histogram was fitted by a single exponential of 20 ms, the initial peak from the first 2 bins being neglected. In mode 2, we obtained 2 exponentials, the long one (12 ms) describing the gaps between the long lasting openings. The short exponential (1.2 ms) shows that some of the long openings are interrupted by short closures.

Bath-application of the Ca-agonist Bay K 8644 (0.5 μM) facilitates the gating-mode 2 (compare Hess et al., 1984, for heart ventricular cells). In the open time histogram (records not selected according to the modes), Bay K 8644 did not modify the lifetime of the open state, i. e. the value of the 2 exponentials remained essentially constant. However, in presence of Bay K 8644 the long openings contributed a larger percentage of the total openings than in the absence of Bay K 8644. In the open-time histogram, the long exponential (7 ms) contributed 6% to the total distribution in absence of Bay K and 22% in presence of Bay K (not illustrated). Both in absence and presence of Bay K 8644, mode-2 gating is recorded more frequently with Ba^{2+} -instead of Ca^{2+}-ions as charge carrier.

The effect of D600 on the single L-type Ca-channel

The effect of 1 μM D600 was tested in the absence of Bay K 8644 and with Ba^{2+}-ions as charge carrier. The comparison of the original tracings (*Fig. 3*) suggests that D600 (1 μM) did not influence the amplitude of the open-channel current. This result is confirmed by the amplitude histogram in (*Fig. 4*), panel A shows that the amplitude of single channel current remained constantly at — 1.1 pA. From i-v plots we evaluated that D600 has no significant effect on the single channel conductance (22±2 pS, see above, versus 21±2 pS, n = 4, in presence of 1 μM D600).

The amplitude-histogram of *Fig. 4* shows that D600 reduces the height of the Gaussian curve to 41% of the control. This reduction suggests that, for the same number of 100 depolarizations, the channel was found less frequently in the open state (open channel current contributing to the histogram) when D600 was present. This reduction of channel openness $N \cdot P_0$ (N = number of channels in the patch, P_0 = open probability), is the most dramatic effect of D600. Reduction of channel openness does not result from a change in the fast gating kinetics; the histographs for the open time (*Fig. 4B*) and the closed time (*Fig. 4C*) are fitted with time constants that are nearly the same before (left) and after (right) addition of the drug. Reduction of channel openness by D600 results from the increase of the number of 'blanks', that is of records in which the depolariz-

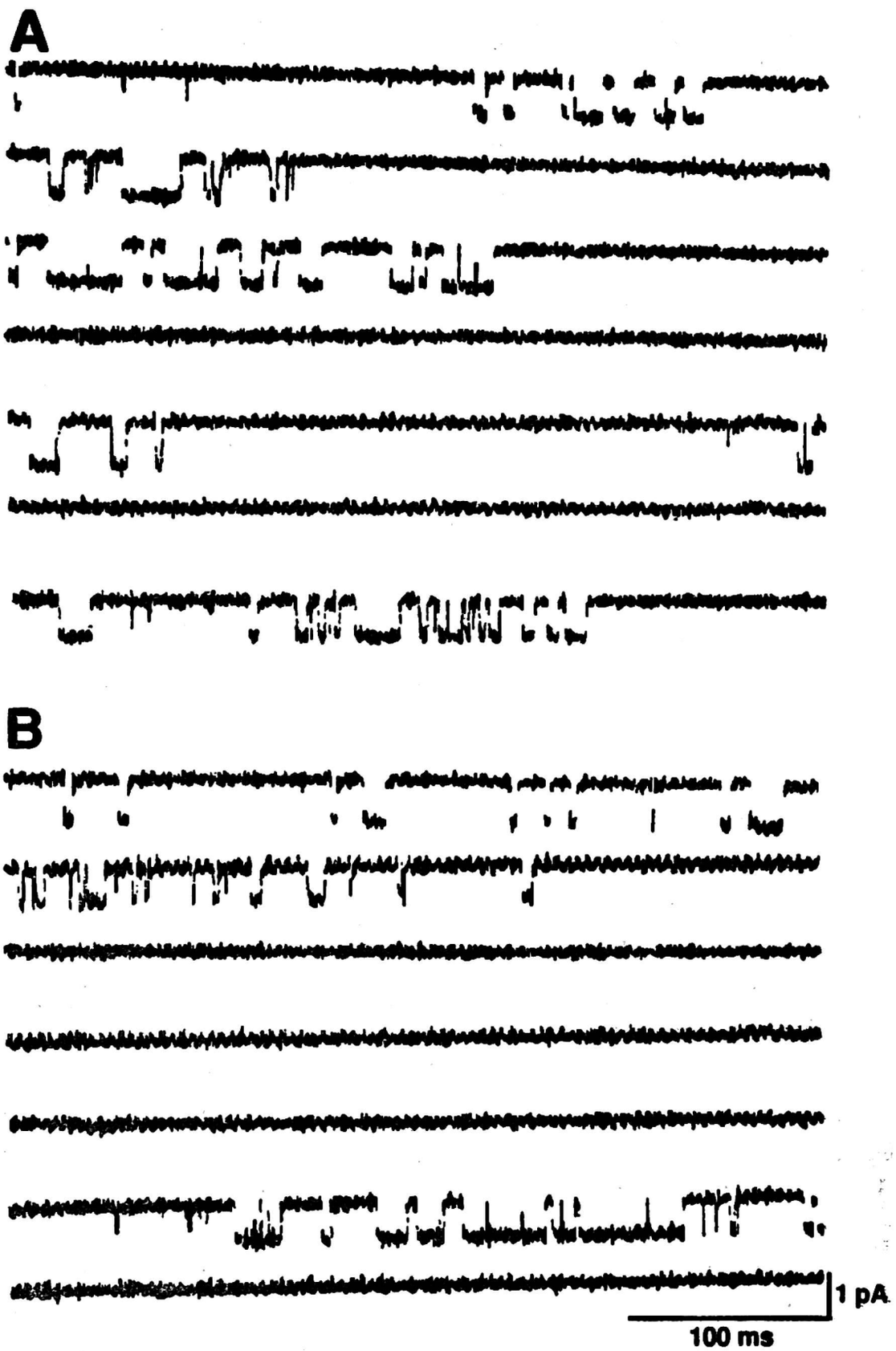

Fig. 3. Effect of D600 on Ba-currents through single Ca-channels, original tracings. 400 ms long steps from —50 to 0 mV (0.5 Hz). **A**: before, **B**: 5 min after bath-application of 1 μM D600.

ation failed to open the channel. The original tracings (*Fig. 3*) show this effect of D600 by a doubling of the blanks. Fig. 3 also shows that the channel, once it has opened, stays open for the same time in the presence and absence of D600, that is, the channel openness per record remained essentially constant. Channel openness, $N \cdot P_0$, is plotted for 100 tracings

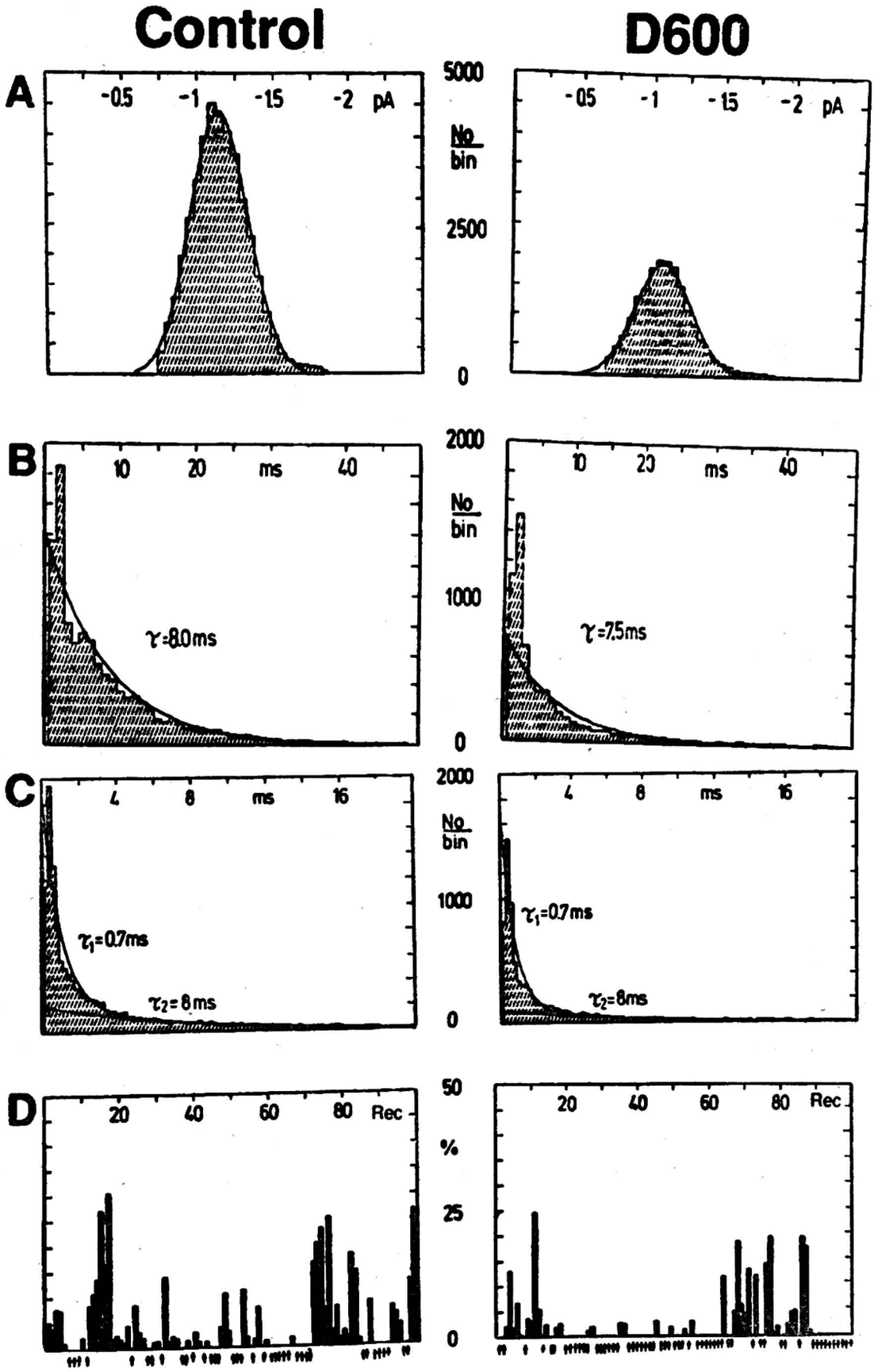

Fig. 4. Effect of D600 on Ba-currents through single Ca-channels, statistical analysis in absence (left) and presence of 1 μM D600 (right). **A**: Amplitude histogram fitted with a Gaussian curve (mean —1.1 pA, σ = 0.1 pA). **B**: Open time histograms, mode 2 gating is suggested by the monoexponential fit with a time constant of 8 ms. **C**: Closed time histograms; the 2 exponentials are attributed to two closed, available states **D**: Channel openness histograms: Channel openness defined as the fraction of period of depolarization during wich the channel is in the open state. Blanks are marked by arrows.

in *Fig. 4D*. In comparing the histogram before (left) and after exposure to D600, we find that D600 increased the number of blanks from 39% to 59% (blanks marked by arrow). It also shows that in case of non-blank records, the openness per record is essentially constant.

Voltage- and use-dependent effects of D600 on whole-cell I_{Ca}

Modulation of whole-cell I_{Ca} by D600 was studied under more physiological conditions, i. e. in cells superfused with a PSS containing 3.6 mM $CaCl_2$ and pre-warmed to 35°C. In heart ventricular preparations, the Ca-antagonistic effects of D600 have been reported to be voltage- and use-dependent (22, 23). Here, we address the question whether analogue properties can be observed in coronary smooth muscle cells.

Initial block. For a period of 2 min, 1 μM D600 was bath-applied to a myocyte that remained unstimulated, i.e. clamped to a holding potential of −65 mV. After this period of exposure-time, the first depolarization evoked an I_{Ca} whose amplitude amounted to 90% of the one measured under identical conditions without D600. That is, the initial block of I_{Ca} by D600 amounts to 10%. We repeated this type of experiment at a holding potential of −45 mV which is close to the resting potential of the porcine coronary myocytes. At −45 mV, the initial block of I_{Ca} by D600 amounted to 35%.

Voltage dependence of the block. By means of the voltage-clamp, we can artificially hyperpolarize the membrane thereby removing most of the blocking effect of D600. For excample, a 20 s long hyperpolarization to −105 mV unblockes the D600-modified current in such a way that the following test-pulse to −5 mV induces an I_{Ca} that coincides with the I_{Ca} before drug application. Since the hyperpolarization has completely 'unblocked' the channel from the effect of D600, the 'tonic block' by D600 is zero. With less negative potentials, this unblocking effect is less complete (*Fig. 5A*).

Starting from a holding potential of −65 mV, we measured the voltage-dependence of peak I_{Ca} with a series of test-steps in the range between −45 and +50 mV. The peak I_{Ca} was plotted in an i-v curve. D600 reduced the peak I_{Ca} by a constant factor over the whole range of potentials, i. e. the voltage-dependence of activation was not modified by this drug (not illustrated).

Use-dependence of the D600 block. In coronary myocytes, the evaluation of this property is difficult since long periods of pulsing at 1 Hz reduce I_{Ca} in coronary myocytes. I_{Ca} recovers from the depolarization-induced inactivation with a slow time constant of 5—10 s. In addition, I_{Ca} shows

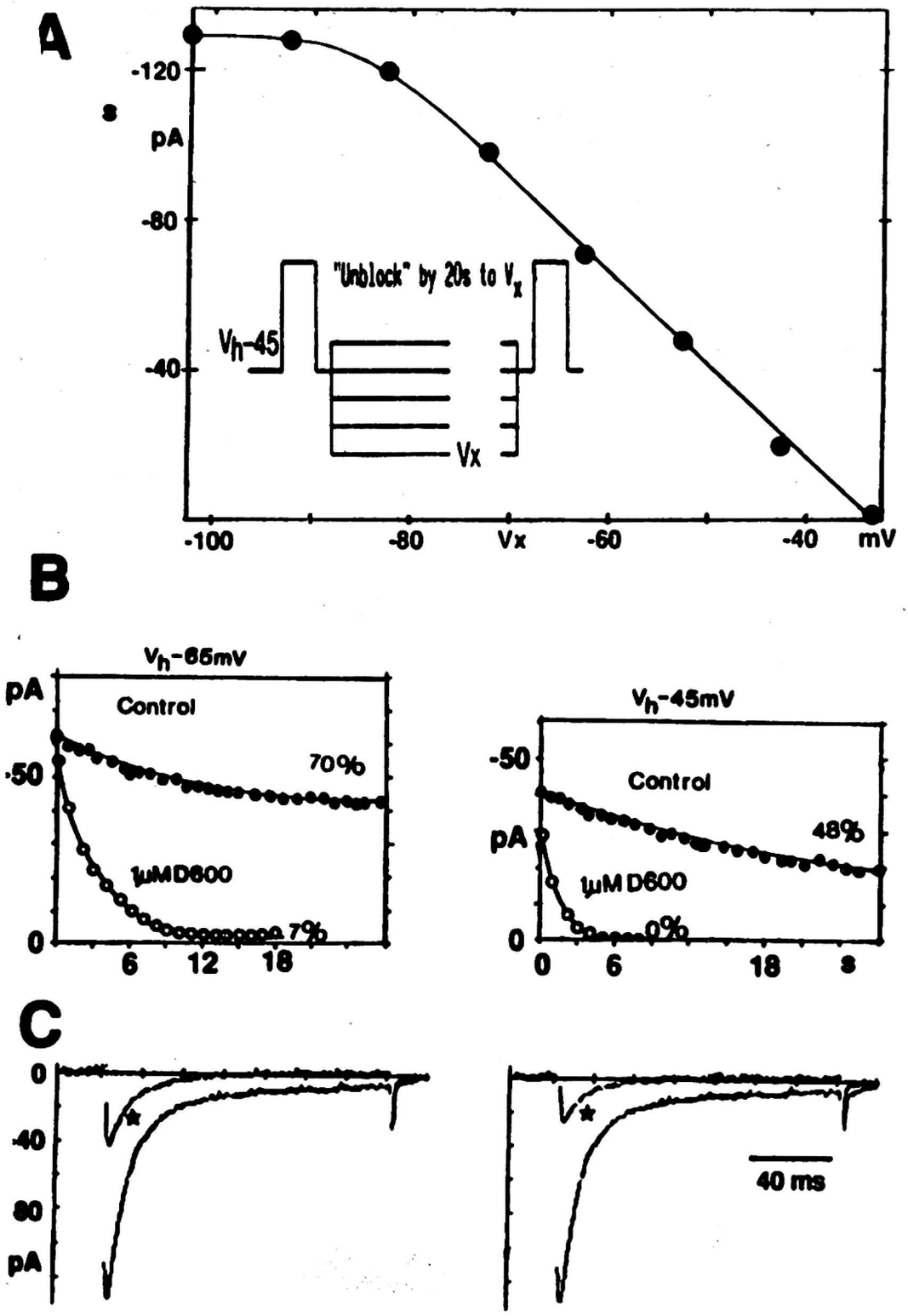

Fig. 5. Reduction of whole-cell I_{Ca} by 1 µM D600, voltage- and use-dependence. **A**: Modulation of the D600-effect by 20 s long holding potentials V_x between —105 and —35 mV. Control and test I_{Ca} were evoked by 170 ms long depolarizations from —45 to —5 mV. **B**: Influence of 1 Hz pulsing (170 ms to —5 mV) on peak I_{Ca}. Holding potential —65 mV (left) or —45 mV (right). Numbers label the ratio of 30th I_{Ca} to 1st I_{Ca} at control (dots) or in presence of D600 (circles). **C**: Test of open channel block: **Left control.** I_{Ca} in regard to a test-step (—65 to —5 mV). Superimposed I_{Ca} (32% amplitude, marked by asterisk) elicited after a 1 min depolarization (—5 mV) and 1 s repolarization (—65 mV) **Right: D600** (0.1 µM), applied 20 s after start of depolarisation to —5 mV. I_{Ca} induced by the following test-step is now reduced to 18% (asterisk).

the 'run-down phenomenon' (9). Therefore, changes of I_{Ca} under the influence of repetitive pulsing alone are discussed first (dots in Fig. 5B). At the holding potential of −65 mV, repetitive pulsing reduced the amplitude of I_{Ca} by 30%, the frequency-dependent reduction reached a steady-state within about one minute. At a holding potential of −45 mV, the 1-Hz pulsing reduced peak I_{Ca} along a faster time course and by a larger extent.

The circles of (*Fig. 5B*) demonstrate the blocking effect of D600 during 1 Hz stimulation. Using a holding potential of −65 mV, peak I_{Ca} fell in a beat-to-beat fashion. After about 12 s the reduction of I_{Ca} was in the steady-state, a 'conditioned block' of 93% was measured. Thus, reduction of I_{Ca} by D600 is largely use-dependent. *Fig. 5B* shows that the conditioned or use-dependent block by D600 is very much stronger than the depression of control I_{Ca} induced by repetitive pulsing alone (compare the dots and circles in *Fig. 5B*).

The use-dependent block I_{Ca} could be unblocked by a 1 min long hyperpolarization to −95 mV (see above and legend *Fig. 5B*). The use-dependent block of I_{Ca} was facilitated by setting the holding potential to the more physiological value of −45 mV. The comparison of the two panels in Fig. 5 B shows that with the holding potential of −45 mV, 1 μM D600 completely suppressed I_{Ca} within 6 seconds (or 6 calcium currents).

Open channel block by D600?

The voltage- and use-dependent reduction of I_{Ca} by D600 had been described firstly in heart ventricular preparations, and they have been interpreted as an 'open channel block' (22, 23). It was postulated that D600 were able to reach its receptor inside the channel only if the channel were in the open configuration. Vice versa, it was thought the closed conformation of the channel (resting or inactivated state) would prevent the drug-channel interaction. The hypothesis of the 'open channel block' can explain some of the above results as follows. For the analysis of the initial block, the membrane was clamped for a long period of time to −65 mV or −45 mV. At those potentials, statistically, very few or some Ca-channels may open, thereby resulting in low or moderate degree of the 'initial block'. Repetitive 1 Hz-pulsing was thought to open the channels and to facilitate the drug-channel interaction, thus resulting in the 'use-dependence' of the D600-effect.

The above experiments, however, did not exclude that D600 could interact also with the inactivated channel (closed state). This question is addressed by the experiment illustrated in *Fig. 5C*. After an initial

test-pulse (170 ms from −65 to −5 mV), the cell was clamped to −5 mV, the duration of 60 s thought to be long enough for complete inactivation of I_{Ca}. Then, after a 1 s repolarization to −65 mV, the test-pulse was applied again. In the absence of the drug, it induced an I_{Ca} which was reduced to 30% (marked by asterisk in the left panel of Fig. 5C). The result suggests that the 1 s repolarization to −65 mV recovered only a 30% fraction of Ca-channels from the inactivated into the available state. The experiment was repeated and 20 s after depolarization to −5 mV D600 was bath-applied. 40 s later, in continuous presence of 0.1 μM D600, the membrane was repolarized to −65 mV for 1 s, and depolarized to −5 mV again. In presence of D600, this test-step evoked an I_{Ca} that was 50% of the I_{Ca} obtained with the same protocol in the absence of the drug (compare the tracings marked by asteriks). Since D600 was applied at −5 mV, where the channels were thought to be in the closed, inactivated state, and since the repolarization to −65 mV does not open them, reduction of I_{Ca} could have resulted from the interaction of D600 with the closed state of the channel. Unfortunately, the result is only suggestive. There is evidence in the literature that the inactivation of I_{Ca} at −5 mV can remaine incomplete, even during the 1 min long period of depolarization. A current of 5% of the peak current was measured after 5 s depolarization to −5 mV by Aaronson et al. (1988). Thus, the reduction of I_{Ca} by D600 at −5 mV could have resulted from an open channel block; Ca-channel that have passed from the inactivated into the open state may have been occupied and blocked by D600. In such a way the result can still be modeled with the idea that D600 is a pure 'open channel blocker'.

Does D600 act from inside or outside on the membrane?

Chemically, D600 is a tertiary amine with a pK_a of 8.5 (24), and at pH 7.4, approximately 7% of the drug is present in an uncharged form. Since the uncharged form can cross the membrane, D600 may bind to its receptor, resulting in block, from either the extracellular or the cytoplasmic side of the membrane. This question has addressed by means of the permanently charged D600-derivative D890. In ventricular cells, D890 was shown to be ineffective from the outside but effective from the inside, the result supporting the hypothesis of an intracellular mode of action (13). In smooth muscle cells isolated from the portal vein, 100 μM D600 was applied intracellularly via the patch electrode and found to be ineffective (25) whereas D890 reduced I_{Ca} (26).

We repeated the above experiments using the protocols of *Fig. 5B*. For intracellular application, D600 was added in concentrations between 1 μM to 100 μM to the electrode solution and dialyzed out of the electrode

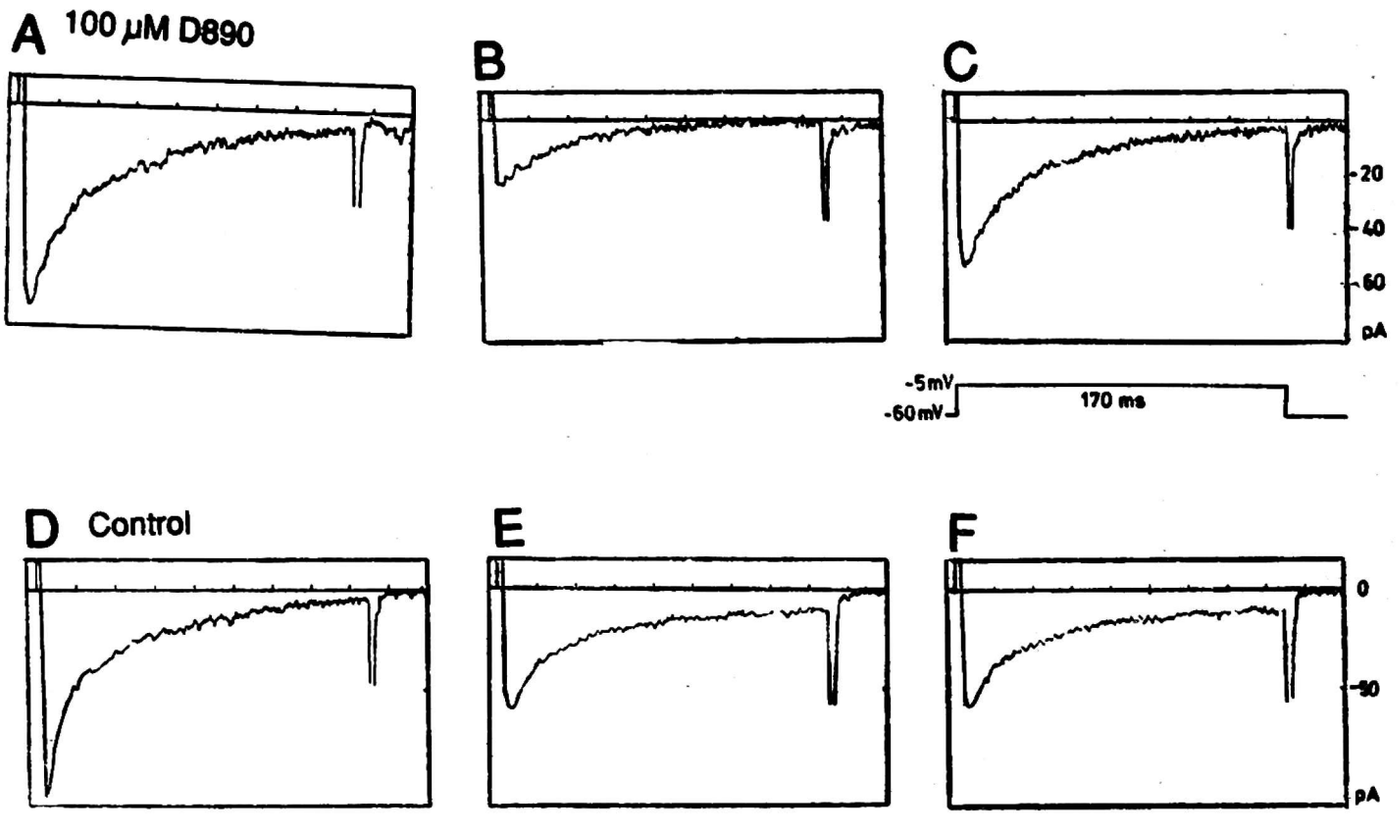

Fig. 6. Effect of intracellularly applied D890 on I_{Ca}. A: I_{Ca} 10 s after rupturing of the patch (control). B: 5 min cell dialysis with 100 µM D890, I_{Ca} decreased by 72%. C: Unblock of the D890-effect by a 60 s long hyperpolarization to —90 mV, the test-step evoked a peak I_{Ca} reduced by 30%. D, E, F: Same experiment but no D890 in the patch piptte. The run-down of I_{Ca} (5 min, E) can not be antagonized by hyperplarization (F).

into the cytosol. We did not observe reduction of I_{Ca}, that is, cytosolic D600 seemed to be ineffective in reducing I_{Ca} of porcine coronary cells. Regarding the impermeable D890, bath application of 1, 10 or 20 µM D890 did not suppress I_{Ca}, only the highest concentration of 100 µM had a small depressing effect on I_{Ca} (block by 20%). Intracellular application of 10 or 100 µM D890 through the patch pipette suppressed I_{Ca} dose--dependently. *Fig. 6* shows a 70% reduction of I_{Ca} by 100 µM D890 within 5 min. The block of I_{Ca} by intracellular D890 was voltage-dependent, i. e. we could largely unblock the effect of D890 (i. e. recover I_{Ca}) by a hyperpolarization to -90 mV for 1 min. In contrast, the 'run-down' of I_{Ca} with time could not be modified by hyperpolarization (*Fig. 6*). Thus, reduction of I_{Ca} by intracellular D890 can be established as a voltage--dependent block that can be separated from other phenomena.

DISCUSSION

In coronary myocytes, we have studied the effect of the Ca-antagonists D600 and D890 on the level of either single-channel or whole-cell currents. Our single-channel data demonstrates reduction of channel openness

by D600. Since D600 was added to the bath outside the cell-attached patch, such a result suggests that D600 must have penetrated the sarcolemma to access the analyzed patch of membrane, either in its uncharged form through the lipid bilayer or in its charged form thorugh the cytosol.

Different to heart ventricular myocytes (13), the coronary myocyte did not respond with reduction I_{Ca} when D600 was intracellularly applied, the result confirming earlier reports in other vascular myocytes (25, 26). Intracellular application of the membrane-impermeable derivative D890 did reduce I_{Ca}, as it was reported for ventricular (13) and vascular myocytes (26).

In contrast to (26) we do not interprete the difference between ventricular and vascular effects of D600 and D890 to suggest different L-type Ca-channels and with different receptors for D600 and D890. Instead, we suggest that difference in the lipid composition of the bilayer may have provided differences in the membrane-diffusion of the intracellularly applied drugs. If the 'escape' of intracellular D600 occurs at a rate fast in comparison to the rate of diffusion out of the patch pipette into the cytosol, the effective D600 concentration may be too low for a block of I_{Ca}. In conclusion, our and the previous results are still compatible with the hypothesis that Ca-antagonists of the verapamil-type block L-type Ca-channels from the inner side of the membrane, in both ventricular and vascular myocytes.

D600 was found not to alter the voltage-dependence of the activation--process as it is indicated by the current-voltage relation of peak I_{Ca}. This result is in line with the observation in heart ventricular preparations (27, 23). It is in contrast to the Ca antagonistic effects of the dihydropyridine--derivatives nifedipine and nimodipine which have been found to modify the i-v curve (28).

The three components of block, initial, use-dependent and tonic have been distinguished in the literature (23). The initial block occurs when resting tissue equilibrates with the drug, and it can be measured only once in a given cell. The present results from porcine coronary myocytes suggest that the initial block is significant if the membrane potential is in the order of -45 mV. Any depolarization is thought to open further Ca-channels, thereby augmenting the block beyond the initial level. The tonic block is defined as the block which could not be removed by a maximally effective hyperpolarization. In the present case, 1 min long hyperpolarizations to -105 mV removed the block almost completely (*Fig. 5A*). This leaves the use-dependent (or conditioned block) as the main mechanism for the D600 effect.

In the coronary smooth muscle cells, the steady state availability of I_{Ca} lies with a half-point of -38 mV close to the resting potential of -45

mV. The proximity of the resting potential to the threshold of the activation curve of I_{Ca} is special for vascular smooth muscle cells. It makes it probable that the channel stays open at the resting potential. Thus, D600 can bind without the conditioning depolarization and produce an initial block that was at -45 mV as large as 35%. If the membrane is depolarized, e. g. by stretch or by neurotransmitters, additional Ca-channels become open and available for the block by D600, thereby.

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