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Involvement of Cl^- in the Increase in Proline Induced by ABA and Stimulated by Potassium Chloride in Barley Leaf Segments

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ABSTRACT

Stimulation by sodium or potassium chloride of the ABA-induced increase in proline was synergistically enhanced by $CaCl_2$ or $MgCl_2$ as well as by 1,3-bis[tris(hydroxymethyl)methylamino] propane chloride (BTP-Cl), *N*-methyl-D-glucamine chloride (NMG-Cl), or 2-amino-2-hydroxymethyl-1,3-propandiol chloride (TRIS-Cl). This enhancing effect did not depend on the osmolarity and occurred when Cl^- was higher than K^+ in the incubation medium, but not vice versa. When $CaCl_2$ or $MgCl_2$ or NMG-Cl were added, the higher the Cl^-:K^+ ratio in the external solution the higher was the increase in proline. When the excess of Cl^- to K^+ was obtained with BTP-Cl the highest enhancing effect resulted with a Cl^-:K^+ ratio of 3:1 while, at a 5:1 ratio, the KCl stimulation was completely suppressed. The inhibiting effect of proline accumulation by NH_4^+ and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene was reversed to varying degrees depending on the magnitude of the excess of Cl^- on K^+ concentration in the medium. Also, the inhibition of proline accumulation obtained by tetraethylammonium chloride, monensin, and D-mannose was similarly reverted. These data suggest that Cl^- elicits an increase in ABA-induced proline which needs the simultaneous presence of K^+ (or Na^+) to take place.

The increase in ABA-induced proline in isolated barley leaf segments incubated in solution occurs in a few hours and is stimulated to the same extent by NaCl and KCl when supplied exogenously (6). The magnitude of the stimulation depends both on Na^+ and K^+ chloride concentrations and on the appropriate anions but, when Na^+ and K^+ are added as salts of a nonpermeating anion, the ABA-induced increase in proline does not occur (4). Also, treatments with ABA alone are practically ineffective in the absence of salts in the external medium (6). A relationship has also been suggested between ion influxes and the accumulation of proline induced by the hormone, because DIDS[1] (an anion transport inhibitor) and TEA (a K^+ channel-blocking agent) inhibit the process (4). The involvement of ion fluxes seems in agreement with the lack of the effect of ABA alone in leaf segments in which, unlike the whole plants or intact leaves, the apoplasmic salt content should be very low because of the effect of washing and incubation in a salt free medium. The stimulating effect of NaCl and KCl is affected either positively and negatively by the simultaneous presence of other sodium or potassium salts in the external medium and it is enhanced by $CaCl_2$ or $MgCl_2$ (4).

On the basis of these findings, the enhancing effect of $CaCl_2$ or $MgCl_2$ on the Na(or K)Cl stimulation of the proline increase was investigated further.

MATERIALS AND METHODS

Plant Material

Sections (6 mm long) from intermediate portions of fully expanded primary leaves of 1 week old barley seedlings (*Hordeum vulgare* cv Georgie) (Sementi Bovo, Isola della Scala, Verona, Italy) grown in a phytotron chamber as previously described (6) were used.

Proline Evaluation Experiments

The samples consisted of 300 mg fresh weight of leaf segments prepared as previously described (6), in 20 mL 10 mM Mes buffer (pH 5.5 with Tris), 0.5 mM $CaSO_4$ and KCl at the desired concentration as specified in the individual experiments. ABA was added at the optimal 0.1 mM concentration (6). Stock solution of BTP-Cl, NMG-Cl, or Tris-Cl were prepared as follows: 1 M (final concentration) HCl solution was brought to pH 5.5 by adding BTP, NMG, or Tris powder. At this pH the concentrations were: BTP, 0.495 M; NMG, 1 M; Tris, 1 M.

Incubation was always carried out for 7 h in the dark at 25°C in a shaking bath.

Proline was extracted by homogenizing the leaf sections in a mixture of methanol:chloroform:water (12:5:1 v/v) without Permutit resin and determined according to the colorimetric method of Singh *et al.* (8) partially modified (5). Other details were as previously described (6). All treatments were run in triplicate and the data are the average (±SD) of at least two experiments.

RESULTS AND DISCUSSION

Effect of $CaCl_2$ and $MgCl_2$

Table I shows that the increase in ABA-induced proline due to KCl stimulation was enhanced in the presence of $CaCl_2$ or $MgCl_2$. The influence of the two salts was nearly the same and depended on their concentration in the external medium.

[1] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; NMG, *N*-methyl-D-glucamine; TEA, tetraethylammonium chloride.

Table I. *Effect of Different Concentrations of $CaCl_2$ or $MgCl_2$ on the Stimulation by KCl of the ABA-induced Proline Accumulation*

The values in brackets are the nmol/g fresh weight of proline detected with different concentrations of $CaCl_2$ or $MgCl_2$ in the presence of 0.1 mM ABA but without KCl. The values are corrected for the nmol of proline measured in the presence of ABA alone (210 nmol/g fresh weight). Values are given as means ± SD. The level of proline in the absence of ABA (about 170 nmol/g fresh weight) was not changed in the presence of the tested salts at all the considered concentrations.

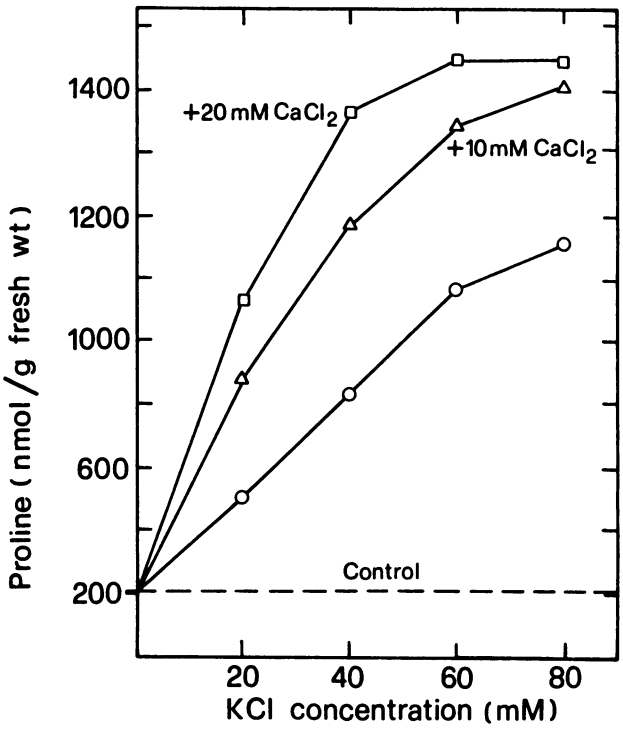
Treatments		Proline Accumulation in 7 h	Difference with Respect to KCl Effect	Increase with Respect to KCl Effect
		nmol/g fresh wt		%
30 mM KCl		480 ± 35	—	—
+10 mM	$CaCl_2$ (25 ± 10)	912 ± 73	+ 432	90
	$MgCl_2$ (18 ± 10)	878 ± 57	+ 398	83
+ 20 mM	$CaCl_2$ (30 ± 15)	1200 ± 90	+720	150
	$MgCl_2$ (33 ± 18)	1152 ± 92	+ 672	140
+ 30 mM	$CaCl_2$ (25 ± 18)	1305 ± 117	+ 825	172
	$MgCl_2$ (35 ± 20)	1358 ± 86	+ 878	183

The increased accumulation of proline obtained in the presence of KCl and $CaCl_2$ (or $MgCl_2$) indicated a synergistic effect of the two salts as the effects of calcium and magnesium chlorides alone (values in brackets in Table I) were negligible.

The increased levels of proline induced by ABA and stimulated by KCl (in the concentration range of 20–80 mM) in the presence of two concentrations of $CaCl_2$ are shown in Figure 1. The synergistic effect of both concentrations of $CaCl_2$ was observed at each tested KCl concentration and was, as a percentage, higher for the lower KCl concentration. In order to determine if the effect of $CaCl_2$ or $MgCl_2$ was due to the divalent cations or rather to chloride, experiments were performed in which chloride was supplied together with an impermeant cation as BTP, NMG (1), or TRIS. The results of Table II show that the synergistic effect was also evident in the absence of Ca^{2+} or Mg^{2+}. Furthermore, chloride in the presence of BTP, NMG, or TRIS as a counterion, had a very negligible effect on the ABA-induced increase in proline in the absence of KCl in the incubation medium. The possibility that an addition of K^+ to the incubation medium containing KCl and ABA (*i.e.* a higher concentration of K^+ with respect to Cl^-) would enhance the KCl stimulation of the hormone-mediated process, was tested. The results of Table III show that the process induced by ABA was positively influenced by the addition of K-Mes to the external medium, while it was inhibited by K-gluconate. Moreover, Table III shows that the increased osmolarity of the medium obtained by mannitol depressed KCl stimulation, thus ruling out the possibility of the involvement of medium osmotic pressure in the case of Ca(or Mg)Cl_2 enhancement.

Effect of Chloride

To test the effect of higher concentrations of Cl^-, with respect to that of K^+ ('Cl^--excess') in the external medium, a low concentration of KCl (20 mM) was chosen to stimulate



Synergistic effect of $CaCl_2$ (%)

	KCl concentration (mM)			
	20	40	60	80
+10 mM $CaCl_2$	+128	+85	+55	+39
+20 mM $CaCl_2$	+210	+141	+76	+52

Figure 1. Enhancing effect of $CaCl_2$ on the accumulation of proline induced by ABA and stimulated by increasing KCl concentrations. The samples were incubated for 7 h with 0.1 mM ABA in the presence of different concentrations of KCl. Control: samples incubated with ABA + 10 or 20 mM $CaCl_2$ (the values are practically the same with both concentrations of $CaCl_2$, see also Table I, and somewhat lower in the absence of ABA). Synergistic effect (%) is calculated on the basis of values corrected from nmol of proline detected in the presence of ABA + $CaCl_2$. SD did not exceed ±7%.

the ABA-induced increase in proline, in order to avoid excessive salt concentration due to chloride addition. The results of Figure 2 show that when Cl^--excess was obtained by means of $MgCl_2$ or NMG-Cl addition, the synergistic effect progressively increased with the increase of the Cl^-:K^+ ratio in the medium. In the case of $MgCl_2$ addition, when the Cl^-:K^+ ratio was 5:1 (*i.e.* 100 mM Cl^- concentration, of which 80 mM was $MgCl_2$ and 20 mM KCl), the proline accumulation increased to a level 30% higher than that detected in the presence of 80 mM KCl alone (Fig. 1). This finding suggested that chloride is a limiting factor in the stimulating effect of KCl. However, the addition of BTP-Cl showed a maximum enhancement when the ratio was 3:1; at higher Cl^-:K^+ ratios the stimulating effect of KCl decreased and was completely abolished at 5:1. Preliminary results show that a synergistic effect similar to that of Cl^- was also obtained with the addition of NO_3^- but not with the less permeating SO_4^{2-}. As the

Table II. *Comparison between the Effect of Ca^{2+} or Mg^{2+} Chlorides and the Effect of Other Chlorides on the Increase in Proline Induced by ABA and Stimulated by KCl*

The values in brackets are the nmol detected with the tested chlorides in presence of 0.1 mM ABA but without KCl. The values are corrected for the nmol of proline measured in the presence of ABA alone (185 nmol/g fresh weight) and are given as means ± SD. The level of proline in the absence of ABA (about 160 nmol/g fresh weight) was not changed in the presence of the salts considered.

Treatments	Proline Accumulation in 7 h	Difference with Respect to KCl Effect
	nmol/g fresh wt	
10 mM KCl alone	223 ± 15	
10 mM KCl + 10 mM $CaCl_2$	(20 ± 9) 650 ± 45	+ 427
10 mM KCl + 10 mM $MgCl_2$	(25 ± 8) 620 ± 56	+ 398
10 mM KCl + BTP-Cl[a]	(30 ± 9) 633 ± 50	+ 410
10 mM KCl + NMG-Cl[a]	(37 ± 12) 651 ± 44	+ 428
10 mM KCl + TRIS-Cl[a]	(50 ± 10) 482 ± 38	+ 259

[a] Cl^- concentration was 20 mM.

Table III. *Effect of K-Mes, K-gluconate and Mannitol on the Stimulation by KCl of the ABA-induced Proline Accumulation*

The values are corrected for the proline detected in the presence of ABA alone (220 nmol/g fresh weight). ABA concentration was 0.1 mM in all the samples. The values are given as means ± SD. The values in brackets are the percent variation with respect to the KCl stimulation. The treatments adopted did not change the level of proline in the absence of ABA (about 170 nmol/g fresh weight).

Treatments[a]	Proline Accumulation in 7 h
	nmol/g fresh wt
20 mM KCl alone	307 ± 18
20 mM KCl + 20 mM K-Mes[b]	330 ± 21 (+8%)
20 mM KCl + 40 mM K-Mes[c]	310 ± 23
20 mM KCl + 20 mM K-gluconate	92 ± 6 (−70%)
20 mM KCl + 40 mM K-gluconate	60 ± 5 (−80%)
20 mM KCl + 40 mM mannitol	135 ± 11 (−56%)
20 mM KCl + 80 mM mannitol	179 ± 16 (−41%)

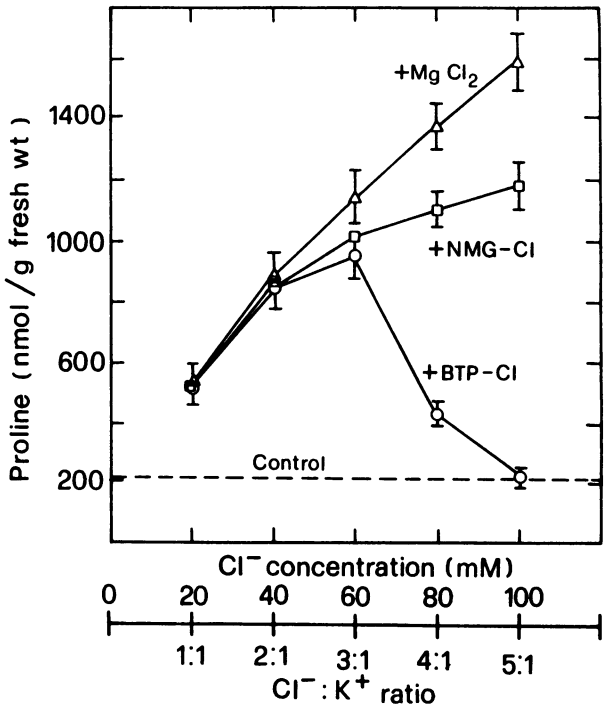
[a] KCl concentration was 20 mM. [b] MES concentration was 75 mM. [c] MES concentration was 170 mM.

permeability of the accompanying anion influences the uptake of the cation (4), one could speculate about a possible involvement of the transmembrane electric potential in the stimulation by Cl^--excess of the ABA-induced and salt-stimulated proline accumulation.

A possible role of Cl^--excess could also be in the regulation of the intracellular pH as described in the squid giant axon (7).

Effect of the Chloride on Proline Accumulation in the Presence of Inhibitors

The possibility was considered of an interference of Cl^--excess with the effect of some substances inhibiting the ABA-induced and KCl-stimulated increase in proline. The results, obtained by a Cl^--excess due to the addition of NMG-Cl, on the effect of NH_4^+ and DIDS, whose inhibition was previously described (4), are reported in Figure 3, A and B. It is shown that the higher the Cl^--excess, the higher the reversing effect was on the inhibition of proline accumulation. The addition of 40 mM Cl^- almost completely restored the effect of KCl alone in both the inhibiting treatments when the inhibition was around 60%, while when the inhibition was lower, the effect of KCl was even enhanced. Other inhibitors of proline accumulation were tested such as TEA (4), D-mannose, and monensin (Fig. 4). While the inhibiting actions of TEA and DIDS on proline accumulation induced by ABA and stimulated by Na(or K)Cl are accompanied by a decrease in the uptake of ions (4), the actions of D-mannose and monensin do not appear to be linked to a change of ions fluxes, at least



Proline level in presence of "Cl^--excess"
Per cent changes with respect to 20mKCl (1:1 ratio)

	Cl^- K^+ ratio 2:1	3:1	4:1	5:1
+ $MgCl_2$	+116	+202	+277	+346
+ BTP-Cl	+103	+138	−28	−94

Figure 2. Influence of the excess of Cl^- with respect to K^+ on the proline accumulation induced by ABA and stimulated by KCl. The samples were incubated for 7 h in the presence of 0.1 mM ABA and 20 mM KCl. The excess of Cl^- to K^+ was obtained with the addition of appropriate amount of $MgCl_2$, NMG-Cl, or BTP-Cl. Control: the average values of proline detected in the presence of ABA alone, ABA plus $MgCl_2$, plus NMG-Cl and plus BTP-Cl but without KCl (all these values ranged between ±6% with respect to ABA alone). SD was not greater than ±11%. The tested salts did not change proline value in the absence of ABA (about 180 nmol/g fresh weight).

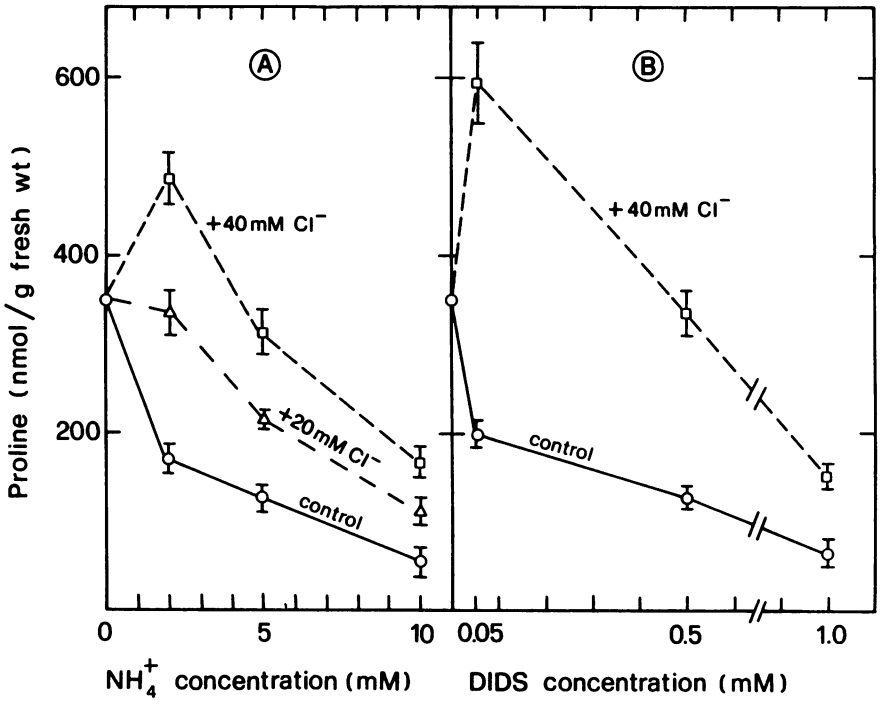


Figure 3. Reversing effect of Cl^--excess on the inhibition by NH_4^+ (A) and DIDS (B) of the ABA-induced and KCl-stimulated increase in proline. The samples were incubated for 7 h in 0.1 mM ABA and 20 mM KCl (control, ○). NH_4^+ was added as ammonia solution brought to pH 5.5 with Mes. 20 mM (△) or 40 mM Cl^- (□) were added as NMG-Cl. The reported values are corrected for the nmol of proline measured in the presence of ABA alone (200 nmol/g fresh weight). SD is shown by bars.

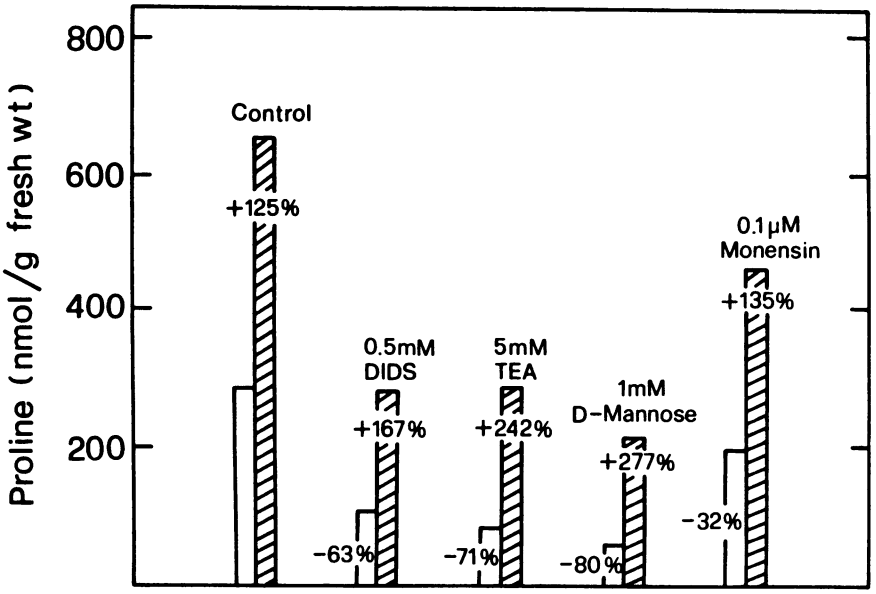


Figure 4. Reversing effect of Cl^--excess on the inhibition by some substances of the ABA-induced and KCl-stimulated increase in proline. The samples were incubated for 7 h. The accumulation of proline induced by 0.1 mM ABA and stimulated by 20 mM KCl (control, □) was tested in the presence of the indicated inhibitors; the reported percent values represent the effects respect to the sample without inhibitor. Dashed bars (▨) indicate the accumulation of proline obtained in the presence of the 40 mM Cl^--excess (as NMG-Cl). The percent values represent the increasing effect due to Cl^--excess on proline level in the presence of the inhibitors. All the values are corrected for the nmol of proline measured in the presence of ABA alone (195 nmol/g fresh weight). SD did not exceed ±9%.

in the conditions adopted here (data not shown). D-Mannose and the ionophore monensin, whose effects are under investigation in our laboratory, are known in many cases to inhibit the protein glycosilation or the movement of vesicles of the Golgi complex respectively (2, 10), when used at the concentrations employed here. If these are their mechanisms of action, the inhibiting effect on proline increase is in agreement with they hypothesis of an involvement of a proteic factor in the proline accumulation induced by ABA (3, 9). The data of Figure 4 show that, in the presence of all these substances, the Cl^--excess (obtained with NMG-Cl addition) reversed their inhibiting effects. Similar results were also obtained with $CaCl_2$ or $MgCl_2$ and BTP-Cl (data not shown). Two suggestions can be made about the reversing effect of Cl^--excess. First, as it is reasonable to think that the various inhibitors act through different mechanisms, the reversing effect of Cl^--excess probably consists in the stimulation of the residual, uninhibited activity of the mechanism (or of one of its components) that leads to the ABA-induced and KCl-stimulated increase in proline. Second, as the inhibition can be reversed by a Cl^--excess, the effect of these inhibitors is not likely due, mostly, to their possible action on the drop of cellular energy charge.

All the data reported in this paper were practically identical when NaCl, as stimulator of ABA-induced proline increase, was substituted for KCl.

CONCLUSIONS

These results show that Ca^{2+} and Mg^{2+} are not involved in the increase in ABA-induced proline due to KCl stimulation, since the enhancing effect of $CaCl_2$ and $MgCl_2$ depends only on the addition of chloride to the medium. As previously shown, the stimulation of proline increase appears only when Na^+ or K^+ and Cl^- are simultaneously present in the external medium (6). The data presented here indicate that this stimulation is enhanced when there is an excess of Cl^- with respect to K^+ (or Na^+), but not vice versa. In the presence of 20 mM KCl, it is possible to increase the proline level in the ABA-treated tissue, by a 'Cl^--excess,' to a higher level than that obtained in the presence of a much higher KCl concentration. Therefore, the increase in ABA-induced proline appears to be dependent on the presence of K^+ (or Na^+) but is stimulated by Cl^- when both ions are present in the incubation medium. The influence of a hormone on the uptake of ions or solutes is usually documented. In contrast to these and the previously reported findings (4), evidence is shown here of the influence of ion uptake on the effect of a hormone.

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