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# A PATCH-CLAMP STUDY OF THE EFFECTS OF CICLETANINE ON WHOLE-CELL CALCIUM CURRENT IN VENTRICULAR MYOCYTES

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**Summary:** *The effects of extracellular application of cicletanine on the voltage-sensitive calcium current ( $I_{Ca}$ ) was studied in isolated cells from frog ventricle. Myocytes were isolated by enzymatic dissociation and  $I_{Ca}$  was measured using the whole-cell configuration of the patch-clamp technique modified to permit intracellular perfusion with various substances. Cicletanine (10 to 100  $\mu$ M) had no effect on control  $I_{Ca}$ . However, when  $I_{Ca}$  was enhanced by superfusion of the cell with saturating doses of  $\beta$ -adrenergic agonist (isoprenaline, 2  $\mu$ M) or by intracellular perfusion with maximal doses of cAMP (20  $\mu$ M), cicletanine exerted a dual effect on  $I_{Ca}$ . At 10  $\mu$ M, cicletanine generally induced a transient or sustained stimulation of  $I_{Ca}$  (5 to 40%), while 100  $\mu$ M of the drug generally reduced  $I_{Ca}$ . The effects of cicletanine were reversible and not voltage-dependent. These results suggest that cicletanine affects  $I_{Ca}$  by acting on a mechanism occurring after cAMP synthesis, by enhancing cAMP concentration (e.g. through an inhibition of cAMP phosphodiesterase) or facilitating cAMP-dependent phosphorylation of the Ca channels.*

## Introduction

The new diuretic and antihypertensive drug, cicletanine, was shown to reduce contraction induced by membrane depolarization (KCl) or  $CaCl_2$  in rabbit aorta, mesenteric artery and rat portal vein (1). Cicletanine was also shown to antagonize the contractions caused by noradrenaline and phenylephrine on isolated rat aorta (2). Since  $Ca^{2+}$  ions play a determinant role in vascular contractility,

it has been suggested that cicletanine may interact directly and/or indirectly with membrane  $Ca^{2+}$  transport (3, 4). Ca influx through Ca channels may be one of several mechanisms by which cicletanine could partially counteract the increase in cytosolic free  $Ca^{2+}$  concentration induced by various agonists and membrane depolarization (3). In some respects, the contractile and diuretic effects of cicletanine resemble those of the atrial natriuretic factor (ANF). As the authors recently reported, ANF has strong inhibitory effects on Ca channels in frog ventricular myocytes (5, 6). Thus, it was of interest to study the effects of cicletanine on Ca current in cardiac cells, where Ca current has been relatively well characterized.

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## Materials and methods

The methods used for cell dissociation, whole-cell patch-clamp recording, superfusion and internal perfusion of the cells, and data analysis have been extensively described in previous papers (6-9) and were used without major modification in the present study.

Briefly, for routine monitoring of calcium current ( $I_{Ca}$ ), the frog (*Rana esculenta*) ventricular cell was depolarized every 8 s from  $-80$  mV holding potential to  $0$  mV for  $200$  ms. To accurately measure  $I_{Ca}$  with no contamination of other ionic currents, the cells were bathed in K-free,  $20$  mM Cs Ringer solution containing:  $88.4$  mM NaCl,  $20$  mM CsCl,  $22.9$  mM  $\text{NaHCO}_3$ ,  $0.6$  mM  $\text{NaH}_2\text{PO}_4$ ,  $1.8$  mM  $\text{CaCl}_2$ ,  $1.8$  mM  $\text{MgCl}_2$ ,  $5$  mM D-glucose,  $5$  mM sodium pyruvate and  $0.3$   $\mu\text{M}$  tetrodotoxin (Sankyo, Japan). The standard internal solution in the patch-electrode ( $1-3$  megohms resistance) contained:  $120$  mM CsCl,  $5$  mM  $\text{K}_2\text{EGTA}$ ,  $4$  mM  $\text{MgCl}_2$ ,  $5$  mM  $\text{Na}_2\text{CP}$ ,  $3$  mM  $\text{Na}_2\text{ATP}$ ,  $0.4$  mM  $\text{Na}_2\text{GTP}$ , adjusted to pH  $7.1$  with KOH. Solutions were applied to the exterior of the cell by placing the cell at the opening of  $250$   $\mu\text{m}$  inner diameter capillary tubing (flow rate of  $10$   $\mu\text{l}/\text{min}$ ) (7). All external solutions were gassed with  $95\%$   $\text{O}_2$  and  $5\%$   $\text{CO}_2$  (pH =  $7.4$ ) and experiments were carried out at room temperature ( $19.5-22.5^\circ\text{C}$ ). Solutions were applied to the interior of the cell via the patch electrode which could be perfused and thus permitted modification of the internal solution (8, 9). Under these conditions, the current traces were digitized at  $10$  KHz (12-bit A/D converter) by a Compaq 286 Desk-Pro computer and  $I_{Ca}$  was measured on-line, using programs written in the Pascal language, as the difference between the peak inward current and the current at the end of the  $200$ -ms pulse (7).

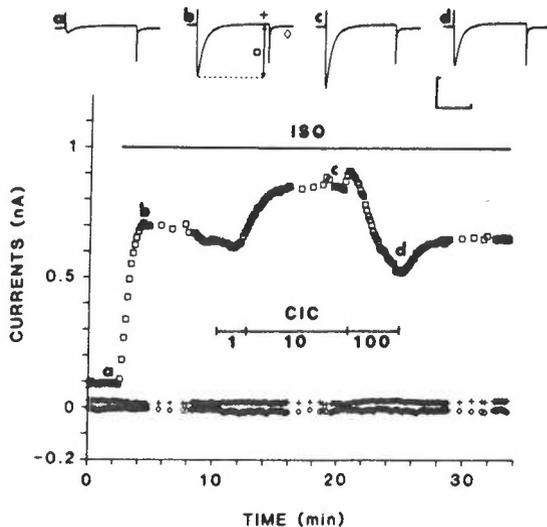
**Drugs.** ( $\pm$ )-Isoprenaline and cyclic AMP were obtained from Sigma Chemical Co. (USA). ( $\pm$ )-3-(4-Chlorophenyl)-1,3-dihydro-7-hydroxy-6-methylfuro(3,4-c)pyridine (cicletanine hydrochlor-

ide) was provided by IHB Laboratories (Le Plessis Robinson, France). Stock solutions ( $0.2$  M) of cicletanine were made from crystalline powder dissolved in DMSO. Control solutions contained identical amounts of DMSO as the cicletanine-containing solutions.

## Results

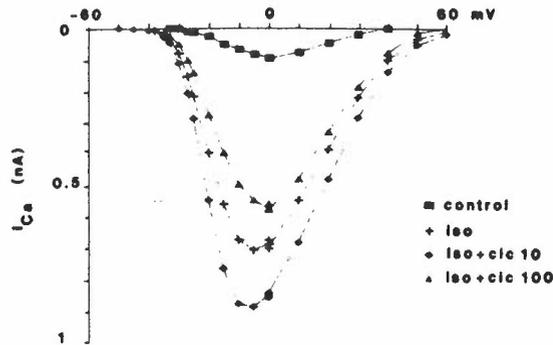
Frog ventricular cells possess only one type of calcium current (10, 11) which corresponds to the high-threshold L-type Ca channels (12), so that the  $I_{Ca}$  current elicited by a voltage-clamp depolarization from  $-80$  mV to  $0$  mV corresponds to a single population of Ca channels. The effects of  $1$  to  $100$   $\mu\text{M}$  concentration of ( $\pm$ )-cicletanine hydrochloride were tested on  $I_{Ca}$  measured every  $8$  s by a depolarizing pulse from  $-80$  mV holding potential to  $0$  mV for  $200$  ms. Cicletanine had negligible effects on the control  $I_{Ca}$ .  $I_{Ca}$  was  $98.24 \pm 9.5\%$  of control value (mean  $\pm$  s.e.m.,  $n = 10$ ) when the cell was superfused with  $10$   $\mu\text{M}$  cicletanine and  $101.13 \pm 10.3\%$  ( $n = 11$ ) with  $100$   $\mu\text{M}$  cicletanine. However, when  $I_{Ca}$  was increased by exposing the cell to  $2$   $\mu\text{M}$  isoprenaline (ISO), cicletanine had more pronounced effects (Fig. 1). At  $1$   $\mu\text{M}$  concentration, cicletanine was without significant effect on  $I_{Ca}$ , but at  $10$   $\mu\text{M}$  cicletanine strongly enhanced ISO-elevated  $I_{Ca}$ . A further increase in the dose of cicletanine to  $100$   $\mu\text{M}$  was accompanied by a strong inhibition of  $I_{Ca}$  following a transient initial stimulatory period. The holding current (at  $-80$  mV) and the quasi-steady-state current at the end of the depolarizing pulse to  $0$  mV were not modified by cicletanine, reflecting the absence of an effect of the drug on the electrogenic Na,K pump. The effects of cicletanine on  $I_{Ca}$  were essentially reversible (Fig. 1).

The current-voltage ( $I-V$ ) relationship for  $I_{Ca}$  was not significantly modified by cicletanine (Fig. 2). The control  $I-V$  curve was symmetrical with a maximal  $I_{Ca}$  amplitude observed around  $0$  mV membrane



**Fig. 1** Effects of cumulative doses of cicletanine (CIC) on membrane currents. The four current traces (top) were recorded at the times indicated by the corresponding letters on the bottom graph: (a) control; (b) 2  $\mu\text{M}$  isoprenaline (ISO); (c) ISO + 10  $\mu\text{M}$  cicletanine (CIC); (d) ISO + 100  $\mu\text{M}$  CIC. Current traces were recorded on and replayed from VCR magnetic tape (bandwidth DC to 20 KHz, 16 bit resolution) and digitized at 10 KHz. Calibration bars: horizontal 100 ms; vertical 400 pA. Each set of symbols (bottom) corresponds to measurements obtained by a depolarizing pulse made every 8 s from  $-80$  mV holding potential to 0 mV for 200 ms. Net amplitude of  $I_{\text{Ca}}$  (squares) was determined by the difference between peak inward current and the current at the end of the depolarizing pulse to 0 mV (i.e. quasi-steady-state current). Holding current at  $-80$  mV (diamonds) and the current at the end of the pulse (crosses) were obtained as indicated on the (b) current trace shown on the top. The cell was externally perfused with 2  $\mu\text{M}$  ISO in the absence or presence of increasing doses of CIC (1, 10 and 100  $\mu\text{M}$ ) during the periods indicated.

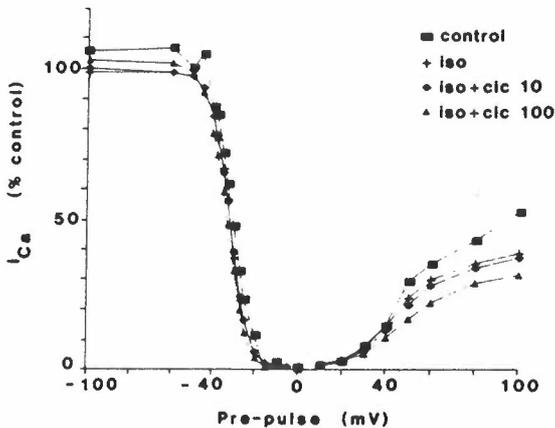
potential; 2  $\mu\text{M}$  ISO increased  $I_{\text{Ca}}$  about 6-fold ( $6.84 \pm 1.49$ ,  $n = 7$ ) and slightly shifted the maximal current potential by  $\sim 5$  mV in the negative direction, most probably due to an incomplete series resistance compensation (7). A 4-min exposure to 10  $\mu\text{M}$  cicletanine induced a further increase in  $I_{\text{Ca}}$  amplitude by  $\sim 25\%$  at each membrane potential, while a 4-min application of 100  $\mu\text{M}$  cicletanine reduced  $I_{\text{Ca}}$



**Fig. 2** Current-voltage ( $I$ - $V$ ) relationships of net  $I_{\text{Ca}}$ . The cell was depolarized from  $-80$  mV holding potential to various potentials for 200 ms, and the net  $I_{\text{Ca}}$  was determined as indicated in Fig. 1. Squares, control conditions; crosses, the cell was superfused with 2  $\mu\text{M}$  isoprenaline (ISO); diamonds, ISO + 10  $\mu\text{M}$  cicletanine (CIC); triangles, ISO + 100  $\mu\text{M}$  CIC.

elevated by ISO by  $\sim 20\%$ . These changes in  $I_{\text{Ca}}$  amplitude induced by cicletanine were not accompanied by any apparent modification in the kinetics of activation and inactivation of  $I_{\text{Ca}}$  (Fig. 1, top).

Inactivation and reactivation properties of  $I_{\text{Ca}}$  were studied in the presence and absence of cicletanine using double-pulse protocols. Inactivation (Fig. 3) was determined by examining the effect of 200-ms prepulses to various potentials on the response to a subsequent test pulse to 0 mV. Prepulses between  $-60$  mV and 0 mV progressively decreased  $I_{\text{Ca}}$  with complete inactivation being reached between  $-10$  and  $+10$  mV. With prepulses above  $+20$  mV, the test  $I_{\text{Ca}}$  progressively increased. The inactivation curve was not significantly modified by either ISO or cicletanine in the negative range of potentials. However, for membrane potentials above  $+20$  mV, ISO increased the amount of inactivation (7, 11);  $I_{\text{Ca}}$  inactivation by prepulses to  $+100$  mV was increased from 50% in the control to 65% in ISO. The addition of 10  $\mu\text{M}$  cicletanine to the ISO solution was without further effect on the inactivation curve. However, a larger dose of the drug (100  $\mu\text{M}$ ) further increased the

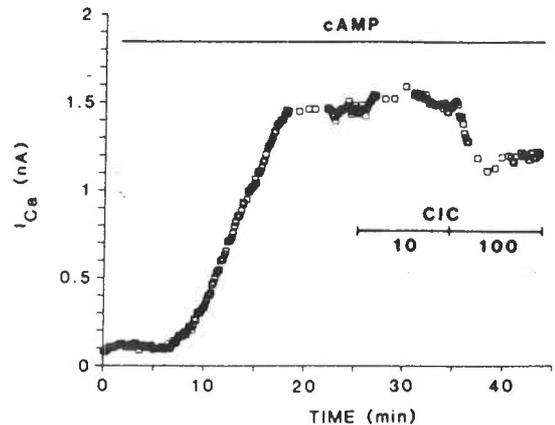


**Fig. 3** Inactivation curves of  $I_{Ca}$ . Inactivation was measured with a double-pulse protocol (see ref. 7 and text for more details). Symbols refer to the same cell and experimental conditions as in Fig. 2.

degree of inactivation induced by strong positive potentials. Such changes in the inactivation curve could result from an enhancement in the Ca-mediated inactivation process (13), possibly due to an increased intracellular  $Ca^{2+}$  concentration near the channels induced by high doses of cicletanine.

Recovery from inactivation was determined by measuring  $I_{Ca}$  elicited by a 200-ms test pulse to 0 mV that followed a 200-ms prepulse to 0 mV and a subsequent variable recovery interval at  $-80$  mV. As already reported (7, 11) reactivation of  $I_{Ca}$  occurred faster in control conditions than when  $I_{Ca}$  was increased by ISO. However, cicletanine (10 or 100  $\mu$ M) added to the ISO solution did not induce any additional modification in the reactivation of  $I_{Ca}$  (not shown).

The observation that cicletanine affected  $I_{Ca}$  only when it had been elevated by  $\beta$ -adrenergic stimulation with ISO raises questions about the mechanism(s) of action of the drug.  $\beta$ -Adrenergic agonists stimulate the activity of adenylate cyclase and increase the level of intracellular cyclic AMP (cAMP). The increase in cAMP is responsible for the  $\beta$ -adrenergic stimulation of  $I_{Ca}$ , by increasing

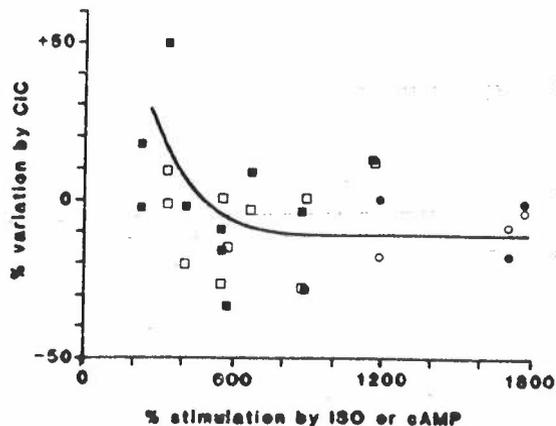


**Fig. 4** Effects of cicletanine on  $I_{Ca}$  enhanced by intracellular perfusion with cAMP. At time zero, the cell was perfused with control intracellular solution (see Materials and methods). After about 2 min, the solution in the patch electrode was switched to one containing 20  $\mu$ M cAMP and the new intracellular perfusion continued throughout the whole experiment. The cell was exposed to 10 and 100  $\mu$ M cicletanine (CIC) during the periods indicated.

the degree of phosphorylation of the Ca channels, since a similar action is seen when cAMP is perfused into the cell (7). In an attempt to clarify whether the action of cicletanine occurs before or after cAMP synthesis, the authors studied the effects of cicletanine on calcium current which was elevated by direct application of intracellular cAMP. In the experiment shown in Fig. 4, after the level of  $I_{Ca}$  had stabilized for a few minutes after disruption of the membrane patch, the solution in the patch-pipette was switched to one containing 20  $\mu$ M cAMP. Five minutes after the beginning of the perfusion with the new intracellular solution, the level of  $I_{Ca}$  started to increase, reflecting the diffusion of cAMP into the cell. When  $I_{Ca}$  stabilized at a value  $\sim 12$ -fold larger than the control, the cell was externally exposed to 10 and 100  $\mu$ M cicletanine; 10  $\mu$ M cicletanine induced a small and transient stimulation of  $I_{Ca}$  (5 to 20%), while 100  $\mu$ M induced a marked diminution.

Although the average stimulatory effects of 10  $\mu\text{M}$  cicletanine were smaller on cAMP-elevated  $I_{\text{Ca}}$  than on ISO-elevated  $I_{\text{Ca}}$ , the differences were not statistically significant. This would suggest that cicletanine acts on a mechanism which occurs after cAMP synthesis in the cascade of events leading to  $\beta$ -adrenergic agonist stimulation of  $I_{\text{Ca}}$ . A possible candidate for the stimulatory action of cicletanine may be an inhibition of cAMP hydrolysis. Such an inhibition would lead to local increases in cAMP levels stimulating the phosphorylation of Ca channels. An inhibition of cAMP phosphodiesterases would be expected to have different effects depending on: (i) the degree of stimulation of cAMP synthesis with  $\beta$ -adrenergic agonists; or (ii) the concentration of cAMP reached near the membrane when the cell is perfused with a cAMP-containing solution (14). Such variations in the increase of local cAMP concentrations would most probably reflect different stimulations of  $I_{\text{Ca}}$ , relative to control levels, by ISO or cAMP. Thus, if inhibition of cAMP phosphodiesterase is one mechanism of action of cicletanine, cells in which ISO- or cAMP-induced stimulations of  $I_{\text{Ca}}$  were the smallest would be expected to exhibit the largest relative stimulations by 10  $\mu\text{M}$  cicletanine (and the smallest inhibitions by 100  $\mu\text{M}$ ).

Figure 5 represents a summary of all the experiments conducted with externally applied ISO (0.1 or 2  $\mu\text{M}$ ) or internally applied cAMP (20  $\mu\text{M}$ ) when the cells were then exposed to 10 and/or 100  $\mu\text{M}$  cicletanine. The relative variations of  $I_{\text{Ca}}$  induced by cicletanine are plotted as a function of the relative stimulatory effect of ISO or cAMP on  $I_{\text{Ca}}$ . Figure 5 represents only steady-state effects of cicletanine, measured 3 to 5 min after exposure to the drugs. The analysis of the response to cicletanine was complicated by the presence of frequent initial transient stimulatory effects of the drug on  $I_{\text{Ca}}$ , probably due to two opposite effects taking place at the same concentration of cicletanine. This, as well as significant cell-to-cell variations in  $I_{\text{Ca}}$  amplitude (10, 11), may be partly responsible for the large scatter of the data. However, a slight negative corre-



**Fig. 5** Plot of the relative variations of  $I_{\text{Ca}}$  induced by cicletanine (CIC) (10  $\mu\text{M}$ , filled symbols; 100  $\mu\text{M}$ , open symbols) vs. the relative stimulations induced by isoprenaline (squares) or cAMP (circles). The line represents a fit by eye to the data obtained with 10  $\mu\text{M}$  CIC (i.e. filled symbols). The data represent only steady-state effects of cicletanine, observed after 3 to 5 min exposure to the drug

lation seemed to exist between the response to cicletanine and the relative stimulation of  $I_{\text{Ca}}$  by ISO or cAMP.

## Discussion

While cicletanine was without any significant effect on basal  $I_{\text{Ca}}$  levels, this antihypertensive drug exerted two opposite effects on Ca channels when their activity had been elevated by either the externally applied  $\beta$ -adrenergic agonist isoprenaline or intracellularly perfused cAMP. At 10  $\mu\text{M}$  concentrations, cicletanine induced a transient or sustained stimulation of  $I_{\text{Ca}}$ , while at 100  $\mu\text{M}$  the drug generally reduced  $I_{\text{Ca}}$ . However, a large scatter in the response of the cells to cicletanine was observed and the overall effect of the drug on  $I_{\text{Ca}}$  was relatively small (< 10% on average).

The dual effect of cicletanine may be due to: (i) the presence of non-specific effects at high concen-

trations; or (ii) the opposite actions on  $I_{Ca}$  of the two enantiomers (+) and (-). The second hypothesis could be verified by analysing the effects on Ca channels of each of the enantiomers in comparison with the action of the racemic form studied here.

An increase in  $I_{Ca}$  by cicletanine seen in some of the present experiments would lead to a more pronounced influx of  $Ca^{2+}$  ions which would result in a positive inotropic effect on the heart. In vascular smooth muscle, such an effect on sarcolemmal Ca transport would lead to an increase in the vascular contraction. This is somewhat surprising in view of the fact that cicletanine has been demonstrated to possess strong antihypertensive properties (1-3). Clearly, a description of the effects of cicletanine on vascular smooth muscle Ca channels would be required in order to resolve this paradox. However, a possible reason for a difference in the action of cicletanine on Ca channels in heart and smooth muscle cells could be due to the recent evidence that the cAMP-mediated regulation of Ca channels is probably absent in smooth muscle cells (15-17). Since it has been observed here that a stimulation of Ca channels activity by cAMP-dependent mechanisms was a prerequisite for the response of cardiac Ca channels to cicletanine, such a response to the drug may be absent in Ca channels from vascular smooth muscle cells.

The enhancement of  $I_{Ca}$  by 10  $\mu M$  cicletanine, seen in some of the present experiments, when the level of cAMP inside the cell was supposedly maximal, most likely reflects either an additional local increase in cAMP concentration (e.g. due to an inhibition of cAMP phosphodiesterase) or a facilitation of cAMP-dependent protein kinase which phosphorylates various proteins, including Ca channels. It should be noted that both of these actions would induce a relaxation of vascular smooth muscle. If cAMP hydrolysis were fast enough to locally reduce the concentration of cAMP available for the phosphorylation of Ca channels, then an inhibition of the cAMP-phosphodiesterase may increase local concentrations of the nucleotide and, thus,

enhance  $I_{Ca}$  (14). Although the authors do not have strong evidence in favour of such an inhibition of phosphodiesterase activity induced by cicletanine, the present data is consistent with such a hypothesis.

The effects of cicletanine on cardiac Ca channels are clearly different from the strong and sustained inhibitory action of ANF (5, 6). Whether these two substances share some common mechanisms at the cellular level in vascular smooth muscle cells remains to be explored.

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