Mechanism of Calcium Channel Block by D600 in Single Smooth Muscle Cells From Rabbit Ear Artery

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This study investigated the action of the calcium antagonist D600 on calcium channel currents recorded in high barium solution from single, enzymatically isolated smooth muscle cells from the rabbit ear artery using the whole cell configuration of the patch-clamp technique. D600 (1–100 μM) was applied by bath perfusion or by a new technique that allowed a concentration jump during the current. Application of D600 at rest (holding potential, −60 mV) did not alter the peak inward current elicited on depolarization, but the activation of the channels led to a marked block and an acceleration of the current decay. The mechanism of block of calcium channels by D600 was studied by using pulse protocols with different pulse length and different interpulse intervals. The results were consistent with the hypothesis that D600 has a low affinity for the calcium channels in the resting state and that they have to pass to the open state before the drug affects the calcium channel current. A fast onset of the calcium channel block by D600 (time constant, 502 msec) could be shown by rapid application of D600 during the sustained current component of the barium inward current. However, experiments did not definitely distinguish whether binding occurred to the open or to the inactivated state (although there was some evidence of a long-lasting binding to an inactivated state). (*Circulation Research* 1989;64:928–936)

Until now, the action of D600 has been examined principally in multicellular smooth muscle preparations. According to these studies,[1] there is much evidence that the relaxant effect of this drug is a consequence of inhibition of voltage-dependent calcium channels.

Detailed voltage-clamp studies on the action of calcium antagonists on the calcium channels in smooth muscle have been complicated in part by methodological problems of voltage clamp in multicellular preparations and also because of residual outward currents occurring simultaneously with the calcium inward currents.[2–4] Calcium or barium inward currents in smooth muscle that are similar to calcium currents in heart muscle with respect to voltage dependence and kinetic properties have recently been described in single smooth muscle cells.[5–10] The total inward current in single smooth muscle cells can be shown using the whole-cell configuration of the patch-clamp technique, composed of fast and slow inactivating components, each having a different voltage dependence[11,12] as has also been demonstrated in cardiac and neuronal cells.[13–15] In heart muscle preparations, D600 has been shown to inhibit calcium currents in a voltage- and use-dependent manner.[16–22] These studies suggest that D600 binds preferentially to the open and inactivated states of the channels. The aim of the present experiments was to examine possible preferential binding of D600 to different calcium channel states in smooth muscle cells from rabbit ear artery.

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Materials and Methods

Single smooth muscle cells were freshly dispersed from rabbit ear artery using a procedure similar to that described previously.[23] Short segments (1–2 mm) of artery were incubated for three 30-minute periods (I, II, and III) in a modified physiological salt solution containing 10 μM calcium, 2 mg/ml bovine serum albumin, and I) 0.6 mg/ml and 1.7 units/ml, II) 0.5 mg/ml and 2.1 units/ml, and III) 0.4 mg/ml and 2.5 units/ml collagenase and elastase, respectively, for sequential incubations. Cells dispersed after mild agitation in this

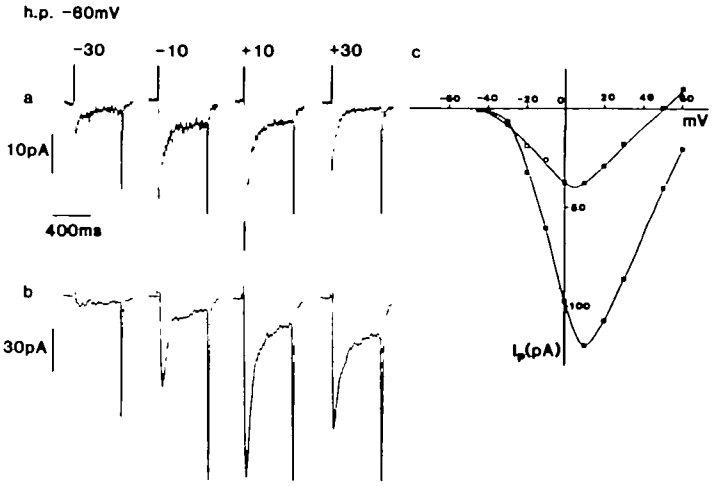

FIGURE 1. *Inward currents recorded in 1.7 mM extracellular $CaCl_2$ (a) and 110 mM extracellular $BaCl_2$ (b) stepping from holding potential of −60 mV to various test potentials. Current/voltage relations are shown for peak inward currents (c): 1.7 mM $CaCl_2$ (□) and 110 mM $BaCl_2$ (■).*

low-calcium physiological salt solution. After centrifugation the cells were resuspended in normal physiological salt solution, stored on coverslips at 4° C, and used within 10–12 hours. The experiments were performed using the whole-cell configuration of the patch-clamp technique[24] by means of a List Model EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG). Patch pipettes had resistances of 3–5 MΩ. The internal (pipette) solution contained (mM) NaCl 126, $MgSO_4$ 3.2, NaH_2PO_4 1, ethylene-glycol-bis(oxyethylene-nitrile)tetraacetic acid (EGTA) 2, glucose 11.5, ATP 2, tetraethylammonium chloride (TEA) 10, and HEPES 5 buffered to pH 7.2 with NaOH. Most experiments were carried out in high barium external solution containing (mM) $BaCl_2$ 110, and HEPES 10 buffered to pH 7.4 with TEA-OH. In some experiments calcium currents were studied when the cells were bathed in the following physiological salt solution composition (mM): NaCl 130, KCl 6, $CaCl_2$ 1.7, $MgCl_2$ 1.2, glucose 14, and HEPES 10.7 buffered to pH 7.4 with NaOH.

Data was recorded on FM tape and later analyzed off-line using a BBC microcomputer that communicated with a 1401 CED-programmable interface. The 1401 provided the pulse sequence and the sample commands through a 12-bit analog-to-digital converter. Since most of the pulse protocols used did not allow signal averaging of the current records, the single current sweeps during recorded pulse trains were low-pass filtered at 0.125 kHz or 1 kHz with an eight-pole Butterworth filter (Barr and Stroud, Glasgow, UK) to reduce the noise. The filtering did not significantly affect the peak values of the recorded inward currents. The leak currents were subtracted either digitally, using average values of steady leakage currents elicited by a 20 mV hyperpolarizing pulse, or electronically by means of an analog circuit. Both methods gave similar results. All recordings were made between 20° and 25° C. The decay of the current was fitted to an exponential function using the algorithm of Marquardt.[25]

D600 (Knoll AG, Ludwigshafen, FRG) was applied by bath perfusion or by a concentration-jump technique previously described in detail.[26] This enabled the application of D600 within less than 10 msec.[26] Stable whole-cell recordings were made from single cells in a microdrop (less than 0.1 μl) formed within an inner bath. This bath was separated from an outer bath by a Sylgard polymer ring, and rapid application of new solution to the single cell from which the recording was made took place when solution from the outer bath flooded over the Sylgard ring and mixed with the microdrop.[26] Tests with solutions with high concentrations of potassium showed that mixing was complete within 10 msec (see Reference 26 for a detailed description of the method). Inward barium currents were evoked by stepping from a holding potential of −60 mV to a test potential of +20 mV for 6 seconds. D600 was applied during the sustained component about 2 seconds after the start of the pulse.

Results

Figure 1 shows inward currents from an isolated ear artery cell bathed in normal physiological salt solution (a) and the inward currents of the same cell after changing the external solution to 110 mM $BaCl_2$ solution (b). In normal physiological salt solution, small calcium inward currents with peak current values from 10 to 40 pA could be recorded. To increase calcium channel currents as well as block residual outward currents,[27] we used barium as the charge carrier through calcium channels. The peak barium current amplitudes were in the range of 40–150 pA. In barium solution, the maximum current occurred at more positive potentials. The observed shift in the reversal potential to more positive potentials (see Figure 1c) may be partially due to an inhibition of contaminating outward currents by extracellular barium.[27]

In normal extracellular calcium (1.7 mM) as well as with barium (110 mM) as the charge carrier, the inactivation of the recorded inward currents could be approximated by a single exponential function at

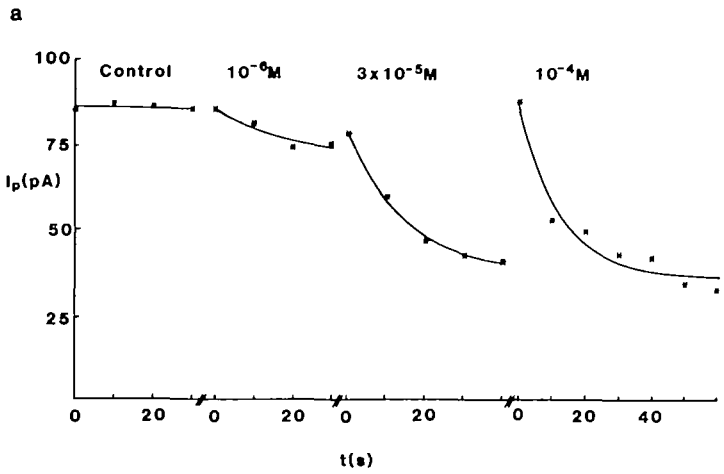

FIGURE 2. *Time course of block by different concentrations of D600 using a frequency of stimulation of 0.1 Hz (pulse length, 500 msec). Onset of block was fitted by single exponential function. Time constants were 19 seconds (1 μM), 15 seconds (30 μM), and 13 seconds (100 μM). No decrease in peak amplitudes was observed under control conditions, and rest periods of 4 minutes (1–100 μM) and 3 minutes (30 μM) were allowed between each pulse train where almost complete recovery of peak current was observed. After a 10-minute washout period, recovery was observed without current decay on repeated stimulation.*

short times, but over longer times two exponentials were required. As pointed out previously in ear artery cells,[9,11] a fast inactivating current component can be distinguished from a slowly decaying current component. Both current components were present in normal calcium solution as well as in high barium solution (Figure 1). We observed large variations in the relative contribution of the slowly inactivating component to the total inward current in different cells, which may reflect peculiarities of cell populations as well as the influence of enzymatic treatment during the isolation procedure.

Affinity of D600 for Resting, Open, and Inactivated Calcium Channels

Initially, the effects of various concentrations of D600 (1–100 μM) were studied on the peak inward barium current amplitude. In the absence of D600, peak current did not decline during a train of 500-msec pulses (stepping from −60 to +20 mV) applied at a frequency of 0.1 Hz (Figure 2). The control current showed a fast inactivating and a sustained component (Figure 3). Stable recordings of barium current could be made under control conditions for 30–40 minutes. The peak current of a postrest pulse was identical with the control current.

The time courses for the block of the peak current amplitude at various concentrations of D600 are shown for stimulation at 0.1 Hz (Figure 2). After treatment with 1 μM D600 for an equilibration period of 1 minute when no voltage step was applied, the peak current decreased during a 0.1-Hz pulse train to a new steady-state level. An increase in D600 concentration from 1 μM to 30 or 100 μM led, after a 1-minute equilibration period, to a greater block of the inward current on repetitive stimulation. The data have been fitted by smooth curves that represent single exponential functions plus a constant. However, a rest period between applied pulse trains led to a rest-dependent recov-

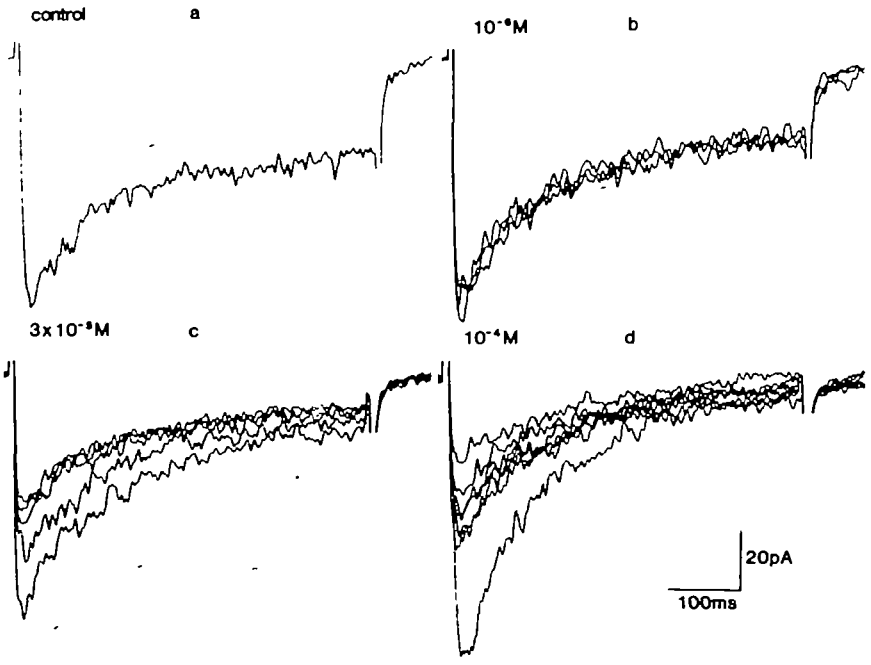

FIGURE 3. *Inward currents showing block with repetitive stimulation at various concentrations of D600. Currents were evoked every 10 seconds by stepping from holding potential of −60 mV to a test potential of +20 mV in all cases, and leakage current was subtracted digitally. Currents correspond to those displayed graphically in Figure 2.* a: *control current.* b: *in 1 μM D600.* c: *in 30 μM D600.* d: *in 100 μM D600. In this and all subsequent figures 110 mM $BaCl_2$ was used.*

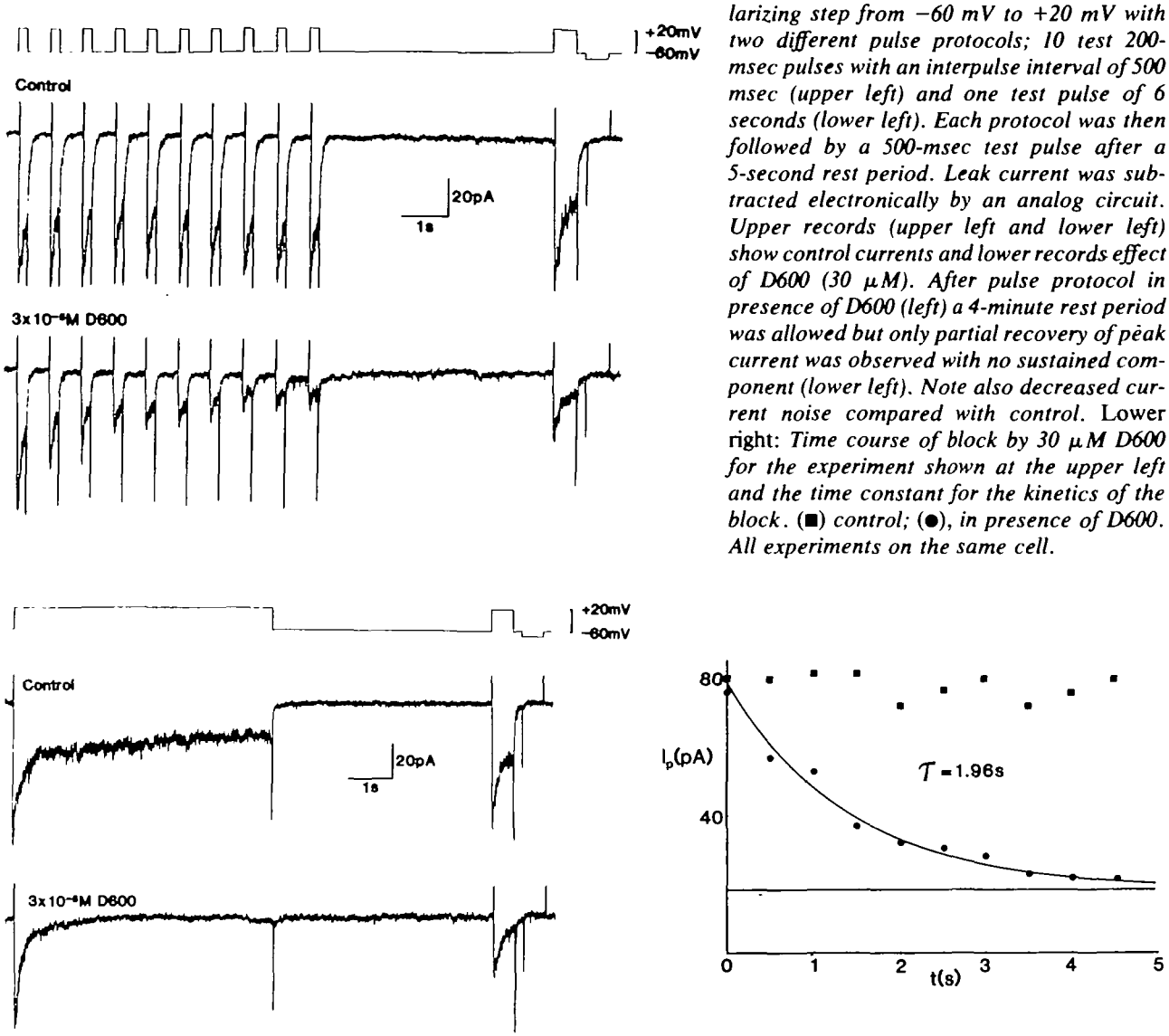

FIGURE 4. *Inward currents evoked by depolarizing step from −60 mV to +20 mV with two different pulse protocols; 10 test 200-msec pulses with an interpulse interval of 500 msec (upper left) and one test pulse of 6 seconds (lower left). Each protocol was then followed by a 500-msec test pulse after a 5-second rest period. Leak current was subtracted electronically by an analog circuit. Upper records (upper left and lower left) show control currents and lower records effect of D600 (30 μM). After pulse protocol in presence of D600 (left) a 4-minute rest period was allowed but only partial recovery of peak current was observed with no sustained component (lower left). Note also decreased current noise compared with control.* Lower right: *Time course of block by 30 μM D600 for the experiment shown at the upper left and the time constant for the kinetics of the block.* (■) *control;* (●), *in presence of D600. All experiments on the same cell.*

ery to about 90% of the control current amplitude after 3 minutes in 30 μM D600 and to a complete recovery of the peak current after 4 minutes in the presence of 100 μM D600 (Figures 2 and 3). The observed complete recovery of the currents at rest at a holding potential of −60 mV after a short period of stimulation at a low frequency (0.1 Hz) was a constant finding during this study. The results in Figure 3 show that the peak current value in 100 μM D600 on the first pulse after a period of 4 minutes without stimulation was almost identical to the control current. However, the current decay during a single pulse was accelerated. The acceleration of the current decay during the first test pulse after a rest period became more evident with increasing drug concentration (Figure 3c and 3d). When two identical trains of stimuli were applied, the decay of the peak currents was usually faster during the second.

The kinetics of the blockade by 1–100 μM D600 of barium currents during different test pulse frequencies were fitted in all experiments by a single exponential function; for example, when stimulating with 200-msec depolarizing pulses and 500-msec interpulse intervals in 30 μM D600, τ_{block} was 1.96 seconds (Figure 4, upper left and lower right). When using pulse trains with longer interpulse intervals (10 seconds), the time course of the decline of peak currents was about one order of magnitude slower in the presence of the same drug concentration (see Figure 2). A monoexponential time course of the decline of peak barium currents during repeated pulsing at different frequencies suggests that D600 was binding to a single binding site or to one calcium channel state. Therefore, the rate at which the block developed was related to the frequency of channel activation by test pulses. Also,

after higher frequency stimulation, a block of 15–30% of the control current remained after a 4-minute rest period, suggesting only partial recovery—an observation not made when using a lower frequency of stimulation.

The absence of initial block in 1–100 μM D600 indicates a low affinity of the drug for calcium channels at negative potentials when the majority would be in the resting closed but available state. The decrease in current amplitude with repetitive stimulation suggests that D600 binds either to the channels in the depolarized membrane or to the open or inactivated states of the channels.

To distinguish between block of the channels in the open or inactivated states we compared the block after a 6-second train of ten 200-msec pulses and after a single 6-second pulse (Figure 4, upper left and lower left). These protocols were chosen because they produce about the same amount of channel open time (current×time, 1:1.17), but the long pulse produces three times as much channel inactivation (current inactivation from peak×time). In the presence of 30 μM D600, block of the peak inward current evoked by short repetitive pulses was observed that could be described by a single exponential function with a time constant τ_{block}=1.96 seconds (Figure 4, upper left and lower right). This suggests a close correlation between the amount of total channel open time and the amount of block. During a 6-second pulse in the presence of 30 μM D600, the current fell to zero after 3 seconds in the same cell (Figure 4, lower left). A block of 45% persisted following a 5-second rest period after the single long pulse, and a 52% block remained at 5 seconds after a train of short pulses despite the marked difference between the protocols of the two experiments (Figure 4, upper left and lower left). As the amount of inactivation × time was much greater with the single 6-second pulse, this result did not imply a high affinity binding of D600 to the inactivated state but rather a binding to the open state; hence, the similar degree of channel block after the two protocols. However, this experiment does not exclude binding of D600 to the inactivated calcium channel state during both pulse protocols.

In order to investigate a possible binding of D600 to inactivated channels, further experiments were done. After a 6-second subthreshold depolarizing step to −40 mV (near threshold voltage), which produced substantial inactivation of peak current (23±8%; n=3) measured at +20 mV (see Figure 5a and 5b), D600 had only a very small (about 3%) blocking effect on peak current (Figure 5c). This suggested little binding to the inactivated state during the single 6-second depolarizing step. As shown in Figure 5 lower, currents recovered under control conditions from inactivation during a 500-msec rest to 90% of their control size (see, also, Figure 4 upper left where almost complete recovery was observed after short test pulses), but if 3 μM D600 was present and the membrane was depolarized to +20 mV, which caused considerable channel opening, recovery was incomplete after 500 msec (Figure 5, lower).

Fast Application of D600 During a Single Voltage-Clamp Step

A newly developed concentration-jump technique (see "Materials and Methods") was used for bath application of 100 μM D600 within a few milliseconds. A step change in drug concentration to a constant known value is essential for the analysis of drug-receptor kinetics.

D600, applied rapidly during a single voltage-clamp step, led to a reduction in the sustained current component (Figure 6b). The next two test pulses applied after 10-second intervals (Figure 6c and 6d) evoked a smaller peak current and current inactivated more quickly. In comparison with the experiment in Figure 3, where 500-msec pulses were applied with 10-second rest intervals, the continuing depolarization during the 6-second pulse in this experiment substantially increased the use-dependent block (compare the 45% inhibition during the third 500-msec step in Figure 3 with the 78% inhibition during the 6-second step in Figure 6d). The time-course of channel block during step application of D600 could be estimated by subtracting current decay after application of the drug from the decay of the control current. A single exponential was fitted to this drug-induced decay, which had a time constant of 502 msec (Figure 6e).

If D600 has negligible affinity for the inward current channel at −60 mV but appreciable affinity at a more positive potential (e.g., +20 mV) then the same rate of block of current might be expected following a voltage step into a potential range where block occurs such as that seen during a concentration jump at that potential. Peak current was unaffected in 100 μM D600 if pulses were separated by 4 minutes or more (Figure 2). However, decay of the current was accelerated (Figure 3). The time course of current decay during a 500-msec pulse in the absence of D600 and the initial change of the current decay after a 1-minute equilibration in 100 μM D600 were studied separately (Figure 7).

The effect of D600 on the current could be fitted by a single exponential. However, the time constant of this averaged 190±68 msec (mean±SEM, n=3), which was about one third the value obtained by the concentration-jump technique. This difference implied that the situation may be more complex than the simple model postulated.

Discussion

Low Affinity of D600 to Resting Calcium Channels

A constant finding during the present study was the low affinity of D600 for calcium channels in the resting available state. An almost negligible initial inhibition of peak inward current on the first pulse

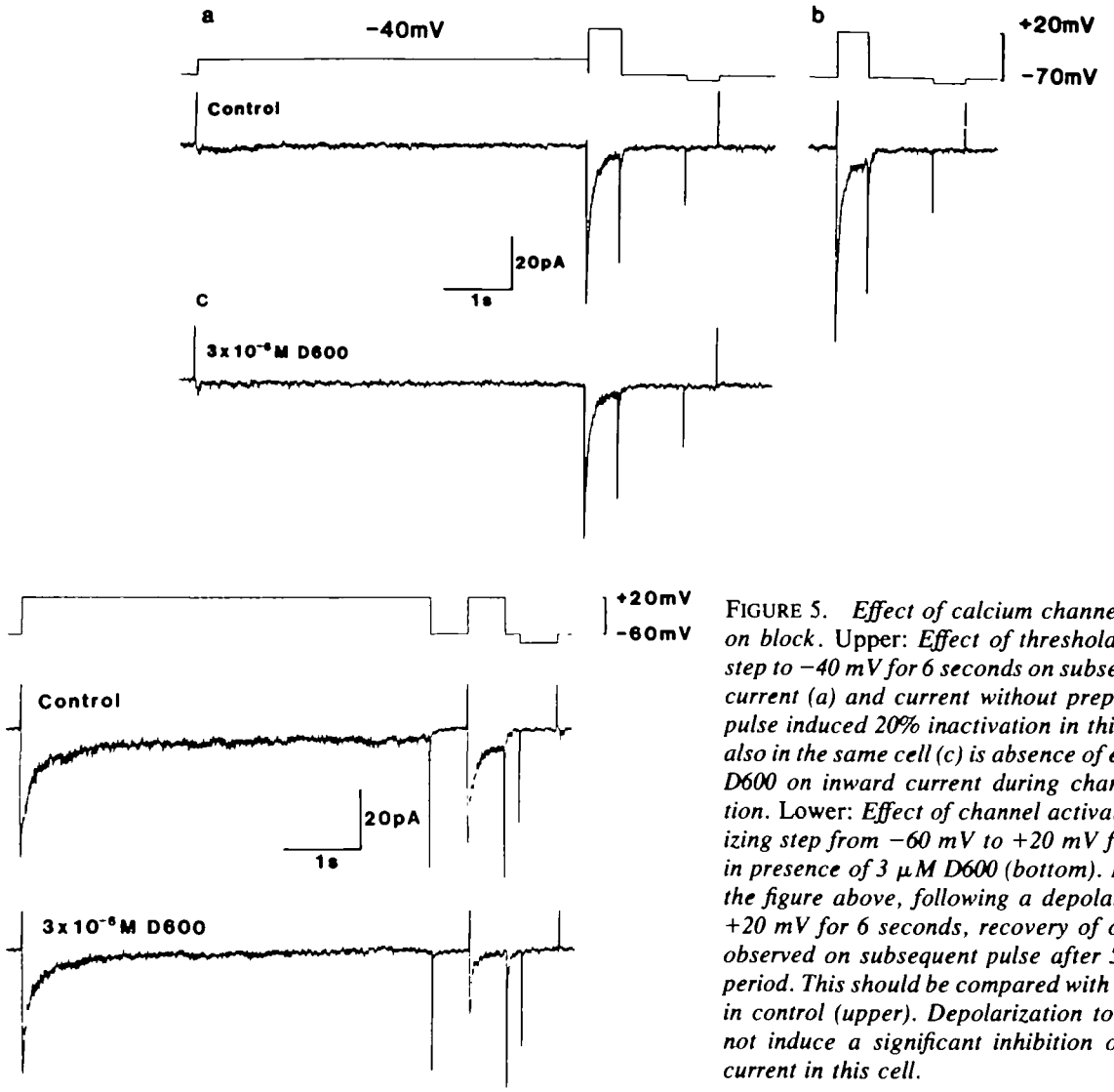

FIGURE 5. *Effect of calcium channel inactivation on block.* Upper: *Effect of threshold depolarizing step to −40 mV for 6 seconds on subsequent evoked current (a) and current without prepulse (b). Prepulse induced 20% inactivation in this cell. Shown also in the same cell (c) is absence of effect of 3 μM D600 on inward current during channel inactivation.* Lower: *Effect of channel activation (depolarizing step from −60 mV to +20 mV for 6 seconds) in presence of 3 μM D600 (bottom). In contrast to the figure above, following a depolarizing step to +20 mV for 6 seconds, recovery of only 50% was observed on subsequent pulse after 500-msec rest period. This should be compared with 90% recovery in control (upper). Depolarization to −35 mV did not induce a significant inhibition of the inward current in this cell.*

after holding at a negative potential was also observed in single and multicellular heart muscle preparations.[19,20,22] Since D600 is basic with a P_{K_a} of 8.7 at pH 7.4, it exists mainly as a charged molecule that is not capable of penetrating the membrane via the hydrophobic pathway.[28,29]

High Affinity of D600 for Open Calcium Channels

As shown by the experiments in Figures 2 and 3, the rate of development of calcium channel block is a consequence of the number of openings with respect to time. A similar observation was made by Klöckner and Isenberg[30] with the verapamil derivative tiapamil. The block of the sustained current component (Figure 4, upper and lower left and Figure 7) leads to changes in shape of the current during the pulse train.

The experiments described suggest that depolarization of the membrane and opening of the channels was required for D600 to exert its blocking action; affinity of D600 for the channels was very low at negative potentials (e.g., −60 mV). However, when block had occurred, then a complete recovery of channel activity was possible after several minutes at a negative potential, but this recovery was much slower than the usual recovery of channels from the normal inactivation process. A possible explanation is that D600 interacts with the open channel (perhaps entering it) to block ionic fluxes through it, the open channel is then held open until D600 dissociates, and dissociation occurs slowly at negative potentials (and perhaps even more slowly at more positive ones), explaining the rather slow recovery from block, which in these experiments took more than 3 minutes.

Role of Inactivation in Calcium Channel Block by D600 and Dependence of Block on Previous Channel Activity

The observed overlap between the voltage at which channel activation begins (threshold at −40 mV) and channel inactivation at the same

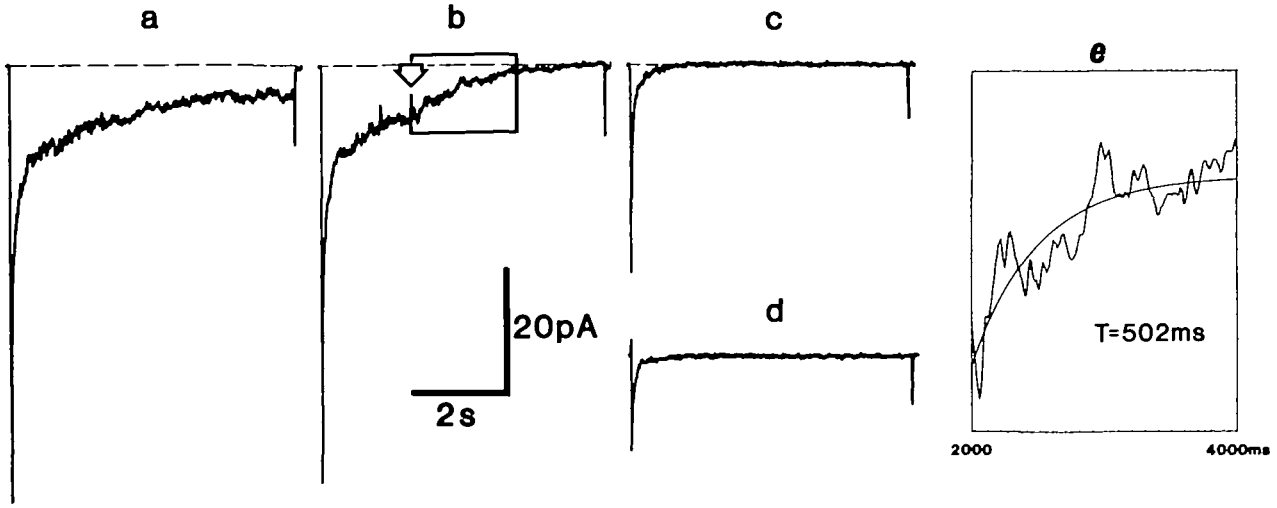

FIGURE 6. *Rapid application of D600 during single voltage-clamp step.* a: *Control current evoked on depolarizing step from holding potential of −60 mV to test potential of +20 mV for 6 seconds. Leakage current was subtracted electronically by means of an analog circuit.* b: *Rapid application of 100 μM D600 (↓). Record in box shown expanded in e.* c *and* d: *Two subsequent steps in presence of D600 after 10-second rest period at holding potential.* e: *Difference current derived by digital subtraction of current in presence of D600 from control with single exponential fit superimposed. Time constant of current block by 100 μM D600, τ_{block}=502 msec.*

voltage (see Figure 5a and 5b) made it impossible to distinguish clearly between binding of D600 to the calcium channels in the open and inactivated states. From the present experiments it is not clear whether there is always an equilibrium between the open and inactivated calcium channels in the rabbit ear artery cells and whether the channels have to open before inactivation takes place (see Figure 5c). However, in the case of high affinity binding of D600 to the inactivated state one would have expected an appreciable block for the experiment shown in Figure 5c, where a very small (2–4 pA) inward current but substantial current inactivation was observed.

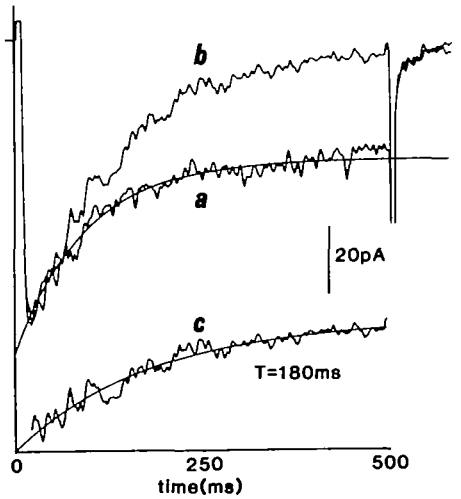

FIGURE 7. *Rate of D600 binding at depolarizing potential.* a: *Control current evoked on depolarizing step to +20 mV for 500 msec from a holding potential of −60 mV. Current decay was fitted by single exponential function which had time constant of 107.8 msec.* b: *First current evoked by a pulse after 4-minute rest period with 100 μM D600 at holding potential.* c: *Difference between exponential fit (a) and current in the presence of D600 (b). This was fitted by single exponential function that had time constant of 180 msec. Experiment in the same cell as Figure 2.*

Further information on the action of D600 on the inactivated state can be deduced from Figure 4. If high affinity binding of D600 to the inactivated channels is the main mechanism of drug action, then there would have been more binding to this state when using pulse protocol of Figure 4, lower left, because the time the channels spend in the inactivated state is much greater and if the off-rates of D600 from the channel in its open and inactivated conformations were different, one might expect different amounts of recovery from block after a 5-second rest at −60 mV in both experiments. This difference would be emphasized by the fact that the channels could recover from inactivation between pulses when using the protocol in Figure 4, upper left. However, a similar amount of recovery was observed for both protocols, which suggests a correlation between the amount of open time and the amount of block that is in favor of the hypothesis that the open state is crucial for the blocking action of D600.

However, a comparison of the frequency-dependent inhibition of the inward current in the presence of 10 μM D600 with different test-pulse length (200 msec in Figure 2, 6 seconds in Figure 6) but with the same interpulse interval (10 seconds) suggests an additional component of block during the long pulse inactivation in Figure 6.

An interesting two-pool concept was proposed by McDonald et al[20] as an explanation for the acceler-

ation of the block during repetitive pulse trains in heart muscle. According to this hypothesis, the drug is translocated during repeated block and unblock from a pool in the intracellular fluid to a pool within the membrane phase. Thus, an increase in intramembrane drug concentration caused by repeated channel opening makes the receptor of the channels more accessible for drug binding and changes the kinetics of the drug-receptor interaction.

Does D600 Differentially Block Different Types of Calcium Channels in Smooth Muscle?

Benham et al[9] have suggested that the "L-type" calcium channel is the dominant channel type in rabbit ear artery smooth muscle cells and that the "T-type" channels are inactivated by about 25–30% at a holding potential of −60 mV. However, using the same cell type, Aaronson et al[31] have found that two components of calcium current are not so clearly divisible by their voltage dependence. However, both studies suggest that two channel types may be activated from a holding potential of −60 mV. The observed changes in barium current kinetics in the presence of D600 may therefore be explained by a selective block of one distinct calcium channel population.

In agreement with studies in single heart muscle cells, we observed marked drug-induced changes in the time course of inactivation of the barium currents through calcium channels in smooth muscle cells, but no changes in the time to peak of the inward current were observed.[19] A selective block with no, or very slow, recovery of the sustained current component (Figure 4, upper left, and Figure 6) may reflect a higher affinity to a separate "high threshold" calcium channel type in smooth muscle cells.[11,12]

However, when using the protocol of Figure 4, lower left, the L-type calcium channels contributed a much larger fraction of the total number of open channels per unit time compared with the transient T-type channels (which are assumed to be completely inactivated after 200 msec at that membrane potential[9]) than when using protocol of Figure 4, upper left, where the T and L types may have been activated repeatedly. As stated above, both protocols gave nearly the same amount of block and recovery from block; this would indicate similar binding of D600 to T- and L-type channels. According to Benham et al,[9] the sustained current component is carried exclusively by L-type calcium channels. As D600 has only low affinity for the channels in their resting state (at −60 mV), the reduction in peak current in Figure 6c should reflect, exclusively, inhibition of L-type channels. However, the block was much larger than expected. A high affinity binding of D600 to inactivated T-type channels without channel opening seems to be unlikely on the basis of the present experiments.

The rate of onset of block obtained by voltage jump was about three times faster than that obtained by concentration jump. The net reduction of current caused by D600 application was very small, 10–20 pA, and the signal-to-noise ratio not sufficient to distinguish two or more exponentials should they have been present. With respect to the observed two channel populations (8 and 25 pS unit conductance, 9) two rates of block may occur. A further complication is the extent to which open channels may become available from other states which may not be the same during voltage and concentration jumps. However, the observations in the present study could equally well be explained by a time-dependent block of a single population of calcium channels.

The role of a single protonable group in the regulation of the channel conductance was recently studied by Prod'hom et al.[32] According to these authors the binding of protons to this part of the channel molecule could trigger conformational changes in the channel molecule, which would reduce the channel conductance or alternatively change local potentials in the channel and repel cations entering the channel. The interaction of the calcium antagonist D600 with the proposed acidic binding site of the channel molecule would be an interesting explanation of the open channel block in the present experiments.

Conclusions

The calcium channel block by D600 in smooth muscle cells was found to be in many respects quite similar to the state dependence of calcium channel block in heart muscle.[19–22,33] In the present experiments we could demonstrate for the first time a low affinity for resting calcium channels and rapid inhibition of the calcium channels by D600 in smooth muscle cells after channel opening. Using the concentration-jump technique of Hering et al,[26] we could show that the acceleration of the current can be explained by a rapid channel block within several hundred milliseconds. Thus, the mechanism of inhibition of the barium inward current by D600 in this cell type can be explained by 1) a rapid open channel block and/or 2) drug-induced calcium channel state transitions from open to inactivated states. Therefore, the action of D600 is quite different from that of the dihydropyridine calcium antagonist nifedipine, which produces a marked initial inhibition of smooth muscle inward calcium channel current (i.e., exhibits a high affinity for the channels in the resting state) and also induces a marked acceleration of the current decay but on rapid application during a voltage-clamp step has no significant effect on the time course of the current decay.[34,35]

References

1. Fleckenstein A: Calcium antagonism: History and prospects for a multifaced pharmacodynamic principle, in Opie LH (ed): *Perspectives in Cardiovascular Research*. New York, Raven Press, vol 9, 1984

2. Mironneau J: Excitation-contraction coupling in voltage clamped uterine smooth muscle. *J Physiol* 1973;233:127–141
3. Jmari K, Mironneau C, Mironneau J: Inactivation of calcium channel current in rat uterine smooth muscle: Evidence for calcium and voltage mediated mechanisms. *J Physiol* (Lond) 1986;380:111–126
4. Kao CY, McCullough JR: Ionic currents in the uterine smooth muscle. *J Physiol* (Lond) 1975;246:1–36
5. Bean BP: Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity and pharmacology. *J Gen Physiol* 1985;86:1–30
6. Klöckner U, Isenberg G: Calcium currents of caesium loaded isolated smooth muscle cells (urinary bladder of the guinea pig). *Pflugers Arch* 1985;405:340–348
7. Ganitkevich VYa, Shuba MF, Smirnov SV: Potential-dependent calcium inward current in a single isolated smooth muscle cell of the guinea-pig taenia coli. *J Physiol* (Lond) 1986;380:1–16
8. Yatani A, Seidell CL, Allen J, Brown AM: Whole cell and single-channel calcium currents of isolated smooth muscle cells from saphenous vein. *Circ Res* 1987;60:523–533
9. Benham CD, Hess P, Tsien RW: Two types of calcium channels in single smooth muscle cells from rabbit ear artery studied with whole-cell and single-channel recordings. *Circ Res* 1987;61(suppl I):I-10–I-16
10. Loirand G, Paccaud P, Mironneau C, Mironneau J: Evidence for two distinct calcium channels in rat vascular smooth muscle cells in short term primary culture. *Pflugers Arch* 1986;407:566–568
11. Aaronson PI, Benham CD, Bolton TB, Hess P, Tsien RW: Two types of single channel and whole cell calcium or barium currents in single smooth muscle cells of rabbit ear artery (abstract). *J Physiol* (Lond) 1986;377:36P
12. Bean BP, Sturek M, Puga A, Hermsmeyer K: Calcium channels in muscle cells isolated from rat mesenteric arteries; modulation by dihydropyridine drugs. *Circ Res* 1986; 59:229–235
13. Carbone E, Lux HD: A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. *Nature* 1984;310:501–502
14. Nilius B, Hess P, Lansman JB, Tsien RW: A novel type of cardiac calcium channel in ventricular cells. *Nature* 1985; 316:443–446
15. Nowycky MC, Fox AP, Tsien RW: Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 1985;316:440–443
16. Ehara T, Kaufmann R: The voltage- and time-dependent effects of (-)-verapamil on the slow inward current in isolated cat ventricular myocardium. *J Pharmacol Exp Ther* 1978; 207:49–55
17. Kohlhardt M, Haap K: The blockade of V_{max} of the atrioventricular action potential produced by the slow channel inhibitors verapamil and nifedipine. *Naunyn Schmiedebergs Arch Pharmacol* 1981;316:178–185
18. Kass RS: Nisoldipine: A new, more selective calcium current blocker in cardiac Purkinje fibres. *J Pharmacol Exp Ther* 1982;223:446–456
19. Lee KS, Tsien RW: Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* 1983;302:790–794
20. McDonald TF, Pelzer D, Trautwein W: Cat ventricular muscle treated with D600: Characteristics of calcium channel block and unblock. *J Physiol* (Lond) 1984;352:217–241
21. Sanguinetti MC, Kass RS: Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ Res* 1984; 55:336–348
22. Uehara A, Hume JR: Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J Gen Physiol* 1985;85:621–647
23. Benham CD, Bolton TB: Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of rabbit. *J Physiol* (Lond) 1986;381:385–406
24. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch clamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pflugers Arch* 1981;391:85–100
25. Marquardt DW: An algorithm for least-square estimation of non-linear parameters. *J Soc Industrial Applied Mathematics* 1963;11:431–441
26. Hering S, Beech DJ, Bolton TB: A simple method of fast extracellular solution exchange for the study of whole-cell or single channel currents using patch-clamp technique. *Pflugers Arch* 1987;410:335–337
27. Benham CD, Bolton TB, Lang RJ, Takewaki T: The mechanism of action of Ba^{2+} and TEA on single Ca^{2+}-activated K^+ channels in arterial and intestinal smooth muscle cell membrane. *Pflugers Arch* 1985;403:120–127
28. Hille B: Local anaesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J Gen Physiol* 1977; 69:497–515
29. Hondegham LM, Katzung BG: Time and voltage dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim Biophys Acta* 1977;472:373–398
30. Klöckner U, Isenberg G: Tiapamil reduces the calcium inward current of isolated smooth muscle cells. Dependence on holding potential and pulse frequency. *Eur J Pharmacol* 1986;127:165–177
31. Aaronson PI, Bolton TB, Lang RJ, MacKenzie I: Calcium currents in single isolated smooth muscle cells from the rabbit ear artery in normal-calcium and high-barium solutions. *J Physiol* (Lond) (in press)
32. Prod'hom B, Pietrobon D, Hess P: Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca^{2+} channel. *Nature* 1987;329:243–246
33. Nawrath H, Ten Eick RE, McDonald TF, Trautwein W: On the mechanism underlying the action of D600 on slow inward current and tension in mammalian myocardium. *Circ Res* 1977;40:408–414
34. Beech DJ, Bolton TB, Hering S: The mechanism of modulation of Ba^{2+} currents by D600, nifedipine or BAY K 8644 in single smooth-muscle cells of rabbit ear artery studied using a new concentration-jump technique. *J Physiol* (Lond) 1987; 390:88P
35. Hering S, Beech DJ, Bolton TB, Lim SP: Action of nifedipine or BAY K 8644 is dependent on calcium channel state in single smooth muscle cells from rabbit ear artery. *Pflugers Arch* 1988;411:590–592

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