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The dihydropyridine niguldipine modulates calcium and potassium currents in vascular smooth muscle cells

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1 Vascular smooth muscle cells were isolated from the portal vein and from pial vessels of the cow. They were voltage-clamped with a single patch electrode technique (whole cell recording) in order to analyse the effects of niguldipine on ionic membrane currents. Due to adsorption of niguldipine to plastic and glass, the effective concentrations are lower than the nominal concentrations by a factor of about 3.

2 Niguldipine reduced Ca-currents (I_{Ca} of the L-type, voltage operated) at nominal concentrations $>0.1\,\mu$M up to a complete block at $1\,\mu$M (50% block at $0.4\,\mu$M). Nominal concentrations between 50 and 200 nM facilitated I_{Ca} ('Ca-agonistic effect'). The Ca-agonistic effects of niguldipine showed modest use- but strong voltage-dependence.

3 Niguldipine increased the outward currents at nominal concentrations >10 nM. The extra outward currents reversed at -85 mV, the result suggesting that niguldipine had increased potassium currents, I_K. Maximal facilitation of I_K by niguldipine was about 400% and was obtained at $1\,\mu$M, half-maximal facilitation was obtained with a nominal concentration of 20 nM.

4 Both reduction of I_{Ca} and facilitation of I_K may contribute to vasodilatation by niguldipine. Due to its greater sensitivity, the effects on I_K may dominate.

Introduction

The compound niguldipine HCl, (±)-3-methyl-5-[3-(4,4-diphenyl-1-piperidinyl)-propyl]-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylatehydrochloride, is a novel antihypertensive drug, structurally related to the dihydropyridine calcium entry blockers (Sanders & Kolassa, 1986). It has been demonstrated that its antihypertensive potency in animals exceeds that of dihydropyridine-congeners, that the onset of action is retarded and that its duration of action is prolonged. Thus, a once daily administration in man may be possible (Sanders & Kolassa, 1986; Sanders *et al.*, 1987).

At the final, postsynaptic level of the vascular smooth muscle cell, an anti-hypertensive drug can relax the vessel via different mechanisms: Ca antagonists hinder the entry of Ca ions from the extracellular space into the cytosol. Furthermore, treatment with Ca antagonists reduces the intracellular Ca load. Both processes reduce the degree of contractile activation via the amount of activator calcium. The principle of Ca antagonists is well established in the treatment of hypertension.

Recently, activation or facilitation of K channels ('K-agonism') has been introduced as another principle of vasodilatation. Drugs that facilitate outward potassium currents can hyperpolarize the membrane, thereby preventing the Ca entry through voltage operated Ca-channels. This may explain the action of BRL 34915, which can relax vascular and tracheal smooth muscle tissue (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b; Allen *et al.*, 1986).

To determine which of the above principles might be responsible for the anti-hypertensive effects of niguldipine, the present study was undertaken. Ca-antagonism or K-agonism induce electrophysiological changes measurable with the voltage clamp technique. This powerful method analyses the transmembrane movement of Ca and K ions by measuring the corresponding ionic currents. It has been successfully applied to isolated visceral and vascular smooth muscle cells (Brown *et al.*, 1986; Benham *et al.*, 1987; Klöckner & Isenberg, 1985a,b,c; 1986). Therefore, in the present study the voltage-clamp technique was used to analyse the effects of niguldipine on the ionic membrane currents and to define its mode of action at the level of the smooth muscle cell.

Methods

The experiments were performed on vascular myocytes isolated from the portal vein or from the pial vessels of the cow. The principles for cell isolation have been described recently for the urinary

bladder (Klöckner & Isenberg, 1985a). The protocol for the bovine vessels was similar, and here only a brief outline is given. The processes of cell isolation as well as the electrophysiological experiments were performed at 35°C.

Portal vein The muscularis was separated from the adventitia and the intima. Chunks of muscular tissue (diameter about 2 mm) were stirred in 25 ml of a Ca-free solution composition, mM: NaCl 90, KH_2PO_4 1.2, $MgCl_2$ 5, glucose 20, taurine 50 and HEPES 5, adjusted to pH 7.1 with NaOH, for 6 periods of 5 min each. Thereafter, the chunks were incubated in the same calcium-free solution but complemented by 1.5 mg per ml collagenase (Sigma, St. Louis, C0130) and 1 mg per ml pronase (Serva, Heidelberg, Germany, Type E). 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 in KB-medium (composition, mM: KCl 85, K_2HPO_4 30, $MgSO_4$ 5, Na_2 ATP 2, Na-pyruvate 5, creatine 5, taurine 20, 5-OH-β-butyrate 5, fatty acid free albumin 1 gl^{-1} adjusted with KOH to pH 7.4) for storage at 5°C. For the experiments, cells were superfused with physiological salt solution (PSS) (composition, mM: NaCl 150, KCl 5.4, $CaCl_2$ 3.6, $MgCl_2$ 1.2, glucose 10, HEPES 5, adjusted with NaOH to pH 7.4). At 35°C they had resting potentials between −55 and −68 mV.

Pial vessels Vessels having diameters between 0.1 and 0.5 mm were selected and cut from the surface of the brain. They were opened from the inside, which resulted in thin sheets 2 mm long and 1 mm wide. The sheets were stirred in the calcium-free solution for 6 periods of 5 min each. For enzymatic cell dissociation, the calcium-free solution was complemented with 0.5 mg per ml pronase (Serva, Heidelberg, Germany, Type E), 0.5 mg per ml collagenase (Sigma, St. Louis, C0130), 10 nM isoprenaline (Sigma, St. Louis) and 2 mg per ml fatty acid-free albumin (Sigma, St. Louis). The specimens were stirred for 3 × 15 min each, and the supernatant with the isolated cells was harvested. The cells were suspended and stored in KB-medium for up to 4 days (at 5°C). In PSS at 35°C the cells had resting potentials between −50 and −65 mV.

For the experiment, a drop of the 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). For the electrical measurements, we used patch electrodes in the 'whole cell clamp mode' (Hamill *et al.*, 1981). The tips were fire polished to an inner diameter of about 1 μm. When filled, they had resistances of about 3 MΩ. The Cs-electrode solution (composition, mM: CsCl 130, Na-pyruvate 5, Cs-oxalacetate 5, Cs-succinate 5, EGTA 1, HEPES 10, adjusted with CsOH to pH 7.4) was used for the experiments in part A, where the Ca antagonistic effects of niguldipine were analysed because intracellular Cs ions depress the potassium currents (Klöckner & Isenberg, 1985a,b). The amplitude of I_{Ca} was defined as the most negative net negative current surge. The K-electrode solution (composition, mM: KCl 140, Na_2ATP 2, HEPES 10, EGTA 0.1, pH adjusted with KOH to 7.4) was used in part B, where the K agonistic effects of niguldipine were investigated. The electrodes were connected via Ag/AgCl wires to an input amplifier with facilities for constant current injection (see Klöckner & Isenberg, 1985a). A PDP 11-23 minicomputer generated the pulse protocol, digitized the recorded membrane currents (1024 points of 12 bit resolution) and stored them. The data were not corrected for leakage and capacitive currents. The illustrations are playbacks from the computer to a graphics terminal with line printer.

The compound niguldipine was obtained from Byk Gulden Pharmaceuticals, Konstanz (batch number UL 19/114). A 2 mM stock solution of niguldipine was prepared by dissolving 13 mg of the drug in 10 ml of DMSO at 40–50°C. Adequate aliquots of this stock solution were dissolved in the PSS to a final concentration between 1 nM and 1 μM. The aqueous solutions were freshly prepared every day.

Dihydropyridine type calcium antagonists and niguldipine adsorb with high affinity to plastic and glass (deJong & Huizer, 1984). Chemical analysis of the PSS containing nominally 1 μM niguldipine from the superfusate in the experimental chamber revealed that the effective free concentration was only 0.3 μM. After washing the chamber with drug-free solution for 5 min, the concentration of niguldipine was below the detection limit (< 5 nM). Chemical analysis of these samples was performed in the laboratories of Byk Gulden, Konstanz, using h.p.l.c. and ultraviolet detection. All concentrations refer to nominal applied concentrations of niguldipine; the 'free' concentrations are approximately one third of these values.

Results

(A) *Calcium currents under the influence of niguldipine*

Calcium currents flow mostly through L-type Ca-channels In vascular myocytes, two types of Ca-channel currents have been described. According to the terminology of Benham *et al.* (1987) a long

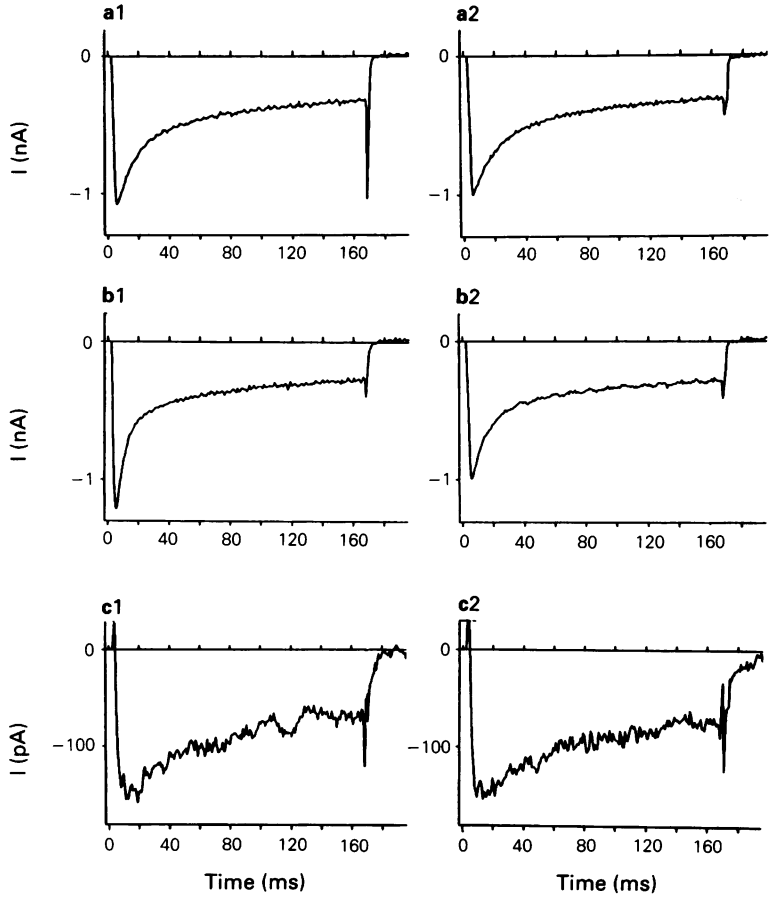

Figure 1 Separation of the Ca-channel current into L- and T-components. (a and b) Myocyte from portal vein, PSS containing 3.6 mM $CaCl_2$ without (a1, b1) or with 50 μM $NiCl_2$ (a2, b2). Pulses, duration 170 ms, to −5 mV were applied from a holding potential of −65 mV (a) or −95 mV (b). (c) Myocyte from pial vessels, PSS containing 20 mM $BaCl_2$. Clamp steps to +5 mV started from a holding potential of −55 mV (c1) or −95 mV (c2).

lasting or L-type current is distinguished from a transient or T-type current. In the myocytes analysed here, the L-type current seems to dominate I_{Ca}. We demonstrate this by blocking the T-channel with 50 μM nickel (Cerbai *et al.*, 1988) or by changing the degree of T-channel inactivation with the holding potential.

Figure 1a shows I_{Ca} of a myocyte of the bovine portal vein. The membrane was held at −65 mV and the depolarizations stepped to −5 mV (0.1 Hz). Under control conditions (panel a1), I_{Ca} peaked with 3 ms to −1.10 nA. Nickel reduced this peak to 1.02 nA (panel a2). If the T-type current is defined as the difference current, it amounts to −0.08 nA or to 7.3% of total I_{Ca}. The record of panel (a) represents an example where the T-component was large. For a total of 10 cells investigated with a holding potential of −65 mV, the contribution of channel current was 4.5 ± 2% of total I_{Ca} on average (mean ± s.e.mean).

Hyperpolarization is known to increase the contribution of the T-channel current (cf. Benham *et al.*, 1987). Therefore, we repeated the analysis with a holding potential of −95 mV (panel b). Peak I_{Ca} was now −1.24 nA before and −1.01 nA after addition of nickel, respectively. Thus the contribution of the nickel-sensitive component to total I_{Ca} was 19% for (b) or 13 ± 4% ($n = 5$) on average. When we used a holding potential of −45 mV, peak I_{Ca} was −0.903 nA and −0.912 nA in the absence and presence of 0.05 mM nickel, respectively. Thus, the more positive holding potential had inactivated the T-channel component.

I_{Ca}, as it is studied in Ca-containing PSS, inactivates along a two exponential time course (time

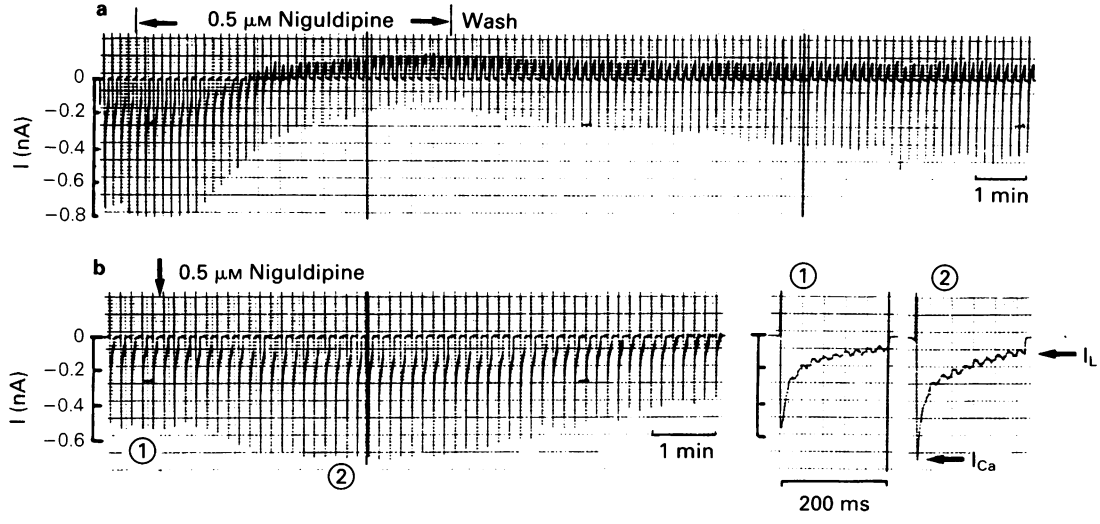

Figure 2 Effect of 0.5 μM niguldipine on the calcium current (I_{Ca}) (myocyte from portal vein). Membrane currents in response to 200 ms clamp steps from −65 mV (holding potential) to −5 mV (pulse potential). Peak I_{Ca} is indicated by the downward deflections. At the end of the pulse, the 'late current' I_L is measured. Pulsing rate 0.2 Hz. The tracings are computer printouts of stored data and not an on line record. For reasons of space, the pulse interval is shortened. (a) Niguldipine reduces within 5 min I_{Ca} from −0.78 nA to −0.15 nA (by 81%) and induces a positive I_L of 0.2 nA. The effect is partially reversed upon washout of the drug. (b) Ca-agonistic effect of niguldipine; 90 s after drug application, I_{Ca} was increased from −0.53 nA (inset 1) to −0.7 nA (inset 2).

constants about 6 and 60 ms) to a non-inactivating component of about 25%. Application of 50 μM nickel reduced the fast exponential but did not block it, i.e. in the presence of nickel the fit of inactivation required two exponentials and the non-inactivating component. The same complex description was necessary when the T-channel component was inactivated with a holding potential of −45 mV. These results suggest that the inactivation time course alone is not an adequate criterion for separation of I_{Ca} into T- and L-channel components.

In myocytes from pial vessels the amplitude of I_{Ca} was small. In order to improve the signal-to-noise ratio and to facilitate the separation of L- and T-type currents (Benham *et al.*, 1987), we substituted in the PSS the 3.6 mM $CaCl_2$ by 20 mM $BaCl_2$ (panel c1 of Figure 1). The intervention is known to shift the gating parameters by more extensive screening of surface charges (see Wilson *et al.*, 1983). We corrected for this shift by setting the holding and step potential to −55 and +5 mV, respectively. In the Ca-free Ba solution, one expects the Ca-mediated inactivation component to be absent. Figure 1 (c1) shows the Ba-current to inactivate slowly and along a single exponential (time constant about 60 ms) to the non-inactivating component. The peak value of this current was not modified when the holding potential was changed from −55 mV (c1: −161 pA) to −95 mV (c2: −159 pA). When we added 0.05 mM nickel to the bath, the current did not change either. A similar ineffectiveness of strong hyperpolarizations or of 50 μM nickel on calcium channel current was observed in 7 other myocytes (either 3.6 mM $CaCl_2$ or 20 mM $BaCl_2$) suggesting that the contribution of T-channel current to the I_{Ca} of pial cells is insignificant.

The results suggest that Ca currents, when elicited from a holding potential close to the resting potential (−65 mV), are mostly L-type currents (Benham *et al.*, 1987). We therefore used a holding potential of −65 mV because it is close to the resting potential and because it effectively removes inactivation of I_{Ca} both in the presence and the absence of the drug.

Ca-antagonistic effects of niguldipine Most experiments were performed on smooth muscle cells isolated from the portal vein of the cow. Examples of the effects of 0.5 μM niguldipine on I_{Ca} are illustrated in Figure 2. Niguldipine (0.5 μM) reduced I_{Ca} within 8 min to 20 ± 8% of control (mean ± s.e.mean; $n = 8$). The reduction of I_{Ca} by 0.5 μM niguldipine did not start immediately after drug administration, but after an initial delay of 1–2 min. During this period, I_{Ca} exceeded the control level by 20–40% ('Ca-agonistic effect', see panel at the bottom of Figure 2). When niguldipine was applied for less than 8 min,

removal of the drug led to a partial recovery of I_{Ca}. The effects of exposure to niguldipine for longer than 10 min could not be reversed by washing for 20 min.

'Use dependence' of the Ca-antagonistic niguldipine effect The reduction of I_{Ca} by Ca antagonists of the verapamil type has been shown to depend on the number of depolarizations applied. This suggests that the channel must open or inactivate before the drug can interact with its binding site (Ehara & Kaufmann, 1978; McDonald *et al.*, 1980; Klöckner & Isenberg, 1986). For such a block, the expressions use-dependence or frequency-dependence are taken to be synonymous.

To evaluate the use-dependence, niguldipine (0.5 μM) was applied under resting conditions for 3 min. The first pulse after the rest evoked an I_{Ca} that was already strongly attenuated; on average the 'initial block' amounted to $62 \pm 7\%$ ($n = 4$, 3–4 min exposure time). The following repetitive pulsing further reduced I_{Ca}. At steady state, niguldipine reduced I_{Ca} by $80 \pm 8\%$ ($n = 8$). Thus, the conditioned block was $18 \pm 8\%$. This number is small for a Ca-antagonist of the dihydropyridine type (compare Sanguinetti & Kass, 1984).

Suppression of I_{Ca} depends on the potential of the clamp step The influence of the clamp step potential on the voltage operated calcium channel is shown in Figure 3. Beyond a threshold potential of −35 mV, the amplitude of I_{Ca} increased at more positive potentials up to −5 mV, but it fell again when the potential became positive. The corresponding I-V curve (Figure 3a) shows a descending branch that reaches maximal inward currents at −5 mV (at 0 mV on average, $n = 15$). At stronger depolarizations, the I-V curve ascended again and intersected the voltage axis of about +50 mV. Niguldipine (0.5 μM) reduced I_{Ca} over the whole range of test potentials and changed the shape of the I-V curve moderately (see below).

Niguldipine shifted the intersection of the ascending branch with the voltage axis by about −5 mV, which could indicate an increase in superimposed outward currents (Figure 3). Thus niguldipine modified not only I_{Ca} but also the late current, I_L, as well as the currents at pulses negative to the threshold potential of I_{Ca}. Under control conditions, the currents negative to −40 mV are almost zero. Five min after application of niguldipine, the I-V curve was shifted in an outward direction (Figure 3b). This increase in outward current also holds true for the potentials positive to −40 mV: the curve of I_L lies above the voltage axis, whereas the control curve was below. Neither the induction of outward currents at potentials negative to the I_{Ca} threshold nor the positivity of net currents can be attributed to Ca-antagonistic effects. Therefore, we postulate that the increase in I_L results from additional effects on a potassium channel. this 'K-agonist effect' will be described in section B.

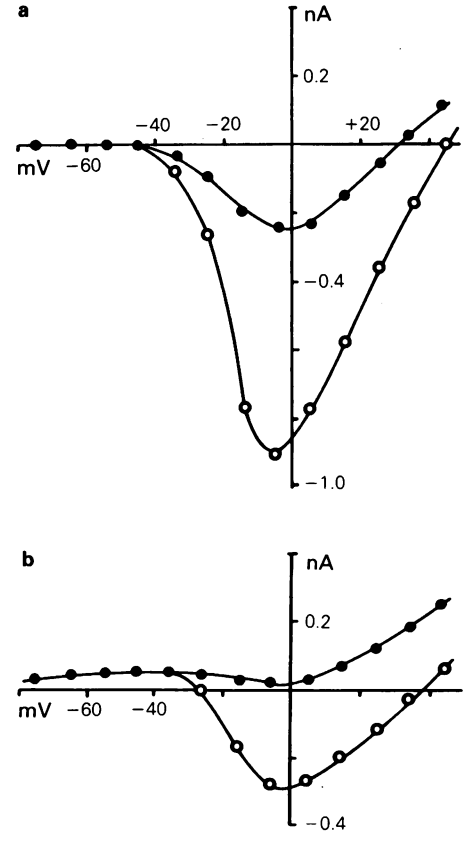

Figure 3 Current-voltage relation of the calcium current (I_{Ca}, ○) and 'late current' (I_L, ●) in control (a) and in the presence of 0.5 μM niguldipine (b). Data obtained with a double pulse protocol. Starting from a holding potential of −65 mV, a conditioning 170 ms long pre-pulse went to voltages between −100 and +50 mV. After a 20 ms repolarization to −65 mV, the 170 ms long test-step to +5 mV started. In this figure, the I_{Ca} and I_L were evaluated as a function of the pre-pulse.

Figure 4 shows the steady-state activation and inactivation curve of I_{Ca} in control solution and during niguldipine. The activation curve was plotted from the data of Figure 3 by dividing the current by the driving force (clamp step potential minus reversal potential) and normalizing the result to give 1 for maximal activation at +35 mV. The activation curve was fitted by the Boltzmann-equation (see legend to Figure 4) adjusting the parameters k (slope of the curve) and V_h (potential of half-maximal activation).

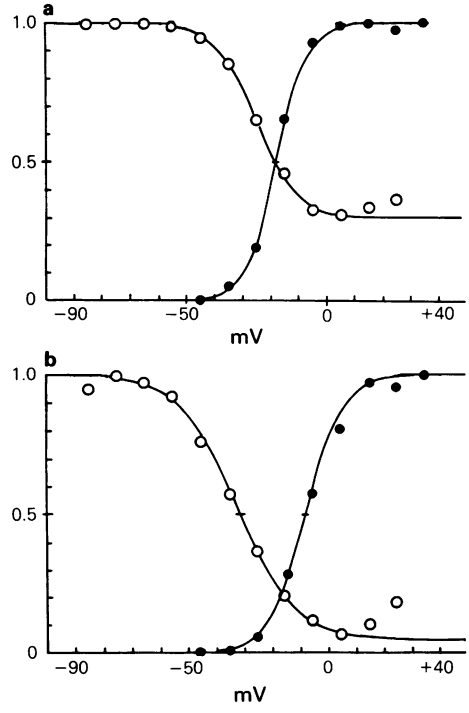

Figure 4 Steady-state activation (●) and inactivation curve (○) of the calcium current (I_{Ca}). (a) Before and (b) after exposure to 0.5 μM niguldipine. The activation curves were fitted with $d_\infty = (1 + \exp[(V - V_h)/k])^{-1}$, where the slope factor k was −7 mV and −8 mV for control and 0.5 μM niguldipine, respectively, and the potential of half-maximal activation (V_h) was −9 mV and −8 mV. The inactivation curve was fitted with $f_\infty = R + (1 - R)(1 + \exp[(V - V_h)/k])^{-1}$. Niguldipine changed k from +7 to +10 mV, V_h from −25 mV to −32 mV, and the residual or non-inactivating fraction R from 32% to 5%.

Niguldipine did not significantly modify these 2 parameters which suggests that the voltage-dependence of activation is not modified.

The inactivation curve was analysed with a paired-pulse protocol. I_{Ca} evoked by a test step to +5 mV (second of the paired pulses) was plotted against the potential of the pre-step. (The curves only approximate the steady state inactivation, since the 170 ms prepulse did not completely inactivate I_{Ca}). Under control conditions, the curve is bell-shaped with a potential of half-maximal availability of −18 mV. Niguldipine 0.5 μM depressed the availability over all of its range, shifted the curve to the left and changed its shape. In terms of the inactivation parameters, the potential of half-maximal inactivation was shifted from -19 ± 7 mV to -37 ± 8 mV ($n = 6$) and the slope factor (of the descending branch) was increased from $+7 \pm 1.2$ to $+11 \pm 2$ mV. Finally, the residual fraction of I_{Ca}, i.e. the fraction that did not inactivate during the 170 ms prepulse to 0 mV, was reduced from $31 \pm 4\%$ to $6 \pm 4\%$ ($n = 6$). These results suggest that niguldipine modifies the voltage-dependence of the steady-state inactivation curve in such a way that membrane depolarizations, evoked either artificially by pre-steps or naturally by transmitters, potentiate the Ca-antagonistic effect of niguldipine. On the other hand, more negative potentials (i.e. membrane hyperpolarizations) attenuate the effects. Thus, niguldipine reduced I_{Ca} by 60% when the pulse started from −85 mV, by 80% after the pre-pulse to −25 mV, and by 94% after the pre-pulse to 0 mV.

The Ca-agonistic effects of niguldipine Figure 2 has already shown that 0.5 μM niguldipine transiently facilitated I_{Ca} by a factor of 1.4. Transient enlargement of I_{Ca} by 0.5 μM niguldipine was seen in 5 of the 8 experiments. Usually, the effect peaked within 1–3 min and disappeared within the following 5–8 min. We attribute the transient 'Ca-agonistic effect' to a slow increase in niguldipine concentration near to the channel, whereby low niguldipine concentrations increase I_{Ca}. Such an argument is supported by the steady-state effect of 50 or 100 nM niguldipine, which consistently increased I_{Ca}. The maximal increase amounts to 30% (Figure 5). Ca-agonistic effects of low concentrations of Ca-antagonists have been found repeatedly for dihydropyridine derivatives (Hess *et al.*, 1984; Brown *et al.*, 1986).

Concentration-response curve for the effects of niguldipine on I_{Ca} Figure 5 summarizes the pooled responses for a total of 17 vascular myocytes (from both portal vein and pial vessels). No material differences were observed in the two cell types. The curve shows no effects of niguldipine at concentrations below 30 nM, a Ca-agonistic effect between 30 and 180 nM (the curve exceeds 1), and a Ca-antagonistic effect at higher concentrations. Due to adsorption of niguldipine to plastic and glass, the effective concentrations are probably lower than the nominal concentrations by a factor of 3 (see Methods).

(B) *Niguldipine facilitates potassium currents*

Niguldipine induces extra outward currents Figure 2 shows that niguldipine, besides reducing the calcium inward current I_{Ca}, induces extra outward currents. The late current (I_L), which was close to zero or inward before application of the drug, became outward. In these experiments, the patch electrodes were filled with the Cs-electrode solution to block the outward potassium currents (Klöckner & Isenberg, 1985b). Therefore, under control conditions,

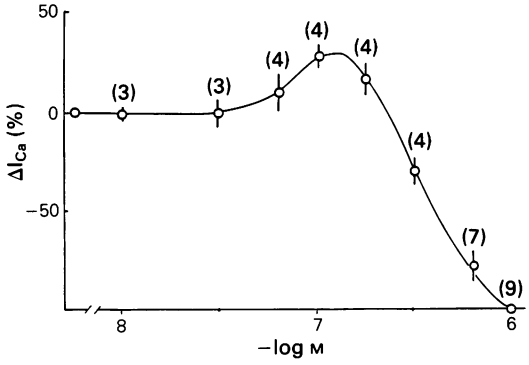

Figure 5 Concentration-response curve for the effect of niguldipine on the calcium current (I_{Ca}). Ordinate scale: current in the presence of niguldipine ($*I_{Ca}$) normalized to $\Delta I_{Ca} = (*I_{Ca} - I_{Ca})/I_{Ca}$. The −log of the drug concentration is plotted on the abscissa scale. The symbols represent the mean of the number of experiments in parentheses; vertical lines show s.e.mean.

the current I_L may result from a fraction of I_{Ca} that had not inactivated within the period of 170 ms. As expected for a 'residual I_{Ca}', I_L was almost zero at all potentials negative to −35 mV (the threshold of I_{Ca}), and at more positive potentials its voltage-dependence followed by voltage-dependence of peak I_{Ca} (Figure 3a).

Under the influence of niguldipine, I_L became outward. A positive or outwardly directed current cannot be explained as non-inactivating, inwardly directed I_{Ca}. Furthermore, these outward currents were also recorded at potentials negative to the threshold of I_{Ca} (−35 mV). Finally, the voltage-dependence of I_L was changed such that it no longer paralleled the voltage-dependence of peak I_{Ca}. Therefore, it is unlikely that the niguldipine-induced outward currents flow through calcium channels. We suggest that niguldipine modified K-channels, and that the current is carried by either K or Cs ions. In order to investigate the effect of niguldipine on the outward potassium currents, additional experiments were performed using electrodes filled with K-electrode solution (see Methods). The effect of 0.1 μM niguldipine on the potassium outward currents is illustrated in Figures 6 and 7. Figure 6 shows the results recorded from a smooth muscle cell of the portal vein. Under control conditions, an outwardly directed current flowed at −65 mV, indicating that the resting potential of the unclamped cell would be negative to −65 mV. The depolarizing clamp step evoked a small time-dependent outward current; the current increased (activated) within about 100 ms to a maximum, from which it slowly decayed (inactivated). A 5 min exposure to 0.1 μM niguldipine

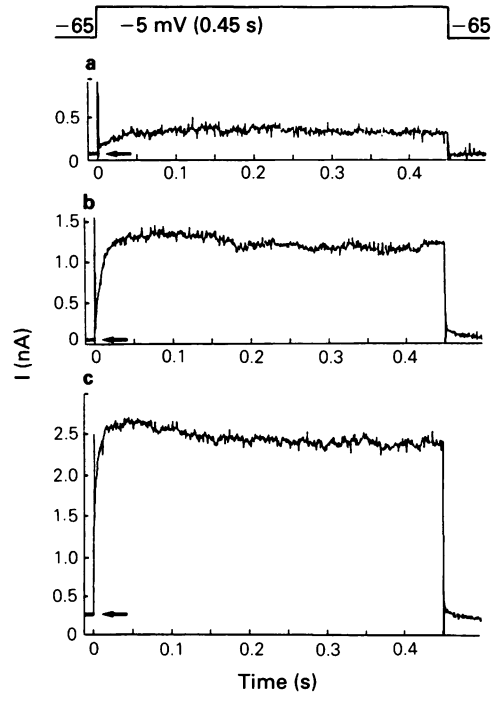

Figure 6 Effect of 0.1 μM niguldipine on the outward currents of a vascular myocyte from the portal vein. The 450 ms clamp steps ranged from −65 mV to −5 mV. (a) Before, (b) 5 min and (c) 10 min after application of the drug.

increased the outward current during the pulse from 0.3 to 1.3 nA and induced a decaying outward directed tail current upon repolarization to −65 mV. The 10 min exposure to niguldipine (Figure 6) increased the holding current (current before the clamp step, marked by arrow), the current during the pulse (maximal 2.5 nA, 50 ms after the start of the depolarization, 1.9 nA for I_L), as well as the tail current upon repolarization. After an exposure time of 15 min, the current was similar to the one recorded at 10 min.

Figure 7 shows the effects of 0.1 μM niguldipine on the outward currents of a pial vascular myocyte. Many of these pial cells are very small in size: the cell of Figure 7 was only 40 μm long and less than 5 μm thick. As expected for such a small cell, the membrane currents were in the range of pA. Thus, the contribution of currents through individual channels can be visualized in the record. A 5 min exposure to 0.1 μM niguldipine induced the following changes: the holding current at −65 mV increased from 1 to 20 pA (marked by the arrow). The current during the pulse was on average larger than before

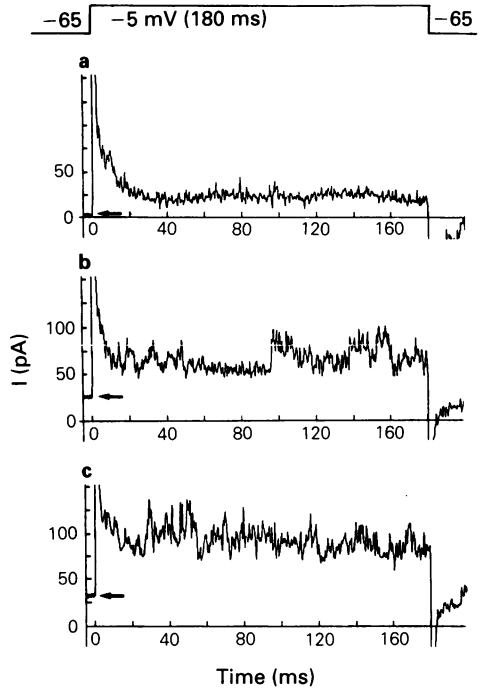

Figure 7 Effect of 0.1 μM niguldipine on the outward currents of a pial vascular myocyte. The 180 ms clamp steps were from −65 mV to −5 mV. (a) Before, (b) 5 min and (c) 7 min after application of the drug.

the application of the drug (50 pA in comparison to 20 pA). In addition, step-like current-jumps with an amplitude of 20–30 pA were evident in the current trace, which can be attributed to the opening and closing of channels with a high conductance. Seven min exposure to 0.1 μM niguldipine further increased the outward currents both at the holding potential (25 pA) and during the pulse (70 pA).

Time course and reversibility of the increase in outward current At all concentrations, niguldipine induced the increase in outward currents with a delay of several min. For a test depolarization to +20 mV and a drug concentration of 0.1 μM, the first significant increments in outward current were recorded 2–4 min after application of niguldipine. This latency is significantly longer than the time required to change the bath fluid completely (20 s). Usually, niguldipine increased the outward current with a 'biphasic' time course: after a first increment, a second, larger increment was frequently recorded about 10 min after drug application. The time course of the increase in outward current did not depend on the frequency of depolarizing clamp-pulses, and in cells that remained 'un-pulsed' at a holding potential of −65 mV, niguldipine increased the outward currents as well.

The niguldipine-induced outward current was largely reversed by drug removal, provided the concentrations remained below 0.1 μM and the exposure time was shorter than 10 min. When niguldipine was applied at 1 μM and/or for 20 min, the effects were only partially reversible after washing in drug-free solution for up to 1 h. Frequently, the cells became 'leaky' during the wash-out, i.e. for the holding potential −65 mV, a rather large negative holding current was required.

The drug-induced current is a potassium current The outward current induced by niguldipine might be carried by K or by Cl ions (an inflow of negative charge would generate an outward current). These 2 possibilities can be distinguished on the basis of the voltage-dependence of the drug-induced current, especially by its reversal potential. The I-V curves from a representative experiment are illustrated in Figure 8. Figure 8a shows an I-V curve from a smooth muscle cell isolated from the portal vein. Under control conditions, the curve is very flat and nearly matches the zero current line between −100 and −20 mV. Positive to −20 mV, the curve bends up showing 'outward rectification'. Five min administration of 0.3 μM niguldipine more than doubled the outward currents at potentials more positive than −20 mV. For more negative clamp potentials, however, the I-V curve was almost unchanged i.e. there was no intersection of the 2 curves and therefore no reversal potential. A 15 min exposure to 0.3 μM niguldipine increased the current further over the potential range +50 mV to −85 mV. At the reversal potential of −85 mV, the 2 curves intersected due to more negative currents at −100 mV. The reversal potential of −85 mV is close to the calculated potassium equilibrium potential of −84 mV. This finding supports the hypothesis that the drug-induced current is a potassium current. Using the nomenclature of Weir & Weston (1986a), we would like to conclude that niguldipine exerts K-agonistic effects on the vascular smooth muscle cells, but, the conclusion has to be proven by single-channel analysis.

Figure 8b supports the above conclusion with an experiment from a pial cell. Seven min after application of 0.3 μM niguldipine, an I-V curve was measured that lies above the control curve within the potential range between +50 mV to −75 mV. For more negative potentials, the I-V curve measured in the presence of niguldipine lies below the control curve. Thus, in this experiment the reversal potential was −75 mV. Again, this value is close to the calculated potassium equilibrium potential, supporting

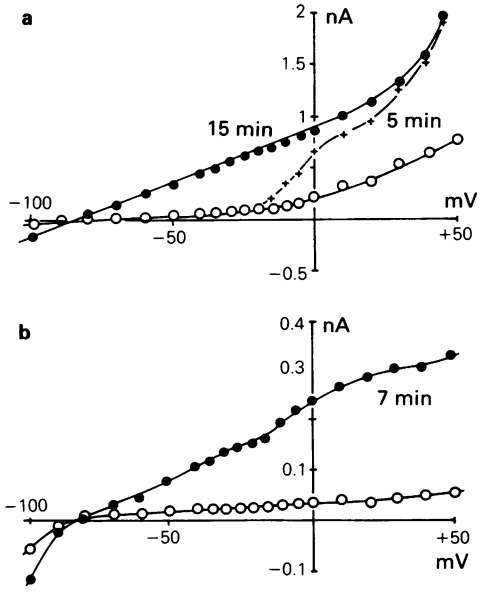

Figure 8 Effect of 0.3 μM niguldipine on the current-voltage curves of the late outward currents. Abscissa scale: pulse step potential. Ordinate scale: current measured at the end of the 180 ms clamp step, the holding potential was −65 mV. (a) Smooth muscle cell from the portal vein; (○) before, (+) 5 min, (●) 15 min after application of the drug. (b) Smooth muscle cell from the pial vessels; (○) before, (●) 7 min after application of the drug.

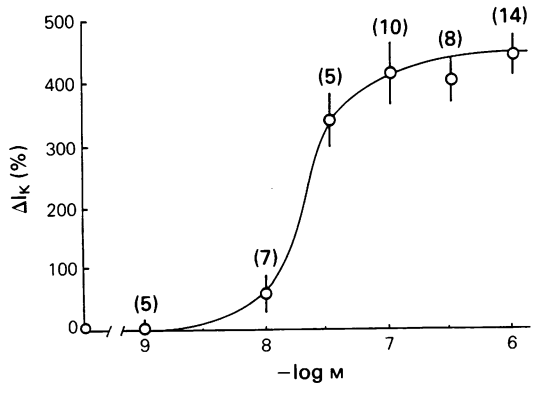

Figure 9 Concentration-response curve for the effect of niguldipine on potassium current. The current was measured with clamp steps to between −5 and +20 mV. Ordinate scale: effect of niguldipine expressed as $(*I_K - I_K)/I_K$ where I_K and $*I_K$ are the currents before and after application of niguldipine. The −log of the drug concentration is plotted on the abscissa scale. Each point represents the mean of the number of experiments indicated in parentheses and vertical lines show s.e.mean.

the view that the drug-induced current is a potassium current.

The concentration-effect curve for the facilitation of I_K by niguldipine The dependence of the increase in potassium outward current on the concentration of niguldipine was evaluated from a total of 49 vascular myocytes. As described above, the effect started with a long latency and needed about 10 min to reach steady-state. Since the small myocyte survives on the patch electrode only 15–30 min, most of the data were taken from a single application of the drug to one cell; only in 8 cells were 2 concentrations tested in a cumulative manner.

The first significant increase in outward current was measured at a nominal concentration of 10 nM. This concentration is considered to be the threshold concentration, it increased I_K of pial myocytes by 55 ± 30% ($n = 3$), I_K from the myocytes of the portal vein by 50 ± 25% ($n = 4$). At a concentration of 100 nM, the increase in I_K was 420% ($n = 3$) and 410% ($n = 7$), respectively. Maximal effects were observed with nominal 1 μM niguldipine, the increase was 460% (pial myocytes, $n = 4$) and 440% (myocytes from the portal vein, $n = 10$), respectively. From the concentration-effect curve of Figure 9 we extrapolated a half-maximal nominal concentration of 20 nM. The curve shown in Figure 9 is based on pooled data. Data were calculated from the current I_L recorded during the clamp step to potentials of −5, +10 or +20 mV. In addition, data from myocytes of the portal vein and of the pial vessels were pooled together since the niguldipine-sensitivity of these two cell types was almost identical (see above).

Discussion

Niguldipine lowers the blood pressure in animals, most probably via a reduction in peripheral vascular resistance (Sanders & Kolassa, 1986; Fischer *et al.*, 1987; Sanders *et al.*, 1987). Our studies on the effect of niguldipine have shown that calcium and potassium currents are modified in such a way that vasorelaxation can be explained at the level of the single myocyte.

Niguldipine influenced I_{Ca} in a similar manner to other dihydropyridines. That is, the Ca-antagonistic

effect showed a steep concentration-dependence with an EC_{50} of nominally 0.4 μM (effective concentrations 0.1–0.2 μM). As shown for nitrendipine and Bay K 8644 (Thomas *et al.*, 1984; Brown *et al.*, 1986), we found a 'Ca-agonistic effect' on niguldipine in concentrations between 50 nM and 200 nM. Thus, the concentration-effect curve of Figure 5 is typical for dihydropyridines.

The Ca-antagonistic effect of niguldipine was characterized by a large initial block (about 62%) on which only a small use-dependent component was superimposed. Thus, block of I_{Ca} by niguldipine (or other dihydropyridines) does not require the Ca-channel to open, whereas block by Ca antagonists of the verapamil-type does (e.g. Klöckner & Isenberg, 1986). Also in line with the known influence of dihydropyridines is the effect on the steady-state inactivation-curve. A similar shift of the activation curve was expected (ct. ref. Lee & Tsien, 1983) but was not found in the present experiments.

In vascular smooth muscle cells, a major part of potassium conductance is activated by an increase in intracellular calcium concentration $[Ca^{2+}]_c$ (Benham *et al.*, 1986). Thus, the facilitation of potassium current could 'secondarily' arise from an increase in cytosolic $[Ca^{2+}]$ as it ought to result from the increase in I_{Ca} (measured between 50 and 200 nM niguldipine). We consider the Ca-agonism unlikely to be the only causal mechanism by which niguldipine stimulates potassium currents, since the concentration-dependence of the two effects is quite different. Stimulation of I_K started at 10 nM niguldipine, which is 5 fold lower than the threshold concentration for the modulation of I_{Ca}. Maximal facilitation of I_K was found at 1 μM niguldipine, a concentration that blocked I_{Ca}. Finally, the increase in I_K by niguldipine was recorded at a constant holding potential of -65 mV, i.e. it was observed under conditions that did not activate I_{Ca}.

The increase in I_K is expected to move the membrane potential towards the potassium equilibrium potential which is thought to hyperpolarize most smooth muscle cells. In addition, facilitation of I_K should stabilize this negative membrane potential, thereby reducing excitability (e.g. in smooth muscle cells of small vessels, such as the pial cells investigated here). In the non-excitable cells of large arteries, the depolarizing effects of neurotransmitters will be reduced. In this paper, we did not demonstrate the expected hyperpolarization, as the isolated myocyte does not suit those measurements. The input resistance of the isolated cell is 3 GΩ (myocytes from portal vein) or 5 GΩ (pial myocytes), thus a small fluctuation in current (e.g 2 pA) changes the membrane potential appreciably (6 or 10 mV, respectively). The fluctuations in current could originate as an artifact of the amplifier system, or physiologically, by the opening of one or two single potassium channels (see Figure 7). Studies with conventional microelectrodes, applied to multicellular tissue where many thousands of cells are coupled to each other, may bypass this problem. The preparations have a much smaller input resistance and the effects originating from the channel fluctuations are bypassed by spatial superimposition. Thus, the postulated hyperpolarization should be tested by future complementary studies.

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