Effects of cicletanine on whole-cell currents of single smooth muscle cells from the guinea-pig portal vein

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1 Smooth muscle cells of the guinea-pig portal vein were dispersed by enzymatic treatment and recordings of membrane currents were made in the whole-cell mode by the patch-clamp technique. The effects of extracellular application of cicletanine-hydrochloride on the whole-cell currents of isolated smooth muscle cells from the guinea-pig portal vein were studied in solutions containing a normal concentration of calcium (2.5 mM).

2 Cicletanine, 10 to 100 μ M, reduced the voltage-dependent inward calcium current with an IC_{50} of 250 μ M. These effects of cicletanine were reversible.

3 The action of cicletanine on calcium currents can be interpreted as a decrease of the availability of calcium channels but not by an alteration of the time course or voltage-dependency of inactivation.

3 The control calcium current was enhanced by application of Bay K 8644. On this enhanced inward current, cicletanine also exerted inhibitory effects which were not use-dependent.

5 Cicletanine, 1 to 100 μ M, did not enhance outward potassium currents.

6 It is concluded that at least one component of the vasorelaxant effects of cicletanine is produced by inhibition of calcium currents.

Keywords: Cicletanine; vascular smooth muscle; voltage-clamp; calcium currents; potassium currents

Introduction

In rabbit aorta and rat portal vein the diuretic and antihypertensive drug, cicletanine, reduces the contraction induced by membrane depolarization (high K^+ concentration) (Baranes *et al.*, 1983). Cicletanine also antagonizes the contractions caused by noradrenaline of rabbit isolated iliac artery and guinea-pig portal vein (Noack, 1992) and relaxes human epigastric arteries precontracted with 25 mM or 80 mM potassium-rich solution (Ebeigbe & Cabanie, 1991). Furthermore in frog ventricular myocytes, cicletanine inhibits isoprenaline-enhanced inward calcium (Ca) currents (Gisbert *et al.*, 1988). Since Ca^{2+} ions play a determinant role in vascular contractility, these data suggest that a reduction of Ca^{2+} entry through Ca-channels could be one of the mechanisms by which cicletanine could counteract an increase in cytosolic free Ca^{2+} concentration.

In this paper we report that cicletanine inhibits the inward calcium current (I_{Ca}) in smooth muscle cells from guinea-pig portal vein and reduces the enhanced Ca-current generated by exposure to (-)-Bay K 8644. Some of the results were previously presented as an abstract (Noack *et al.*, 1991).

Methods

Cell preparation

All experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male guinea-pigs, previously killed by stunning and bleeding. The portal vein (about 20 mm length) was carefully cleaned of fat and connective tissue with fine scissors and microscopic control. The tissue was incubated in a nominally Ca^{2+} -free physiological salt solution (PSS) for 30 min. Consecutive treatment of the tissue with 1 ml of a modified PSS (for details see Lammel *et al.*, 1991) containing purified collagenase (Sigma, Type VII, 100 units ml^{-1}) and the same solution containing papain (Sigma, 20 units ml^{-1}) and a collagenase inhibitor (phosphoramidon, Sigma, 200 μ g ml^{-1})

provided isolated contractile cells. The cells were used for experiments within 12 h after isolation. During this time they were stored at 4°C in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). During all experiments the organ bath temperature was held at 26°C.

Single-cell voltage-clamp

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was employed to voltage-clamp the smooth muscle cells. Patch pipettes were pulled from Pyrex glass (Jencons H 15/10) and had resistances of 3–4 M Ω when filled with the intracellular solution.

Low-pass filtered signals (10 kHz) were digitized at 5 kHz with AXOLAB 1100 hardware and an IBM microcomputer in conjunction with pCLAMP 5.5 software (Axon Instruments). Currents were displayed on the computer monitor and hard copies were obtained with a plotter (Taxan). Calcium currents were assumed to be completely and specifically blocked by addition of 0.5 mM cadmium to the extracellular solution. Unless indicated, the cadmium (0.5 mM)-resistant current (leakage and background current) was not subtracted either from the current traces or from the current-voltage relationships presented in this paper. Only for calculation of the effect of cicletanine on the calcium current, was the current recorded in the presence of cadmium (0.5 mM) digitally subtracted from total current. Recordings were evaluated from a total of 25 cells and from each of these continuous measurements were made for at least 30 min.

Drugs and solutions

The PSS in the bath had the following composition (mM): NaCl 125, KCl 4.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, HEPES 10, pH 7.3. The pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, pyruvic acid 2, succinic acid 5, pH 7.3. In those experiments in which calcium currents were recorded, potassium in the pipette solution was replaced by equimolar amounts of caesium. The reagents used were ($\pm$)-cicletanine hydrochloride (IHB,

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Paris, France), nifedipine and (-)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer AG, Germany) and cadmium chloride (Sigma). To reduce any solvent effects on membrane currents, cicletanine was dissolved in dimethyl sulphoxide (DMSO) as a concentrated stock solution (2 M). From this, dilutions with water were prepared immediately before application to the cells to reduce the possibility of cicletanine precipitation.

Results

Interaction of cicletanine with net whole-cell currents

In those experiments in which the pipette solution did not contain caesium ions (potassium pipettes), the whole-cell currents were mainly carried by calcium and potassium ions. Representative examples of a whole set of current recordings are shown in Figure 1a which illustrates the typical size and time course of the whole-cell currents in a bathing solution containing 2.5 mM Ca^{2+} . The currents were elicited with 500 ms depolarizing voltage-clamp pulses from a holding potential of -90 mV to test potentials in the range -80 to $+50$ mV in increments of 10 mV. At potentials positive to -30 mV, the initial capacitive surge was followed by a

transient net inward current that peaked in about 10 ms. This inward current then declined and reversed to become an outward current. The major part of outward current in these cells was also present in a calcium-free bathing solution (Noack *et al.*, 1990). The family of current traces shown in Figure 1a illustrates the general voltage-dependency of the inwardly- and outwardly-directed current in this tissue.

Within 3 min of adding cicletanine ($10 \mu\text{M}$) to the bathing solution, inwardly-directed currents became smaller than under control conditions (Figure 1b), whereas the outward currents elicited at each potential were little affected. A more detailed analysis of the effects of cicletanine in this vascular preparation was derived from the construction of the current-voltage relationships ($I-V$ curves) for the peak inwardly-directed (Figure 2a), the peak outwardly-directed (Figure 2b) and the current measured 500 ms after the depolarizing step (Figure 2c). As can be seen from Figures 2a–c the effects of cicletanine were exerted on the peak inwardly-directed currents and with essentially no effect on either the peak outwardly-directed currents or on the current at the end of the 500 ms depolarizing step.

The inhibitory effect of cicletanine on the peak inwardly-directed current could be due to inhibition of calcium channels. To evaluate this hypothesis, further experiments were carried out with pipettes in which the potassium ions were replaced by equimolar amounts of caesium ions.

Figure 1 Examples of whole-cell currents (potassium pipette) generated by a smooth muscle cell from guinea-pig portal vein (a) and the effect of cicletanine (b). The cell was bathed in a physiological salt solution containing 2.5 mM calcium. It was held at -90 mV and stepped for 500 ms to the test potentials (TP) as indicated. The arrows indicate zero current.

Figure 2 Current-voltage relationships for (a) peak inwardly-directed current, (b) peak outwardly-directed current and (c) current measured after 500 ms of the depolarizing voltage step. Mean values obtained with potassium pipettes and derived from 3 cells are shown. In (a) the control values (●), the effects of cicletanine 10 μ M (Δ) and 50 μ M (■) are given. In (b) and (c), the effects of cicletanine 50 μ M were essentially the same as those for 10 μ M and have been omitted from the diagrams for clarity.

Interaction of cicletanine with inward calcium currents

Representative records illustrating the size and time-course of the whole-cell currents in a bath solution containing 2.5 mM Ca^{2+} and with a caesium-loaded pipette are shown in Figure 3a. Note the four fold difference between the current calibration in Figures 3 and 1. The current traces were elicited with 500 ms depolarizing voltage-clamp pulses from a holding potential of -90 mV to test potentials of -80 to $+50$ mV in increments of 10 mV. At potentials positive -30 mV, the initial capacitive surge was followed by a transient net inward current that peaked in about 10 ms and then declined to a steady current level. This family of current traces illustrates the voltage-dependency of the inward current generated by this tissue and shows that the maximum inward current was obtained at test potentials between $+10$ mV and $+20$ mV. Within 3 min of adding cicletanine (10 μ M) to the bathing solution, inwardly-directed currents were visibly reduced compared with control conditions (Figure 3b).

As can be seen from the current records shown in Figure 3a and b, the current at positive potentials became outward indicating a contamination of the net current by current components which were not carried by calcium ions. To identify the true calcium inward currents and to eliminate contaminating background currents from current traces, extracellular application of cadmium (0.5 mM) was used to produce complete block of calcium currents. The values of the peak inward currents under control conditions, and in the presence of cicletanine and of cadmium were transferred to a current-voltage relationship to allow the individual inhibitory effects of both cicletanine and of cadmium on inward currents to be determined (Figure 4). Cadmium inhibited the inward current throughout the voltage range allowing the CdCl_2 -sensitive component of the net inward current to be isolated by subtracting the CdCl_2 -insensitive current (background and leakage current) from the net current. Previous studies in various smooth muscle tissues have shown that the cadmium (0.5 mM)-sensitive current represents calcium current flow through voltage-gated calcium channels (Matsuda *et al.*, 1990; Noack *et al.*, 1992).

For calculation of the inhibitory effect of cicletanine on inward calcium currents, cadmium-insensitive currents were subtracted from current values. As illustrated in Figure 4, cicletanine inhibited the inward calcium current in a concentration-dependent manner, over the potential range from -30 mV to $+50$ mV. Although the peak current was reduced, the time-course of its inactivation was not greatly affected by cicletanine (Figure 3a and b). The reduction of inward current induced by cicletanine (10–100 μ M) was marked but not complete. The concentration of cicletanine necessary to produce 50% inhibition of the peak inward current was estimated to be approximately 250 μ M (Figure 5).

To determine whether cicletanine produced a change in the voltage-dependency of inactivation, three different holding potentials were chosen (-90 mV, -30 mV and -10 mV) from which current traces were elicited with 500 ms depolarizing voltage-clamp pulses to test potentials of -80 mV to $+50$ mV. Inactivation of I_{Ca} occurred when the chosen holding potentials were -30 mV and -10 mV (Figure 6a–c). This experimental protocol was performed either under control conditions or in the presence of cicletanine (30 μ M). When the peak inward current of each curve was plotted against each applied holding potential, the steady-state inactivation curves of I_{Ca} under both conditions could be constructed. Under control conditions the current was half available at $V_h = -30$ mV, and the slope, V_s , was 7.1 mV, when a Boltzmann equation of the form

$$I/I_{\text{max}} = (1 + \exp((V - V_h)/V_s))^{-1}$$

was fitted to the inactivating component of the current. V is the holding potential which conditions the inactivation process. I/I_{max} is the fraction of current available relative to that available from -90 mV. Cicletanine (30 μ M) reduced the

Figure 3 Examples of whole-cell currents (caesium pipette) of a smooth muscle cell from guinea-pig portal vein (a) and effect of cicletanine (b). The cell was bathed in a physiological salt solution containing 2.5 mM calcium. The cell was held at -90 mV and stepped for 500 ms to the test potentials (TP) as indicated. The zero current is indicated by arrows.

Figure 4 Current-voltage relationship for net peak inward current (caesium pipettes) activated from a holding potential of -90 mV. Control ($\circ$), effect of cicletanine 3 μM ($\blacksquare$) and 30 μM ($\square$) and effect of cadmium 0.5 mM (Δ). Ordinate scale: peak inward current. Abscissa scale: test potential. Data points are means derived from four cells. The curves are fitted by eye.

peak current evoked at each potential by stepping from each of the three holding potentials, with little change in V_h (-37 mV) or V_s (8.2 mV) (Figure 6d). This indicates that cicletanine exerts its inhibitory effects on I_{Ca} by reducing the availability of calcium channels and not by changing their inactivation parameters.

Interaction between cicletanine and Bay K 8644

In guinea-pig portal vein, L-type channels are the predominant type of calcium channel present. To test more directly whether cicletanine inhibits such channels, (-)-Bay K 8644, was used to increase calcium channel availability (Figure 7a). Peak inward currents increased two or three fold when (-)-Bay K 8644 (100 nM) was applied to the bathing solution. With a double pulse protocol used in the same cells, a pair of two inward currents was elicited every 15 s and the peak inward currents obtained at the two test potentials (0 mV and $+20$ mV) were plotted against time (Figure 7b). When the double pulse protocol was applied to the cells, the inward current which developed under control conditions at $+20$ mV was smaller than that seen at 0 mV, although the peak of the $I-V$ curve was near $+20$ mV (Figure 7a). This indicates that the chosen time interval between the two depolarizing pulses was short enough to produce partial inactivation of I_{Ca} (50%).

When (-)-Bay K 8644 (100 nM) was added to the bathing solution, the inward current at both depolarizing pulses increased, although the increase associated with the first pulse was larger than that produced by the second stimulus (Figure

Figure 5 Inhibition by cicletanine of peak inward (cadmium-sensitive) current evoked at +10 mV test potential when stepped from a holding potential of -90 mV. Data points are mean values with s.e.mean. The dashed vertical line indicates the IC_{50} concentration when data points were fitted by eye and the curve was extrapolated. The numbers near data points indicate numbers of cells investigated.

7b). As expected, the inward current elicited by the first depolarizing pulse was of a similar magnitude to that obtained with the experimental protocol of Figure 7a. However, the current elicited by the second pulse was only approximately 50% of the magnitude expected from the $I-V$ curve obtained with a single pulse protocol. This indicates that under control conditions and in the presence of Bay K 8644 the portion of channels being inactivated is similar and that Bay K 8644 neither changes the voltage-dependency of the steady-state inactivation nor the time for the recovery from inactivation at this membrane potential (-60 mV) which was in both cases 730 ms. In the presence of Bay K 8644 cicletanine (30 μ M to 100 μ M) inhibited the Bay K-augmented inward calcium current. This result indicates that cicletanine interacts with dihydropyridine-sensitive L-type calcium channels. Furthermore inhibition of the partially-inactivated inward current by cicletanine indicates that there is some affinity of cicletanine for the inactivated state of the channels. An increase of block by repeatedly applying such depolarizing pulses (use-dependency) was not observed in the presence of cicletanine (Figure 7b).

Discussion

The results described in this paper indicate that cicletanine blocks the calcium currents in single isolated vascular smooth muscle cells. However, it does not seem to act as a vasodilator via the opening of potassium channels which was suggested by Ebeigbe & Cabanie (1991). The potency of cicletanine was 10–250 times lower than that of nifedipine, verapamil, diltiazem, flunarizine or pinaverium (Terada *et al.*, 1987a,b; Beech *et al.*, 1990).

Only a very small negative shift in the steady-state inactivation curve of inward current was observed with cicletanine. Under voltage-clamp conditions dihydropyridines like nifedipine cause a distinct negative shift of the steady-state inactivation curve (Noack *et al.*, 1992). Such a lack of

Figure 6 Effect of holding potential and of cicletanine (30 μ M) on calcium current. (a–c) Current-voltage relationships for peak inward current elicited from the three holding potentials (HP: -90 mV, -30 mV and -10 mV respectively) under control conditions (●) and in the presence of the drug (▲). Data points for these three plots represent mean values from three cells (s.e.mean in each case within the size of symbols). The calcium current was isolated from the total current by subtraction of the cadmium-resistant current. (d) Effects of cicletanine (30 μ M) on the voltage-dependence of the steady-state inactivation of the calcium current. Peak values of currents were plotted against holding potentials. To each set of data points a Boltzmann equation was fitted (continuous curves). Voltages for half-inactivation (V_h) and slope factors (V_s) characterizing these equations are given at each curve.

Figure 7 Effects of (a) (-)-Bay K 8644, 100 nM and (b) cicletanine in the presence of (-)-Bay K 8644 on whole-cell calcium currents in isolated cells from rat portal vein. Each point shows the mean value derived from two cells. (a) A current-voltage relationship for the peak inward current under control conditions (●) was determined using depolarising steps from a holding potential of -60 mV to the different test potentials as indicated. (b) The cells were then stimulated every 15 s with a double pulse protocol (see insert for details) and the peak inward currents generated by the first (Δ) and the second pulse ($\blacktriangle$) were determined. With this double pulse protocol, the currents elicited at each first test pulse were comparable in magnitude to those obtained with the experimental protocol of (a). However, the current elicited at each second pulse was partially inactivated. In the presence of (-)-Bay K 8644, a second current-voltage relationship was determined (a, $\square$). The double pulse protocol was then used to determine the effects of cicletanine on (-)-Bay K 8644-enhanced calcium currents (b, Δ , $\blacktriangle$).

shift could indicate that cicletanine has similar affinities for both the closed, available state and for the inactivated state of the calcium channel. Holding the cell at a depolarized potential, which increases the fraction of channels in the inactivated state with a corresponding reduction of those in the closed state, did not increase the affinity for the channel. This is in contrast to the action of nifedipine on calcium current (Noack *et al.*, 1992). In the presence of nifedipine, an increasing affinity of the drug for the channel is characterized by an increasing reduction of current at depolarizing holding potentials. Such differences between drugs might be of great importance in their therapeutic use, in respect to the different patterns of electrical activity reported for a variety of smooth muscle organs.

In some respects, the actions of cicletanine on guinea-pig portal vein, are similar to those reported for pinaverium on the longitudinal muscle layer of the rabbit jejunum (Beech *et al.*, 1990). Both substances have little effect on the Ca^{2+} inactivation curve, neither is a dihydropyridine nor exhibits the use-dependency characteristic of D600, verapamil and diltiazem (Terada *et al.*, 1987b; Hering *et al.*, 1988).

In some smooth muscles two or more types of voltage-dependent calcium channel have been distinguished on the

basis of their different unitary conductances and their modulation by several drugs (Bean *et al.*, 1986; Loirand *et al.*, 1986; Benham *et al.*, 1987; Ganitkevich & Isenberg, 1990). It has also been reported that different calcium current components can be identified by use of different holding potentials (Bean *et al.*, 1986). In the present study, however, distinct populations of calcium channel were not observed and no specific component of inward current was identified in the presence of cicletanine. From this and from the action of cicletanine in the presence of (-)-Bay K 8644 it seems reasonable to conclude that cicletanine acts on the L-type calcium channel.

The results of the present study indicate that cicletanine is a calcium channel blocking drug. In vascular smooth muscle, current flow through this channel is responsible for contractions initiated by spike discharges. Although cicletanine also inhibits nifedipine-resistant contractions of guinea-pig isolated portal veins, precontracted with noradrenaline (Deitmer *et al.*, 1991), the inhibitory action of cicletanine on L-type calcium channels observed in the present study seems likely to contribute to its vasorelaxant action previously described in several *in vitro* studies (Baranes *et al.*, 1983; Ebeigbe & Cabanie, 1991; Noack, 1992).

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