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# Effects of Heavy Metals on Stomatal Movements in Broad Bean Leaves[1]

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**Abstract**—How do heavy metals affect stomatal movements and whether water channels are involved in stomatal movements was investigated in broad bean (*Vicia faba* L.) leaves. Three-week old fully expanded leaves were harvested. Leaf epidermis was peeled off and soaked in the Mes–KOH buffer containing the salts of heavy metals. Stomatal aperture was measured under the microscope. The tested heavy metal ions, such as $Hg^{2+}$, $Pb^{2+}$, $Zn^{2+}$, and $La^{3+}$, partly inhibited stomatal opening in light or closing in darkness at submillimolar concentrations, while $K^+$, $Na^+$ and $Mg^{2+}$ had no visible effects on stomatal movements. As compared to $La^{3+}$, $Hg^{2+}$ affected stomatal movements more significantly. Stomatal movements were almost completely inhibited under a combined $Hg^{2+}$ and $La^{3+}$ treatment. Apparently, $La^{3+}$, a $Ca^{2+}$ channel blocker, inhibits the changes in the cytosolic $Ca^{2+}$ concentration in guard cells, thus affecting stomatal movements. The inhibitory effect of $Hg^{2+}$ on stomatal movements may be explained by the inhibition of water channels. Like $Hg^{2+}$, $Zn^{2+}$ and $Pb^{2+}$ interfered with stomatal movements. It is concluded that heavy metals at submillimolar concentrations inhibit stomatal movements. They may affect water fluxes through guard cell membranes in different ways, i.e., $Hg^{2+}$, $Pb^{2+}$, and $Zn^{2+}$ inhibit water channels, whereas $La^{3+}$ block ion channels. Water channels may be involved in stomatal movements by regulating water fluxes and play a dominant and primary role in stomatal movements.

*Key words: Vicia faba - heavy metals - water channels - ion channels - stomatal movements*

## INTRODUCTION

Numerous experiments have demonstrated that plants responded to the environmental stimuli, such as water deficit, $CO_2$ enrichment, and high temperature, by the regulation of stomatal movements, i.e., opening and closing [1–3]. It has been well studied that heavy metals at high concentrations generally caused lethal or sublethal effects on plants [4–6]. Whether heavy metals at 0.2–1.0 mM concentrations affected stomatal movements and in which way still awaits further exploration. A network of ion channels, which may control stomatal movements, has been well characterized in the plasma and vacuolar membranes of guard cells [6–8]. Water channels, which can specifically and rapidly promote the water transport, have been found in the plasma and vacuolar membranes of guard cells [9–11]. It is still not clear whether water channels were involved in stomatal movements and what is the role of water and ion channels in stomatal movements. $HgCl_2$ can inhibit water channels to regulate water transport [12–17]. There is a possibility that heavy metals may affect stomatal movements by inhibition of water channels and limit water fluxes into guard cells.

In this study, broad bean leaf peels were treated with chlorides of various heavy metal ions, such as $Hg^{2+}$, $Zn^{2+}$, $Pb^{2+}$, and $La^{3+}$. Stomatal movements were observed to evaluate the effects of heavy metals. It was hypothesized that (1) heavy metals significantly affect stomatal movements, including stomatal opening and closing; (2) water channels may be involved in the influence of heavy metals on stomatal movements; (3) water channels and ion channels may play important but different roles in stomatal movements.

## MATERIALS AND METHODS

*Plant material and growth conditions.* Broad bean (*Vicia faba* L., cv. Dabaican) seeds were first soaked in water for 48 h and then sowed in pots with soil. The seedlings were watered every 3 days to keep 80 ± 5% field water capacity and grown at the irradiance of 829 μmol/($m^2$ s) at an average day/night temperature of 23.7/11.2°C. Three-week old fully expanded leaves were cut, and the abaxial epidermis was peeled off. In order to maintain the activities of stomatal guard cells, leaf peels were put in the Mes–KOH buffer, which contained 10 mM Mes (pH 6.0), 50 mM KCl, and 0.1 mM $CaCl_2$.

[1]This article was submitted by the authors in English.

*Abbreviation*: ME—mercaptoethanol.

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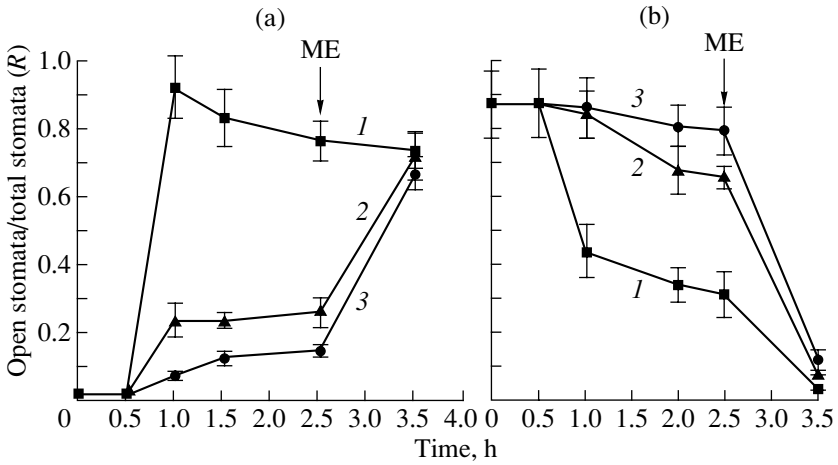


**Fig. 1.** Inhibition of stomatal (a) opening in the light and (b) closing in the dark by $HgCl_2$, and reversal of inhibition by ME (arrow). (*1*) Control leaf peels; (*2*) 0.4 mM $HgCl_2$, and (*3*) 0.8 mM $HgCl_2$.

*Treatments with salts.* Leaf peels were soaked in the Mes–KOH buffer in 10-ml containers and kept in darkness for 3 h to induce the stomata closing. $HgCl_2$ was added, and the material remained in darkness for another hour. Then, the containers were transferred to the light and 5 mM 2-mercaptoethanol (ME) was then added to overcome the inhibition. Observations and counting were performed before and after the ME addition. In other experiments, the peels soaked in the Mes–KOH buffer were first kept in the light to induce stomata opening. The next step was to observe the inhibition of stomatal closing by $HgCl_2$ in darkness followed by the reversion of inhibition by ME at 5 mM concentration. The protocol and time scales were the same with other salts. $ZnCl_2$, $PbCl_2$, $LaCl_3$, KCl, NaCl, and $MgCl_2$ were also tested at 0.8 mM concentration ($LaCl_3$ at 0.5 mM).

*Measurements and statistical analyses.* Three samples per treatment were used. In each sample, 6–8 fields were randomly selected and observed under the microscope. The number of the open stomata and the total stomata number were recorded, and the open stomata/total stomata ratio (*R*) was calculated. The stomatal pores were not fully open or closed after the treatments. The stomatal aperture was classified as follows: if no visible distance was seen between the guard cells surrounding the pore, the stoma was judged to be closed; other stomata were taken to be open.

Standard deviation (S.D.) for each treatment was calculated. The significance of the differences was calculated using *t*-test at $P < 0.05$ with Duncan's Multiple Range Test.

## RESULTS

The time-course of the ratio of open stomata to total stomata (*R*) under $HgCl_2$ treatments radically differed from that in the control leaves (Fig. 1). The addition of $HgCl_2$ largely inhibited stomatal movements. In the presence of $HgCl_2$, *R* was less than that in the control leaves in Fig. 1a, but greater than in Fig. 1b. The addition of $HgCl_2$ inhibited stomatal opening in light or closing in darkness, respectively. The inhibition by 0.8 mM $HgCl_2$ was more pronounced than by 0.4 mM $HgCl_2$ (Fig. 1), *R* being less at 0.8 mM $HgCl_2$ than that at 0.4 mM $HgCl_2$ in the light, but greater in the dark. The addition of 5 mM ME could significantly (from 50 to 90%, detailed data not shown) increase *R* in the light (Fig. 1a) or decrease to the control values in the dark (Fig. 1b). The inhibition of stomatal movements by $HgCl_2$ was largely reversible by ME.

The stomatal movements in the case of KCl, NaCl, and $MgCl_2$ treatments occurred like in the control (Fig. 2). The incubation in $ZnCl_2$, $HgCl_2$, and $PbCl_2$ inhibited stomatal opening in the light (Fig. 2a) or closing in darkness (Fig. 2b). Some small differences were observed between $Mg^{2+}$ treatment and $K^+$, $Na^+$ treatments, as well as between $Hg^{2+}$ and $Pb^{2+}$ and $Zn^{2+}$ salts.

The changes in the stomatal aperture varied with $HgCl_2$, $PbCl_2$, or $ZnCl_2$ concentrations (Fig. 3). The inhibition of stomatal movements became more powful at the greater concentrations of $HgCl_2$, $PbCl_2$, or $ZnCl_2$. R could not decrease to 0 even if the heavy metal concentration increased to 1.0 mM.

After $LaCl_3$ treatments, the ratio significantly differed from control values and changed with time (table). The $LaCl_3$-induced inhibition of stomatal movements attenuated with time. The effect of $LaCl_3$ treatments differed from that after $HgCl_2$ treatments (table). Combined $HgCl_2$ and $LaCl_3$ treatment, unlike $HgCl_2$ or $LaCl_3$ treatments, resulted in the values very close to 0 or 1, i.e., stomatal movements were almost completely inhibited.

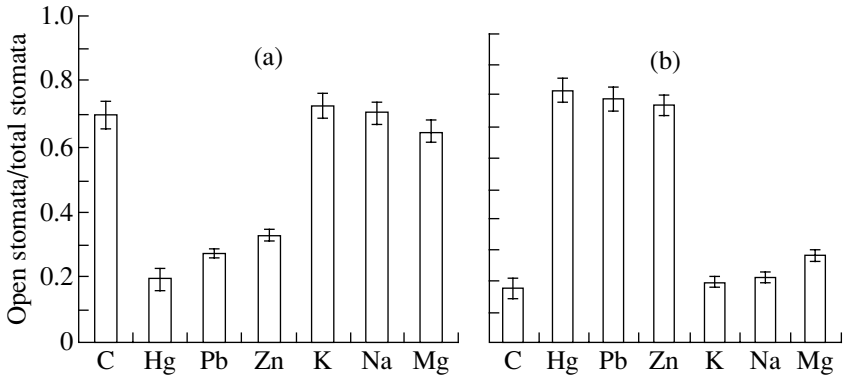


**Fig. 2.** Effects of leaf peel treatment with various cations on stomatal (a) opening in the light and (b) closing in the dark. All salts are tested at 0.8 mM concentration. C—control leaf peels.

## DISCUSSION

### *Effects of Heavy Metals on Stomatal Movements*

The experiments have shown that heavy metals strikingly affected stomatal movements at submillimolar concentrations, probably in different ways. $LaCl_3$, a $Ca^{2+}$ channel blocker, apparently affected the changes in the cytosolic $Ca^{2+}$ concentration in guard cells, thus indirectly influencing the activities of other ion channels, such as $K^+$, $Cl^-$, and malate$^{2-}$ channels, and finally, stomatal opening or closing [18]. The inhibition of water flow through water channels by lower concentrations of mercurial reagents has been tested in a lot of experiments [12–17]. Though they can affect plants via other pathways, for instance, the metabolic pathway or enzymes, $Hg^{2+}$ affected stomatal movements mainly by blocking water channels as follows from the significant differences between its effect on stomatal movements and that of $La^{3+}$. The reversal of inhibition by ME addition confirmed this conclusion. In addition, since the exposure duration was not so long, $HgCl_2$ could exert slight effects on other channels or enzymes. $Pb^{2+}$ and $Zn^{2+}$ also significantly affected stomatal movements (Fig. 2) and the inhibitions became more obvious as the concentrations increased (Fig. 3). Stomatal aperture after incubation with $Pb^{2+}$ and $Zn^{2+}$ was close to that after incubation with $Hg^{2+}$ (Fig. 2). $Pb^{2+}$, $Zn^{2+}$, and $Hg^{2+}$ have similar atomic semi-diameters and valency and share the same mechanism to affect water channels. On the whole, it may be suggested that heavy metals affected stomatal movements at least in two different ways, i.e., via water channels or ion channels.

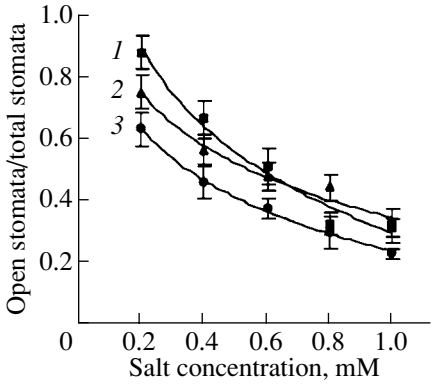


**Fig. 3.** Concentration-dependent inhibitions of stomatal movements by (*1*) $HgCl_2$, (*2*) $PbCl_2$, and (*3*) $ZnCl_2$.

The concentrations of heavy metal ions used in this study are a little higher than those reported by other researchers [14, 16] due to some properties of other epidermal cells and the cell wall of guard cell in broad bean leaves.

### *Inhibition of Water Channels in Guard Cell Membranes by $HgCl_2$*

It was suggested that aquaporins in guard cell might be involved in stomatal movements [11, 18, 19]. Here, the direct evidences confirm this possibility. In previous reports, it was shown that mercurial agents at submillimolar concentrations inhibited plant water channels [12–14, 16], although there are some exceptions [15]. We demonstrated a significant difference between $HgCl_2$ and $LaCl_3$ effects on stomatal movements (Table and Fig. 2), which may be explained by different mechanisms. $HgCl_2$ inhibits water channels in *Vicia faba* leaves [18, 19]. The substantial effects at very high $HgCl_2$ concentrations have been interpreted as the toxicity of $Hg^{2+}$ [15], but at lower concentrations it can exert the direct or indirect effects on water channels [13, 14, 16]. Some researchers considered the possibilities that $Hg^{2+}$ may affect the metabolic process or cause the membrane depolarization [15, 16] that would more or less affect stomatal movements. In our opinion, $Hg^{2+}$ directly inhibited water channels to suppress stomatal movements. β-mercaptoethanol could specifically reverse the effects of $Hg^{2+}$. Such reversion of $HgCl_2$ inhibition by ME further proves that water channels were really involved in stomatal movements. Slight effects of $Mg^{2+}$ on stomatal movements are in

Effects of the addition of 0.8 mM $HgCl_2$ and 0.5 mM $LaCl_3$ on the open stomata/total stomata ratio ($R$) in the light or in darkness

| Treatment | $R$ in the light | | $R$ in the dark | |
|---|---|---|---|---|
| | 2 h after salt addition | 3 h after salt addition | 2 h after salt addition | 3 h after salt addition |
| Untreated | 83.2 ± 3.7[a] | 87.5 ± 3.8[a] | 12.3 ± 1.5[d] | 3.3 ± 0.1[d] |
| $HgCl_2$ | 6.3 ± 0.4[c] | 28.7 ± 1.1[c] | 70.9 ± 2.7[b] | 67.3 ± 2.5[b] |
| $LaCl_3$ | 29.7 ± 1.3[b] | 58.3 ± 2.1[b] | 39.4 ± 1.4[c] | 32.1 ± 1.6[c] |
| $HgCl_2 + LaCl_3$ | 0.3 ± 0.1[d] | 0.5 ± 0.1[d] | 84.2 ± 3.2[a] | 78.5 ± 2.3[a] |

Note: After the addition of $HgCl_2$, or $LaCl_3$, or both, the peels were incubated for another hour and then transferred to the light or dark. An hour later, observations and measurements were performed (2 h after salt addition). Within columns, values mean average percent ± standard deviation ($n = 8$) and values followed by different letters are significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

part similar to those reported for $Ca^{2+}$, which also inhibited water channels [20]. This effect also confirmed that water channels may be involved in stomatal movements.

### *Involvements of Water and Ion Channels in Stomatal Movements*

Water is known to enter the cells not only via water channels but also directly by diffusion through membrane matrix. The former pathway is regulated by water channel activity and the latter is controlled by the gradient of water potentials between the inner and outer sides of the membranes. Addition of $HgCl_2$ inhibited more than 70% of stomatal movements (Fig. 1), and the inhibition became stronger at a higher $HgCl_2$ concentration (Fig. 3), whereas $LaCl_3$ induced only 20% inhibition (table). Combined $HgCl_2$ and $LaCl_3$ treatment almost completely inhibited stomatal movements (table). In our experiments, the $HgCl_2$ concentration used inhibited water channels; meanwhile, $LaCl_3$ affected the major ion channels in the membranes. As a result, the osmotic potential and water potential were affected outside and inside the guard cells, water fluxes were limited, and guard cell volumes could not change. With time, the extent of inhibitions of stomatal movements decreased in light and in darkness (table). Thus, water diffused directly through the membrane lipid matrix even when water channels were inhibited, guard cell volumes changed and stomatal aperture also changed. Therefore, water channels and ion channels together controlled stomatal movements, and the former may be of primary importance. In addition, the changes of the cytosolic $Ca^{2+}$ concentration may partly regulate the activities of water channels [21–23] and thus control stomatal movements. Water channels may be parallel to or the downstream element in $Ca^{2+}$/calmodulin signal transduction pathway, or both. We preferred the third way on the basis of data obtained.

The proportion of opened stomata did not reach 1 or 0 in this study, and stomatal movements were not inhibited completely by $HgCl_2$, $LaCl_3$, or $HgCl_2$ + $LaCl_3$ treatments. Not all stomata were in the same state, either open or closed. Some of them appeared open, while others were closed. It was supposed that stomatal oscillations increased water use efficiency of plants by controlling stomatal state [24]. Water channels would be involved in stomatal oscillations, a special stomatal movement.

Plants could adapt to their environments or respond to the stresses by regulating cell water relations through water channels [2, 17, 25, 26]. In previous works, the ways on regulating stomatal movements were considered mainly with respect to ion aspects. Now, more attention would be paid to water channels to look for a new path to resist to the environmental stresses.

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