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Effect of external calcium on the control of stamen movement in *Berberis vulgaris* L.

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Abstract

The mechanical stimulation of the sensitive internal lower part of *Berberis vulgaris* stamen resulted in its rapid bending. In the present study we have examined the influence of external Ca^{2+} concentration on stamen movement. The external Ca^{2+} reduced the extent of the response and the effect was dependent on Ca^{2+} concentration and duration of the treatment. Addition of calcium ionophore A 23187 to the medium reduced the response and the effect was dependent on the external Ca^{2+} concetration. This result might suggest an increase in Ca^{2+} level in cytosol. The inhibitory effect of higher Ca^{2+} concentration on stamen bending was cancelled by Ca^{2+}-chelating agents. Ca^{2+}-channel blockers prevented the stamen response in higher external Ca^{2+} concentration with different effectiveness. In these conditions, during 4-h experiments, La^{3+}, verapamil and nifedipine gave ability of stamen movement at about 85 %, 58 % and 10 %, respectively. An energy-dependent Ca^{2+} efflux was confirmed in experiments by using vanadate, a non-specific ATPase inhibitor. The lack of inhibition of stamen bending after application of calmodulin antagonists suggests that it might not be directly involved in regulation of the response. The inhibitory effect of higher Ca^{2+} concentration on stamen movement might result from: (a) the binding with cell wall materials, (b) changes of structure of cytoplasm and metabolic activity and (c) influence on transport processes.

Introduction

The mechanical stimulation of the sensitive internal lower part of the filament of *Berberis vulgaris* stamen performs a rapid movement, which results in striking the anther against the pistil (Millet and Thibert 1976, Fleurat-Lessard and Millet 1984). The perception of the mechanical signal and its transmission are not known.

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Abbreviations used: CaM - calmodulin; CPZ - chlorpromazine; CTC - chlortetracycline; EDTA - ethylenediaminetetraacetic acid; EGTA - ethylene glycol-*bis*(beta-aminoethyl ether)*N,N,N',N'* - tetraacetic acid; ER - endoplasmatic reticulum; MES - (2-[*N*-morpholino]) ethanesulfonic acid; TFP - trifluoperazine; W-7 - *N*-(6-aminohexyl)-5-chloro-1-naphtylenesulfonamide.

After mechanical stimulation, a receptor potential is generated in sensitive cells of *Berberis*. The increasing receptor potential accounts for rapid changes in the permeability of membranes. Transmembrane flux of ions, chiefly K^+, Cl^-, and Ca^{2+}, is closely associated with decreasing pressure potential and brings about a movement response (for review see Białczyk and Lechowski 1988). It is supposed that ER may be included in the perception of mechanical stimuli. In sensitive cells of *Berberis canadensis* stamen ER shows a close structural connection with plasmalemma. Pairs of ER cisterns are located at a distance of 10 - 20 nm from plasmalemma (Fleurat-Lessard and Millet 1984). ER may store Ca^{2+} ions and release them in the case of stimulation (Poovaiah and Reddy 1987). Ca^{2+} may be involved in the regulation of the mechanism of stamen bending by controlling ionic and water fluxes through membranes (plasmalemma, tonoplast, membrane of cell organelles) of motor cells after mechanical stimulation.

The importance of Ca^{2+} as a second messenger in various cell functions is well known and the influx of Ca^{2+} is presumed to trigger morphogenetic events (Kauss 1987, Steer 1988). In many excitable cells the influx of Ca^{2+} can be inhibited by calcium antagonists. The inhibitory action of divalent cation-chelating agents and $LaCl_3$ (Ca^{2+}-channel blocker) suggests that extracellular Ca^{2+} is involved in the seismonastic reaction of *Mimosa pudica* (Campbell and Thomson 1977).

In the present study we examined the influence of external Ca^{2+} concentration on stamen bending. Using vanadate (an inhibitor of membrane ATPases) and Ca^{2+}-channel blockers, it was attempted to characterize the system of Ca^{2+} transport in motor cells. In order to explain this problem different Ca^{2+}-channel blockers (nifedipine, verapamil, La^{3+}), A 23187 calcium ionophore, and Ca^{2+}-chelating agents (CTC, EGTA) were used. The possible action of CaM, which is activated by internal Ca^{2+} level, was tested by external application of CaM antagonists of a different kind. It is suggested that high external Ca^{2+} concentration affects the extent of stamen response.

Material and methods

Plant material: Shoots of *Berberis vulgaris* L. with inflorescences were collected in natural conditions at 8.00 and dipped in water for 2 h in room conditions at 27±1 °C. Single flowers with petioles were separated from inflorescences by razor blades and pincers and used in the experiments. For checking the rate of transport of the solution through petioles to flowers, the petioles were dipped in a 1 % aqueous neutral-red solution. Preliminary experiments with about 500 flowers showed that after about 15 min the stain reached petals, stamens and pistils with transpiration current as it was manifested by the change of their colour to red. In further experiments excised ends of petioles were placed in tested solutions. The basal medium consisted of 10 mM KCl, 5 mM $Mg(SO_4)_2$ and 10 mM MES-buffer. Depending on the kind of experiments different concentration of $CaCl_2$ (from zero to 100 mM) and drugs were added. pH of medium was adjusted to 6.6 immediately before use.

Stamen bendings: The movement response of stamen was tested by irritating the internal surface of the filament of stamens with a hair. Each stamen which resulted in a complete bending after irritation was counted. When the investigated effect was tested in the irritated stamens, the flower was eliminated from further experiments. The reaction was tested after incubation in solutions at 15 or 30 min intervals. The results illustrated by curves represent means from 8 independent series, in each case the response of about 200 stamens having been investigated (n = about 1500-1600 stamens).

Chemicals: All chemicals were purchased from Sigma.

Results

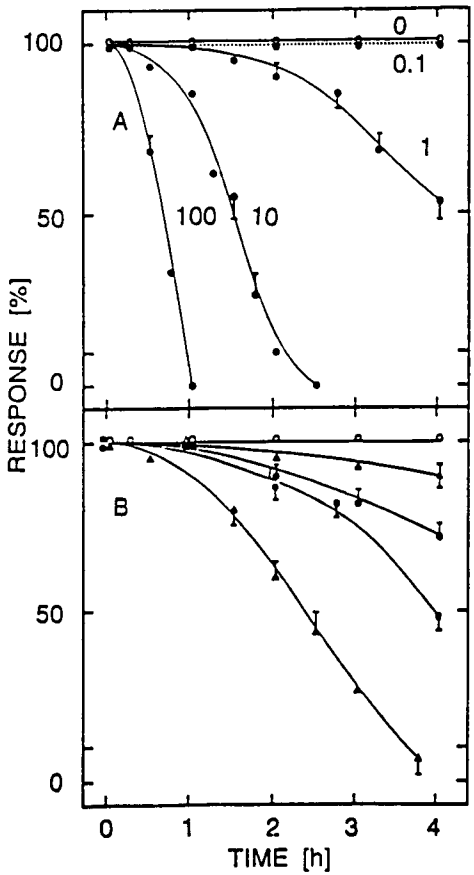

Fig.1. The stamen bendings during the treatment of *Berberis vulgaris* flowers with different concentration of $CaCl_2$ (A) or 1 mM vanadate (B). Numbers beside the curves represent the concentration of $CaCl_2$ in mM. The symbols in Fig. B represent: 0.1 mM $CaCl_2$ (*open circles*), 1 mM $CaCl_2$ (*open triangles*), 1 mM vanadate (*closed circles*) given alone (control) or in combination with 0.1 mM $CaCl_2$ (*closed boxes*). 1 mM $CaCl_2$ (*closed triangles*) was applied together with 1 mM vanadate. Bars represent standard errors.

Effect of external Ca^{2+} concentration: The supply of external $CaCl_2$ to the medium, in which the excised flowers were placed, reduced the extent of stamen bending caused by mechanical stimuli. The effect was dependent on $CaCl_2$ concentration and duration of the treatment. The 0.1 mM concentration of $CaCl_2$ in external medium had no significant effect on the movement of stamens during a 4 h experimental period, similar to the action of basal medium (control) (Fig. 1 A). The highest concentration of Ca^{2+} influenced the reduction of per cent response of the movement. Fairly rapid effects were observed within the first hour of treatment at the concentration of 100 mM, the treated stamens becoming completely unresponsive to mechanical stimuli. A further increase in $CaCl_2$ content (to 500 mM) in external medium had no significant effect on a decrease in the numbers of stamens bending (the data not shown). This result shows that Ca^{2+} concentration modulates the mechanism of the response.

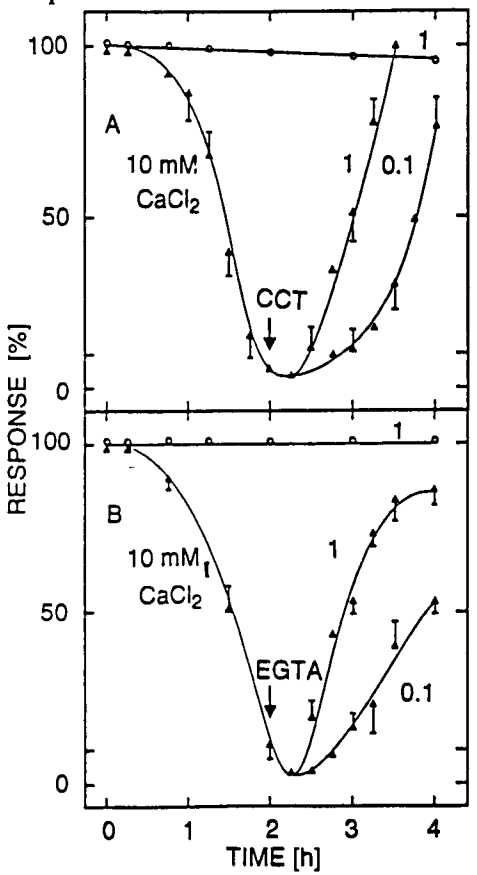

Fig.2. Reversion of stamen bendings inhibition after pretreatment with 10 mM $CaCl_2$ by Ca^{2+}-chelating agents. *Open symbols* represent the response during treatment of flowers with Ca^{2+}-chelating agents given alone. *Close symbols* indicate the response after pretreatment of flowers with 10 mM $CaCl_2$. The *arrows* indicate the time of transfering preparations to CTC (A) or EGTA (B). Numbers beside the curves represent the concentration of Ca^{2+}-chelating agents in mM. Bars represent standard errors.

Effect of vanadate and Ca^{2+} concentration: 1 mM vanadate, a non-specific ATPases inhibitor, applied to the medium without Ca^{2+}, had no effect on the inhibition of stamen movements (Fig. 1 B). This result might suggest that at a low external Ca^{2+} concentration a energy independent Ca^{2+}/H^+ extrusion operates and it is sufficient to keep a constant and very low level of Ca^{2+} in cytoplasm. In the case of vanadate applied together with 0.1 mM or 1 mM $CaCl_2$ the response was significantly inhibited as compared with the control. This result clearly indicates an energy-dependent Ca^{2+} efflux at higher Ca^{2+} concentrations in cytoplasm.

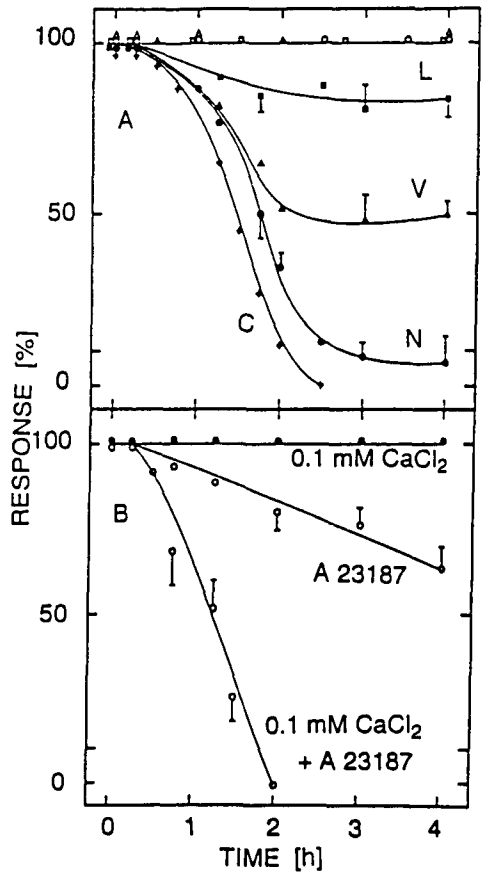

Fig.3. Effect of Ca^{2+}-channel blockers (A) and A 23187 ionophore (B) on stamen bendings. The 0.01 mM Ca^{2+}-channel blockers (L - $LaCl_3$, N - nifedipine, V - verapamil) were given alone (*open symbols*) or together with 10 mM $CaCl_2$ (*close symbols*). Control represent the effect of 10 mM $CaCl_2$ (C). A 23187 ionophore (0.01 mM) was given alone or applied in combination with 0.1 mM $CaCl_2$. Bars represent standard errors.

Effect of Ca^{2+} chelating agents: The complete cessation of stamen bending in higher Ca^{2+} concentrations could be reversed by applying the known Ca^{2+}-chelating agents, *i.e.*, EGTA or CTC. The 4 h treatment of flowers with a 1 mM CTC or EGTA solution did not change the sensitivity of stamen filaments to mechanical stimuli

(Fig. 2). The pretreatment of flowers with 10 mM $CaCl_2$ solution for 2 h brings about an almost 90 % inhibition of response. When the preparations from treated plant were transferred to CTC (Fig. 2A) or EGTA (Fig. 2B) solutions, the ability of stamen filaments to move was restored. In the case of the two chelating agent solutions, the stamen bending depended on their concentration. With 1 mM CTC the ability to a full response was restored after 1.5 h while with EGTA a 90 % response was obtained during that time.

Effect of Ca^{2+}-channel blockers: In order to check how the triggering of a mechanical signal in stamen bending of *B. vulgaris* is blocked by an influx of external Ca^{2+} excess, inorganic (La^{3+}) and organic Ca^{2+}-channel blockers (nifedipine, class: 1.4 dihydropyridine and verapamil, class: phenylalkylamine) were used. As shown in Fig. 3 A no Ca^{2+}-channel blockers (at 0.01 mM concentration) tested under a calcium-free solution have no effect on stamen movement. In this case Ca^{2+} transport systems in cell organelles (*e.g.* mitochondria, vacuoles, ER) must probably be involved in controlling the mechanism of intracellular Ca^{2+} concentration. If flowers of *B. vulgaris* were simultaneously treated with 10 mM $CaCl_2$ and Ca^{2+}-channel blockers, the response was different in each group of blockers. La^{3+} was most effective in inhibiting Ca^{2+} inward current. At the concentration of 0.01 mM $LaCl_3$ the response was saturated and within of 1.45 min of treatment the response of stamen was reduced to about 15 % only. The prolongation of La^{3+} treatment to 4 h had no signigicant effect. This result may suggest that some part of Ca^{2+} may be influxed through Ca^{2+}-channels other than the La^{3+} sensitive ones. The verapamil and nifedipine at 0.01 mM concentration prevented about 58 % and 10 % response during 4-h experiments, respectively (Fig. 3 A).

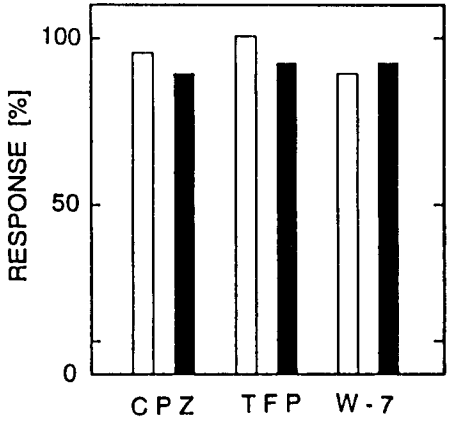

Fig. 4. Effect of CaM-antagonists on stamen movements. Data obtained after 4 h treatment with 0.01 mM (*open columns*) or 0.1 mM (*closed columns*) CaM - antagonists.

Effect of calcium ionophore A 23187: The application of a calcium ionophore A 23187 solution (0.01 mM) alone resulted in a loss of stamen movement by about

35 % during experiments prolonged to 4 h. The effector can bring about a partial cessation of response without an alteration of Ca^{2+} concentration in external medium. In this case it is probable that calcium ionophore enhanced the influx of Ca^{2+} from apoplastic solution or external/internal deposites. In the presence of external Ca^{2+} at 0.1 mM $CaCl_2$ this concentration of Ca^{2+} given on its own had no effect on stamen bending and the addition of A 23187 ionophore caused a complete inhibition of response during 2 h experiments (Fig. 3B). This result suggests that A 23187 ionophore enhanced the Ca^{2+} concentration in cytosol of stamen sensitive cells.

Effect of calmodulin antagonists: CaM is suggested to play an important role in response to different kinds of stimuli. It is activated by Ca^{2+} after its concentration in cytosol is enhanced to a micromolar level (Poovaiah and Reddy 1987). CaM antagonists of the phenothiazine class (CPZ and TFP) and naphtalenesulfonamide class (W-7) had no significant effect on stamen bending at the concentrations tested (0.01 mM, 0.1 mM) (Fig. 4). In particular, the effects of CaM on Ca^{2+} transport may be eliminated because Ca^{2+} transport antagonists are effective if added in 2-h experiments (Fig. 3A) while CaM antagonists are ineffective if added in experiments of the same duration or prolonged to 4 h. The lack of inhibition of stamen movements in the case of application of CaM-antagonists suggests that CaM might not be directly involved in regulating the response.

Discussion

External Ca^{2+} concentration: The rapid bending of stamen filaments in *Berberis vulgaris* and the immediate initiation of the recovery processes are associated with a great osmotic fluctuation in motor cells (Białczyk and Lechowski 1988). It is well known that Ca^{2+} is necessary to maintain the permeability and integrity of membranes (Clarkson and Hanson 1980) and it can control the K^+ flux (Schwartz 1985). The lower concentration of Ca^{2+} (about 5-10 μM) induced responses of many kinds of stimulation and was indicated as a "second messenger" (Poovaiah and Reddy 1987). The inhibitory effect of a higher Ca^{2+} concentration on stamen movements might result from: (a) the binding of Ca^{2+} with cell wall molecules of motor cells *e.g.* pectins and cellulose; (b) an increase in their concentration in cytoplasm and changes of its structure and metabolic activity, and (c) the influence on transport processes by membranes. The cell walls of sensitive inside part of stamen filaments have a deeply plicate external surface, this allowing efficient changes of the volume of cells of this area (Fleurat-Lessard and Millet 1984). The binding of Ca^{2+} with structural materials of the cell walls may bring about their stiffening and, hence, exclude the motor response. High concentrations of external Ca^{2+} are known to cause a local collapse of the transmembrane Ca^{2+} gradient (Shina and Tazawa 1987, Felle 1988). It has also a distinct effect on various membrane properties of the plasmalemma such as surface charge density, selectivity and regulation of ion transport (Ballanyi and Deitmer 1984). According to Armstrong and Matteson (1986) higher concentration of Ca^{2+} are also able to interact directly with channel proteins and bring about the closing of K^+-channels.

High concentration of Ca^{2+} cancelled the dark-induced closure of open stomata (Schwartz 1985) and inhibited H^+ extrusion (Schwartz 1985, Inoue and Katoh 1987) in *Commelina communis*. Inoue and Katoh (1987) suggest that Ca^{2+} inhibits the activity of ATPase, which translocates H^+ and K^+. In a similar way high concentration of Ca^{2+} should directly inhibit the electrogenic H^+-pump and K^+ efflux during the seismonastic movement of stamens

Ca^{2+} transport systems: The response of stamen bending may be connected with the central regulation of calcium level in the cytoplasm. Therefore, the changes of Ca^{2+} level in cytoplasm may be associated with: (*a*) the degree of opening of Ca^{2+}-channels decisive for the amount of Ca^{2+} influxed into cytoplasm; (*b*) the feed-black reaction in which Ca^{2+}-channels close at certain Ca^{2+} concentration in the cytoplasm until the influxed Ca^{2+} extrusion through an energy driven Ca^{2+}/H^+ dependent on Ca^{2+}-ATPase activity (Carafoli 1987) or by a Ca^{2+}/H^+ exchange (Felle 1988). The widely studied Ca^{2+} ionophore A 23187 was used to promote absorption of Ca^{2+} from cell walls and from $CaCl_2$ solution. Addition of A 23187 reduced the stamen bendings (Fig. 3B) and the effect was dependent on the external Ca^{2+} concentration. An increase in Ca^{2+} concentration after addition of A 23187 was documented in animal cells by Artelejo and Garcia-Sancho (1988). Ca^{2+} increase in cytosol may inhibit the activity of ATPase which translocate H^+ and K^+, and also the extrusion of Ca^{2+} by Ca^{2+}/H^+ system (Inoue and Katoh 1987).

The effect of EGTA and CTC which chelate Ca^{2+} (Fig. 2) might be directly related to an increase in H^+-pump activity (Rasio-Caldogno *et al.* 1985). Results similar to our observation were reported by Roblin and Fleurat-Lessard (1987) in experiments concerning the influence of EGTA and EDTA (0.1 to 1 mM) which had effect on the seismonastic reaction of *Mimosa pudica* leaflets. Apart from the outward removal of excessive Ca^{2+} from cytoplasm of sensitive cells of *B. vulgaris*, it is possible to transport and store it in vacuoles and other cell organelles. Felle (1988) directly measured Ca^{2+} and vacuolar pH and stated that the alkalization was coupled with a massive increase in Ca^{2+} content. He suggested that Ca^{2+}/H^+ antiport located at the tonoplast might serve as a system to regulate cytoplasmic Ca^{2+} concentration. In a variety of plant tissues the antiport mechanism at tonoplast vesicles has been demonstrated as having different membrane-bound Ca^{2+} transport systems in plant cells (Evans 1988). Despite abondant information relating to the different systems of isolated Ca^{2+} transport, not much is known about role in intact cells.

Ca^{2+}-channel blockers: Ion channels, elementary parts of energy-excitable membrane, are indispensable for the generation and transduction of electical stimuli in living cells. Ca^{2+}-channels seem to be widely distributed both in animal and plant cells, playing an essential role in signal transduction. Lee and Tsien (1983) electrophysiologically examined the effect of several kinds of Ca^{2+}-channel blockers on Ca^{2+} flux in cardiac cells. They proposed that inorganic and organic blockers operate in different ways.

In our experiments La^{3+} was very effective in prevention of the stamen bending in the case of high external Ca^{2+} concentration (Fig. 3A). The La^{3+} blocker does not penetrate plant cells (Thomson *et al.* 1973) and its action may be attributed to the

inhibition of Ca^{2+} uptake by binding the external part of the channel (Dos Remedios 1981). Effects similar to our data were obtained by Takagi and Nagai (1988) in experiments with cession of cytoplasmic streaming in *Vallisneria gigantea* protoplasts. Our data on an organic Ca^{2+} -channel blockers in *B. vulgaris* flowers show a stronger effect of verapamil than nifedipine in prevention of the inhibition of stamen movements. In general, the binding of organic inhibitors depends to a great degree on external Ca^{2+} concentration (Lee and Tsien 1983, Dolle and Nultsch 1988). Ca^{2+} can occupy binding sites in the Ca^{2+}-channel near the receptor sites of organic inhibitors. In this case the inhibitory reaction of these agents is strongly reduced (Lee and Tsien 1983). Therefore, Ca^{2+}-channel blockers from different chemical classes may vary in their effects on different organisms and tissues. Contraindicatory results concerning the application of these inhibitors to plant cells were obtained (Shina and Tazawa 1987). For example, in contrast with verapamil neither specific binding sites for dihydropyridines nor an inhibition of calcium fluxes could be confirmed in carrot protoplasts (Graziana *et al.* 1988). Fleckenstein (1977) proposed that verapamil competed with calcium for a common site. However, in his experiments the restorative effect of a higher external Ca^{2+} concentration might be attributed to increased Ca^{2+} influx through a constant fraction of unblocked channels. As regards this point, some experimental evidence is contradictory. Lee and Tsien (1983) described a non-competitive interaction between Ca^{2+} and verapamil in rat ventricular muscle. On the other hand, with respect to calcium fluxes, and inhibition of calcium-dependent processes by dihydropyridines could be demonstrated (Dolle and Nultsch 1988, Takagi and Nagai 1988, Roberts and Haigler 1990) while verapamil had a poor or no effect. On the basis of our experiments one may postulate that apart from the putative Ca^{2+}-channel blocked by verapamil, there exists another kind of Ca^{2+}-channel through which the influx of Ca^{2+} may partly occur. The lack of their action may be explained by the inability of drug charged-molecules to penetrate cell membranes and to bind external receptors (Kass and Arena 1989).

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