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Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel

(toxins/contraction/cardiac cells/vascular relaxant)

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ABSTRACT **The venom of the black mamba contains a 60-amino acid peptide called calciseptine. The peptide has been fully sequenced. It is a smooth muscle relaxant and an inhibitor of cardiac contractions. Its physiological action resembles that of drugs, such as the 1,4-dihydropyridines, which are important in the treatment of cardiovascular diseases. Calciseptine, like the 1,4-dihydropyridines, selectively blocks L-type Ca^{2+} channels and is totally inactive on other voltage-dependent Ca^{2+} channels such as N-type and T-type channels. To our knowledge, it is the only natural polypeptide that has been shown to be a specific inhibitor of L-type Ca^{2+} channels.**

Voltage-activated Ca^{2+} channels play a central role in the generation of electrical signals by neurons and other excitable cells such as cardiac or smooth muscle cells. They have a key function in excitation–contraction and excitation–secretion coupling (1–3).

It has now become clear that there is a great diversity of Ca^{2+} channel types (4–10). At least three of them are now well characterized on the basis of both their biophysical and pharmacological properties (4–7).

The L-type Ca^{2+} channel has a rich pharmacology. Blockers of this type of channel can abolish contractions in cardiac and smooth muscle cells. A high number of relatively simple organic molecules [1,4-dihydropyridines (DHPs), phenylalkylamines, benzothiazepines, etc.] have now been developed by pharmaceutical industries for treatment of cardiovascular disorders and other diseases (7, 11, 12).

The N-type Ca^{2+} channel is present in neuronal cells (3, 6, 9, 13). This channel (or one subtype of N-type Ca^{2+} channel) is blocked by a natural polypeptide toxin isolated from *Conus geographus* and called ω-conotoxin (9, 14). This toxin thereby inhibits neurotransmitter secretion (13, 15). T-type Ca^{2+} channels are found in most excitable cells. However, although the pharmacology for this type of channel is being studied (16), high-affinity and high-specificity blockers of T-type Ca^{2+} channel are still lacking. A funnel-web spider toxin has been recently reported to block P-type Ca^{2+} channels that are present in dendrites of cerebellar Purkinje cells (17).

Well-established polypeptide toxins specific for voltage-sensitive Na^+ channels (18), for voltage-sensitive K^+ channels and Ca^{2+}-activated K^+ channels (19), and for N-type Ca^{2+} channels (13) have become essential research tools. While there have been reports suggesting the presence of polypeptide toxins specific for L-type Ca^{2+} channels in different venoms (for a review, see refs. 7 and 20), none of these toxins, until now, has been completely purified and sequenced and definitely proven to be a Ca^{2+} channel effector. This paper describes the structural and functional properties of a toxin that we have called calciseptine; to our knowledge, it is the only natural polypeptide toxin that has been shown to block L-type Ca^{2+} channels.

MATERIALS AND METHODS

Venom from the black mamba *Dendroaspis polylepis polylepis* was obtained from Latoxan (Rosans, France). Calciseptine was purified from the crude venom in three steps: (*i*) gel filtration, (*ii*) ion exchange on TSK SP 5PW, and (*iii*) reverse-phase chromatography on RP18. Details can be found in ref. 21, which describes the isolation of all peptides from *D. polylepis polylepis* venom. In this work, calciseptine is referred to as peptide E3 (see table I of ref. 21). It represents 2.8% of the total venom components.

The primary structure of calciseptine was determined twice by Edman degradation of the complete peptide using Applied Biosystems 470A and 477A microsequencers. The structure was further confirmed by sequencing fragments generated by cyanogen bromide cleavage at positions 25 and 19.

Muscle contraction measurements were carried out as described elsewhere (21). The following medium was used for blood vessels and uterus: 118 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/2.5 mM $CaCl_2$/25 mM $NaHCO_3$/11 mM glucose. For atria, it was 127 mM NaCl/4 mM KCl/0.5 mM NaH_2PO_4/1 mM $MgSO_4$/1.8 mM $CaCl_2$/12 mM $NaHCO_3$/5 mM glucose. Left atria were stimulated by an electric field of 45 V/cm. Stimulation duration and frequency were 2 ms and 1 Hz, respectively.

RINm5F and HIT rat insulinoma cells, N1E-115 mouse neuroblastoma cells, and A7R5 rat aortic smooth muscle cells were grown as described (22–26). Rat cardiac ventricular cells and chicken dorsal root ganglion cells were dispersed in a Ca^{2+}-free medium containing 0.01% collagenase, followed by gentle mechanical agitation. Details of the methods have been described elsewhere (27, 28). Cells were plated at a density of 20,000 cells per 35-mm-diameter Petri dish.

Whole-cell currents and membrane potentials were recorded by techniques described in ref. 29. The pipette solution used in current-clamp experiments was as follows: 150 mM KCl/1 mM $MgCl_2$/2 mM EGTA/2 mM ATP/10 mM Hepes·KOH, pH 7.2. The pipette solution in voltage-clamp experiments was as follows: 80 mM cesium sulfonate/20 mM CsCl/2 mM $MgCl_2$/100 mM glucose/2 mM EGTA/2 mM ATP/10 mM Hepes·CsOH, pH 7.3. The compositions of the extracellular solutions used in current-clamp and voltage-clamp experiments were as follows: low Ca^{2+} solution; 40 mM NaCl/1 mM $MgCl_2$/2 mM $CaCl_2$/10 mM Hepes·KOH, pH 7.3; Ca^{2+}-free solution, 140 mM NaCl/2 mM EGTA/10 mM Hepes·NaOH, pH 7.3. All voltage-clamp experiments

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Abbreviation: DHP, 1,4-dihydropyridine.

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were carried out in the presence of 0.5–1.0 μM extracellular tetrodotoxin. Membrane potentials and currents were recorded by a voltage-clamp amplifier (Biologic, Grenoble, France), digitized at intervals of 0.1–50 ms by a digital oscilloscope (Nicolet) and stored on hard disc by a computer (Hewlett–Packard) for further analysis. Pipettes were coated with Sylgard resin to reduce capacitive currents. Experiments were carried out at room temperature (28°C–30°C).

RESULTS

Calciseptine has been purified from the venom of the black mamba *D. polylepis polylepis*. It is a 60-amino acid polypeptide with 8 cysteines forming four disulfide bridges. Its primary structure is indicated below.

$$\overset{1}{R}\,I\,C\,Y\,I\,H\,K\,A\,S\,\overset{10}{L}\,P\,R\,A\,T\,K\,T$$
$$C\,V\,E\,\overset{20}{N}\,T\,C\,Y\,K\,M\,F\,I\,R\,T\,\overset{30}{Q}\,R\,E$$
$$Y\,I\,S\,E\,R\,G\,C\,\overset{40}{G}\,C\,P\,T\,A\,M\,W\,P\,Y$$
$$Q\,\overset{50}{T}\,E\,C\,C\,K\,G\,D\,R\,C\,N\,\overset{60}{K}$$

Another similar peptide with an unknown function was previously isolated and sequenced (30). It is present in black mamba venom and differs from our sequence in three positions: a serine instead of isoleucine in position 5, a histidine instead of glutamine in position 30, and a glutamine instead of glutamic acid in position 22.

Fig. 1 shows that calciseptine blocks spontaneous portal vein contractions. It also blocks K^+ (40 mM)-induced contractions of aortic smooth muscle and spontaneous contractions of uterine smooth muscle. This blockade is similar to the inhibitory effect of PN200-110, one of the most potent DHPs (data not shown). Calciseptine also blocks contractions induced in all these different smooth muscle preparations by the Ca^{2+} channel activator Bay K8644 (7, 11) (data not shown). Calciseptine block of uterine and portal vein contraction could not be reversed after a 15-min washing.

Aortic smooth muscle cells from the A7r5 cell line are electrically active in a medium containing 2 mM Ca^{2+} (31), generating action potentials once every 1–2 s (Fig. 1*D*). Upon extracellular application of 1 μM calciseptine, the action potential amplitude decreases rapidly and activity finally disappears (n = 7). After washout of the toxin, almost full-sized action potentials reappear. A similar progressive inhibition of electrical activity is observed with the L-type channel blocker PN200-110 (data not shown), suggesting that calciseptine is an L-type channel blocker. The latter is directly demonstrated by voltage-clamp experiments shown in Fig. 1*E*, which were carried out in 2 mM Ca^{2+}. If extracellular Ca^{2+} is raised to 10 mM (n = 4) or if Ca^{2+} is replaced by a solution containing 95 mM Ba^{2+} (n = 4), calciseptine fails to block the L-type current. Similarly, 1 μM calciseptine is without effect on action potential amplitude in the presence of 10 mM Ca^{2+}, suggesting that high extracellular concentrations of divalent cations suppress the inhibitory action of calciseptine. Binding of ω-conotoxin to the N-type Ca^{2+} channel protein is also antagonized by Ca^{2+} (7, 32).

It has been shown previously that, although the permeability of Ca^{2+} channels to Na^+ is much less than their permeability to Ca^{2+}, large membrane currents can be recorded due to the high flux rate of Na^+ through Ca^{2+} channels (33). The voltage dependence of the DHP-sensitive membrane current in A7r5 cells is shifted 20 mV in the hyperpolarizing direction if Ca^{2+} is omitted from the extracellular solution (now containing 140 mM Na^+). Calciseptine blocks both the Ca^{2+} and Na^+ modes of the L-type channel (Fig. 1*G*). Dose–response curves for the effects of calciseptine on

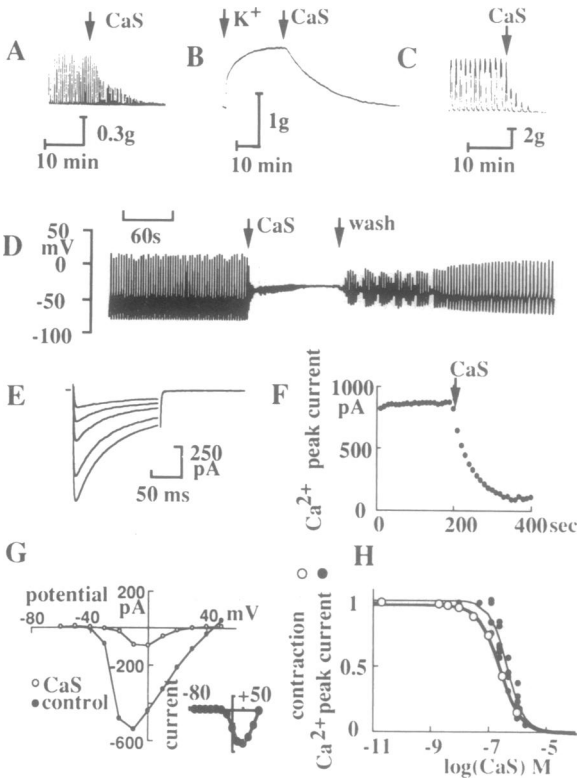

FIG. 1. Effect of calciseptine on smooth muscle preparations. Calciseptine (CaS) relaxes spontaneous contractions of rat portal vein (*A*), contractions of the rat thoracic aorta induced by 40 mM K^+ (*B*), and spontaneous contractions of rat uterus (*C*). Calciseptine was applied at a concentration of 0.2 μM (*A* and *C*) or 1 μM (*B*). Spontaneous electrical activity of A7r5 smooth muscle cells ceases after application of 1 μM toxin and returns after washout (*D*). The L-type Ca^{2+} current evoked in voltage-clamped A7r5 cells by a depolarizing step from a holding potential of −80 to −20 mV is rapidly and extensively inhibited by 1 μM calciseptine (*E* and *F*). Traces in *E* at 0, 10, 30, 60, and 120 s after application of calciseptine. Ca^{2+} channel inhibition by calciseptine could not be reversed after a 5-min washing of the toxin. Toxin action on the L-type current in its Na^+ mode (Ca^{2+}-free medium, 140 mM Na^+). The current activates at more hyperpolarizing potentials than in 2 mM Ca^{2+} (*G Inset*). It is largely inhibited by 1 μM calciseptine within the whole range of potentials tested. Holding potential was −60 mV. Dose–response curve (in M) for the action of calciseptine on the L-type Ca^{2+} current (measured in 2 mM Ca^{2+}) in A7r5 cells (●) and the K^+-induced contraction of rat thoracic aorta (○).

both K^+-induced contractions of rat aorta and L-type Ca^{2+} channel activity measured by the whole-cell voltage-clamp technique are presented in Fig. 1*H*. They are nearly superimposable, having IC_{50} values of 230 and 430 nM, respectively.

Calciseptine also affects cardiac function. The toxin (1 μM) abolishes electrically stimulated left atrial contractions in both the absence and presence of the L-type Ca^{2+} channel activator Bay K8644 (Fig. 2). The dose–response curve of the inhibitory action displays an IC_{50} of 15 nM.

Dissociated rat ventricular myocytes contract spontaneously in 2 mM external Ca^{2+}, firing one or two action potentials per s. Extracellular application of 1 μM calciseptine stops spontaneous contractions and drastically decreases both action potential frequency and amplitude (n = 6; data not shown). Cardiac cells express both L-type and T-type Ca^{2+} channels (34, 35). The T-type Ca^{2+} current is transient and resistant to DHPs, while the L-type Ca^{2+} current is slowly inactivating and blocked by DHPs such as PN200-110. Fig. 2*C* shows that, similarly to PN200-110,

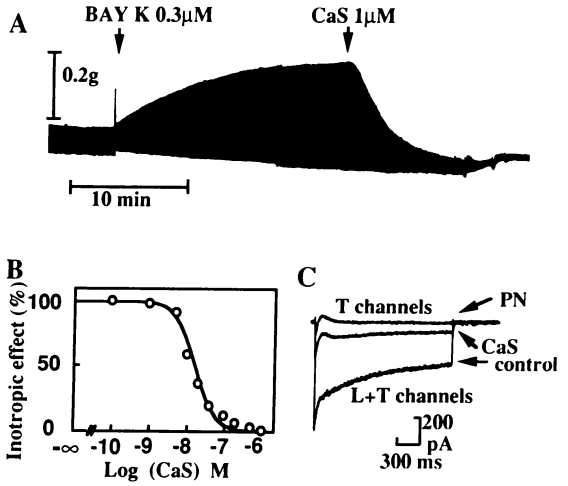

FIG. 2. Effect of calciseptine on rat cardiac preparations. The contraction amplitude of rat left atria, stimulated electrically at a frequency of 1 Hz, increased considerably after application of the Ca agonist Bay K8644 (0.3 μM) (*A*). This positive inotropic effect of Bay K8644 is reversed dose dependently by subsequent addition of calciseptine (*A* and *B*). Calciseptine (1 μM) blocks ≈80% of the Na^+ transport activity of the L-type Ca^{2+} channel in rat ventricular cells (*C*) but does not affect the T-type Ca^{2+} channel. Ventricular cells were voltage clamped at −70 mV and stepped to 0 mV in Ca^{2+}-free Na^+-containing medium.

calciseptine blocks the L-type current but is inactive on the T-type Ca^{2+} current (n = 5).

Ca^{2+} channels are present in neuroblastoma cells (36). Undifferentiated rat NIE115 neuroblastoma cells selectively express a T-type Ca^{2+} channel (Fig. 3*A*) that is insensitive, as expected, to the L-type blocker PN200-110 (0.5 μM) and to the N-type blocker ω-conotoxin (0.5 μM). The T-type Ca^{2+} current in neuroblastoma cells is unaffected by 1 μM calciseptine (n = 8) (Fig. 3*A*).

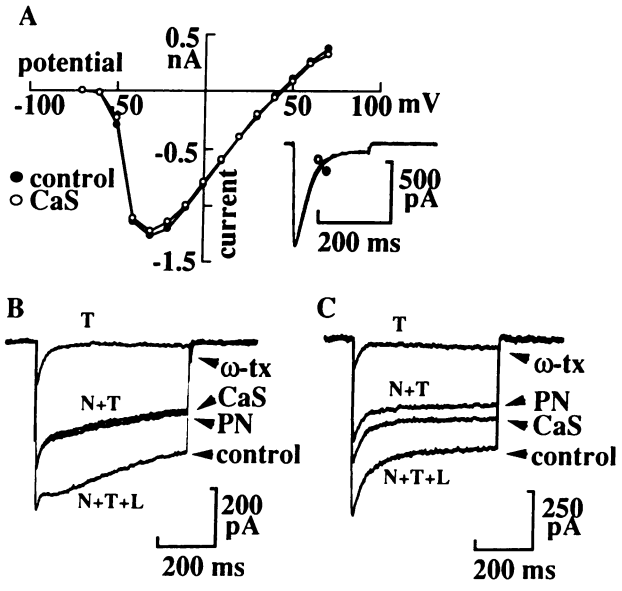

FIG. 3. Effect of calciseptine on neuronal Ca^{2+} channels. Undifferentiated N1E115 neuroblastoma cells (*A*) and chicken dorsal root ganglion cells (*B* and *C*) were voltage-clamped at −80 mV in Ca^{2+}-free Na^+-containing medium (Na^+ mode of the Ca^{2+} channel). Calciseptine (1 μM) has no effect on the T-type current recorded in neuroblastoma cells. (*Inset*) T currents elicited by a depolarizing step to 0 mV in the presence (○) or absence (●) of the toxin. PN200-110 (PN) (0.5 μM; incubated 1 min) blocks the L-type current in dorsal root ganglion cells (*B*). Subsequent application of 3 μM calciseptine does not affect the ω-conotoxin (ω-tx; 0.5 μM)-sensitive current component (N-type current) or the T-type current. Traces were recorded in the following order: control, PN, CaS, ω-tx (*B*). Calciseptine (3 μM) inhibits ≈70% of the L-type current. Traces in *C* were recorded in the following order: control, CaS, PN, ω-tx.

The chicken dorsal root ganglion cell membrane contains three types of Ca^{2+} channels (T, N, and L) (5, 6). They all carry Ca^{2+} as well as Na^+ (37, 38). The L-type component is blocked by PN200-110 (0.5 μM) (n = 8). The N-type current, which was dominant in most tested cells, is blocked by 0.5 μM ω-conotoxin (n = 9). The T-type current is resistant to both types of inhibitors (Fig. 3 *B* and *C*). Neither T- nor N-type currents are affected by calciseptine (1 μM). The L-type current is 50% inhibited by 1 μM toxin (data not shown). The concentration used in Fig. 3 *B* and *C* is 3 μM.

L-type Ca^{2+} channels are also expressed in secretory cells such as β-pancreatic cells (39). Whole-cell voltage-clamp studies of insulinoma cells (RINm5F cell line, n = 8; HIT cell line, n = 6) have shown that calciseptine inhibits 50% of the L-type Ca^{2+} channel activity in these cells at a concentration of 1 μM (data not shown).

Skeletal muscle cells also express both T-type and L-type Ca^{2+} channels (40). The L-type Ca^{2+} channels in these cells are atypical compared to cardiac or smooth muscle Ca^{2+} channels (40). Moreover, it probably serves a dual function as a channel and as a voltage sensor (41–43)—i.e., an electromechanical device essential for excitation–contraction coupling that transduces surface membrane depolarization into Ca^{2+} liberation from the sarcoplasmic reticulum. Both functions (L-type channel and voltage sensor) are blocked by the different families of classical L-type Ca^{2+} channel blockers (44). Neither T-type nor L-type channel activity nor contraction (associated with the voltage-sensor function) is affected (<10%) by 1 μM calciseptine (data not shown).

DISCUSSION

Calciseptine is a natural polypeptide that affects the L-type Ca^{2+} channel. It clearly does not affect N-type or T-type Ca^{2+} channels. Other voltage-sensitive cationic channels (voltage-sensitive Na^+ and K^+ channels in cardiac ventricular cells, N1E115 neuroblastoma cells, RINm5F and HIT insulinomas, chicken dorsal root ganglia) were also found to be totally insensitive to 1 μM calciseptine.

The L-type Ca^{2+} channel sensitivity is clearly tissue dependent. The sensitivity is higher in cells of the cardiovascular system. Neuronal L-type Ca^{2+} channels are more resistant. A partial inhibition is also observed in insulinoma cells. A total resistance is found in skeletal muscle cells. These results are not surprising, since it is now well established that there are different genes for L-type Ca^{2+} channels in neuronal, muscle, cardiac, and vascular smooth muscle cells (45–48) and that these different genes encode L-type Ca^{2+} channel proteins with different sensitivities to classical L-type Ca^{2+} channel blockers (7).

It seems (with the exception of the skeletal muscle) that those channels that are most sensitive to DHPs (in vascular and cardiac cells) are also more sensitive to calciseptine. Neuronal L-type Ca^{2+} channels are known to be more resistant to this class of drugs; they are also more resistant to the polypeptide toxin.

Other polypeptide toxins that are active on Ca^{2+}-activated K^+ channels or voltage-sensitive K^+ channels such as apamin or MCD peptide have been shown to have endogenous equivalents in the brain (49, 50). Moreover, and more recently, endothelin, a vasoconstricting peptide from vascular endothelial cells was shown to be very homologous to the snake neurotoxin sarafotoxin (51). On the other hand, there have been claims for the possible existence of small brain peptides with L-type Ca^{2+} channel activity (52) and a number

of research groups are looking for possible endogenous equivalents of DHPs. It would of course be particularly interesting to see whether there exist endogenous equivalents of calciseptine in mammalian tissues. If such equivalents were found in vascular endothelial cells they could be candidates to act as endothelial relaxing factors (53), since intravenous injections of calciseptine to rats lead to very significant decreases of arterial pressure with only small effects on cardiac rhythm.

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