The rôle of calcium in the hatching of *Globodera rostochiensis*

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SUMMARY

Experiments indicating that free Ca^{2+} is not essential for the hatching of *Globodera rostochiensis* Woll. eggs, are described. *G. rostochiensis* eggs were stimulated to hatch by solutions of decationised potato-root exudate to which a range of 0.5 to 10mM of Na^+, K^+, Ca^{2+} or Mg^{2+} chlorides were added, but only the solution containing 0.5mM $CaCl_2$ caused the emergence of significantly more juveniles from cysts than the exudate alone. When cysts were extracted with the chelating agent EGTA to remove Ca^{2-} and other polyvalent cations the number of juveniles which emerged in dilute exudate solutions containing up to 2.0mM $CaCl_2$ reached a maximum at 0.1mM $CaCl_2$, probably because some of the hatching factor ionically bound to inert materials such as the cyst walls was displaced by Cl^-. Ca^{2+} is extracted from *G. rostochiensis* cysts by decationised potato-root exudate. Concentrations of 1 to 12 mM EGTA did not significantly inhibit the hatching induced by root exudate. We found that the calcium ionophore A23187 inhibited hatching and that Ca^{2+} did not activate juveniles immobilised by 0.4M trehalose. It is suggested that the initiation of hatching may be the result of changes in eggshell permeability brought about by the effect of the hatching factor on bound Ca^{2+}.

RÉSUMÉ

Rôle du calcium dans l'éclosion de Globodera rostochiensis

Les auteurs décrivent des expériences indiquant que le Ca^{2+} libre n'est pas essentiel pour l'éclosion des œufs de *Globodera rostochiensis* Woll. L'éclosion de ces œufs a été stimulée par des solutions d'exsudats de racines de pomme de terre auxquelles des chlorures de Na^+, K^+, Ca^{2+} et Mg^{2+} étaient ajoutés en quantités variant de 0.5 à 10mM, mais seule la solution contenant 0.5mM $CaCl_2$ a provoqué la sortie d'un nombre de juvéniles significativement supérieur à celui obtenu avec l'exsudat seul. Quand les kystes étaient extraits avec de l'EGTA, agent de chélation, pour enlever Ca^{2+} et autres cations polyvalents, le nombre de juvéniles émergeant dans des solutions d'exsudats contenant jusqu'à 2mM $CaCl_2$ était maximum pour 0,1mM $CaCl_2$, probablement parce qu'un facteur stimulant l'éclosion et lié à un matériel inerte, tel que la paroi du kyste, était déplacé par Cl^-. Ca^{2+} est extrait des kystes de *G. rostochiensis* par l'exsudat décationisé. Des concentrations de 1 à 12mM d'EGTA n'ont pas inhibé significativement l'éclosion induite par l'exsudat de racine. Les auteurs ont observé que l'ionophore A23187 du calcium inhibait l'éclosion et que Ca^{2+} n'activait pas les juvéniles immobilisés par 0,4 mM de tréhalose. Ils émettent l'hypothèse que l'initiation de l'éclosion peut être le résultat de changements dans la perméabilité du tégument de l'œuf dus à l'action du facteur stimulant de l'éclosion sur le Ca^{2+} lié.

Robinson and Neal (1956–1959)—Ellenby and ——— to have a similar effect. Clarke and Hennessy (1981)

ment in *G. rostochiensis* hatching. We re-examined the effects of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} on the hatching properties of decationised potato-root exudate (DPRE) using both untreated cysts, and cysts extracted with chelating agents to remove divalent cations. We also determined the amounts of Ca^{2+} extracted from cysts by DPRE and chelating agents. We repeated hatching tests (Atkinson & Ballantyne, 1979) of the ionophore A23187. The compound is reported to be a hatching agent and might act by carrying Ca^{2+} through the egg-shell. Furthermore, we tested the effect of Ca^{2+} on juveniles immobilised in 0.4M trehalose (Clarke, Perry & Hennessy, 1978), *i.e.* with the juveniles exposed to the conditions

tests contained 0.13mM KCl, 0.21mM NaCl, and either 0.29mM $MgCl_2$ and 4.06 mM $CaCl_2$ ("Salts AA") or 0.13mM $MgCl_2$ and 2.10M $CaCl_2$ ("Salts AB"), depending on whether the concentrations (given in μg/ml for "Salts A") refer to the anhydrous or hydrated salts of Mg^{2+} and Ca^{2+}. We therefore compared (Tab. 1) the number of juveniles which emerged from cysts in a 1:4 dilution of DPRE, in 1:4 dilutions of DPRE containing "Salts AA", and "Salts AB", and in 1:4 dilutions of DPRE containing 4.06mM or 2.10mM $CaCl_2$.

To observe changes in the pH of test solutions, batches of about 100 cysts were prepared as for hatching tests (see above). After soaking the water

addition of $CaCl_2$, and also at one and two days after dilution with water to obtain a trehalose concentration less than 0.1M.

The ionophore 23187 is only sparingly soluble in water and so is often used as a colloid, prepared by adding a concentrated solution in organic solvent to a vigorously agitated aqueous phase. We found that organic solvents tended to inhibit hatching and so used suspensions instead : the solvent was removed from a small volume of 0.01M ethanolic solution of A23187 by a current of air. The film of ionophore was scraped from the glass with a spatula and the particles suspended in the aqueous medium. Ultraviolet light showed any A23187 left in the beaker.

The solubility of A23187 in aqueous solutions was determined by spectroscopy. The aqueous phase

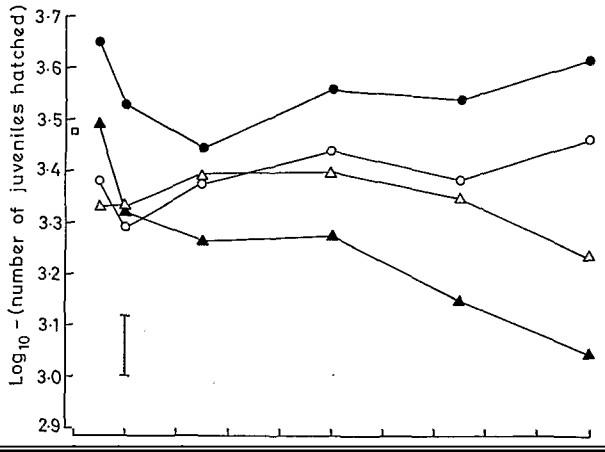


Table 1

Emergence of juveniles from cysts of *G. rostochiensis* treated with (A) a 1:4 dilution of decationised potato-root exudate (DPRE) and with similar dilutions of DPRE containing "Salts AB" or 2.10mM $CaCl_2$; (B) a 1:4 dilution of DPRE, and similar dilutions of DPRE containing "Salts AA" or 4.10mM $CaCl_2$. Three-fold replication, 100 cysts/test.

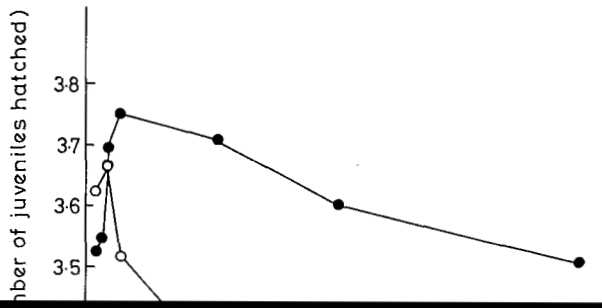


Table 3

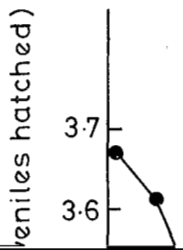
Total calcium (ppm), given as the mean of three replicates together with the standard error, in four successive extracts at 25° of *G. rostochiensis* cysts (100 cysts/1.5 ml solution) with 30mM EGTA, and three with glass-distilled water (GDW) and then with either a 1:4 dilution of decationised potato-root exudate (DPRE) or 2mM EGTA. Mean juvenile emergence from 100 cysts, in 1:4 DPRE (8a) and 2 mM EGTA (8b), 3 700 and 150, respectively.

Extract	*Extracting solution*	*Period of treatment (days)*	*Calcium content (ppm)*
1	EGTA	2	21.53 ± 1.258
2	EGTA	2	2.30 ± 0.367
3	EGTA	2	2.15 ± 0.377
4	EGTA	2	1.53 ± 0.150
5	GDW	1	1.18 ± 0.048
6	GDW	1	0.40 ± 0.071
7	GDW	1	0.53 ± 0.184

for Ca^{2+} removed from cysts by four successive EGTA extractions over eight days was 27.5 ppm (= 0.69mM).

There was little Ca^{2+} in the 1:4 DPRE (0.7 ppm) or in the 2 mM EGTA (1.7 ppm) removed from around the cysts after the 10-day treatment (Tab. 3) at 25°.

Figure 3 shows the number of juveniles which emerged from EGTA-extracted cysts in a 1:4 dilution of DPRE containing 1 to 12mM EGTA at pH 7.2. Although hatching decreased with increase in EGTA concentration, there was no significant difference ($p > 0.05$) between the hatch in DPRE and in EGTA



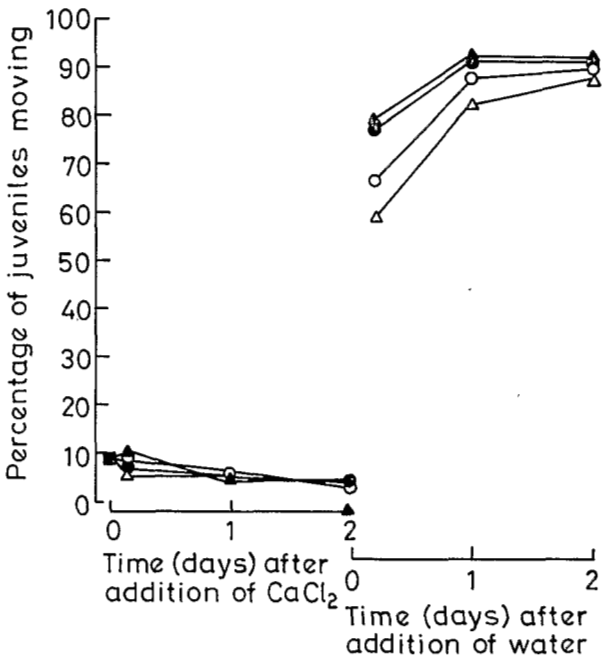


Fig. 4. Percentage of juveniles moving in 0.4 M trehalose (black square) solutions after seven days, and at 6, 24 and 48 h after adjustment of the solutions so that they contained 0 (black circle), 0.2 (white circle), 2.0 (black triangle), or 4.0 mM (white triangle) $CaCl_2$, and thereafter at 6, 24 and 48 h after addition of water.

Table 5

Emergence of juveniles from cysts immersed in potato-root exudate diluted 1:64 with distilled water or 0.4 mM $CaCl^2$, and in similar dilutions containing suspensions of the ionophore A23187 (0.10 μmol/10 ml). Five-fold replication, batches of 100 cysts for each test. The hatch obtained in a test marked * was significantly less ($p < 0.05$) than the hatch obtained in a test with the comparable solution without A23187.

Test Solution	*Mean Hatch*
Potato-root exudate diluted 1:64 with distilled water	2 205
Potato-root exudate diluted 1:64 with distilled water and containing a suspension of A23187 (0.10 μmol/10ml)	1 145*
Potato-root exudate diluted 1:64 with 0.4mM $CaCl_2$	2 580
Potato-root exudate diluted 1:64 with 0.4mM $CaCl_2$ and containing a suspension of A23187 (0.10μmol/10 ml)	930*
0.4mM $CaCl_2$	1 455
A suspension of A23187 (0.10 μmol/10 ml) in distilled water	375*
Distilled water	1 360

treated with A23187 suspended in water or with 1:64 dilutions of PRE containing A23187, were significantly less ($p < 0.05$) than the numbers which emerged from cysts in the appropriate solution without A23187.

DPRE alone, although the increase were not significant ($p > 0.05$). Our experiments with a mixture of cations ("Salts A", Robinson & Neal, 1959) showed (Tab. 1) that similar numbers of eggs were hatched by DPRE containing "Salts A" (with concentrations

with the hatching factor anion ; when cysts are treated with solutions of DPRE, some of the hatching factor binds to basic groups present in the cyst walls, and elsewhere. If $CaCl_2$ is present, the Cl^- competes more effectively than the hatching factor anion for the basic sites and hence the concentration of the hatching factor in solution is increased. The chlorides of other metals will behave similarly (in proportion to the Cl^- concentration). The effect is however more readily detectable with $CaCl_2$ because of the less pronounced inhibitory action of Ca^{2+}. In experiments with $MgCl_2$ for example (Fig. 2) the Cl^- concentrations, at which displacement of hatching factor from basic sites occur, are also those at which there is marked inhibition by Mg^{2+}. Na^+ and K^+ also inhibit more strongly than Ca^{2+} (Fig. 1).

to the test solutions. Atkinson, Taylor and Ballantyne (1980) reported that eggshells removed from stimulated eggs contained more Ca^{2+} than those from non-stimulated eggs.

Our observations on the influence of cations on the hatching of *G. rostochiensis* are clearly relevant to the assay of isolates of the natural hatching factors.

According to Atkinson and Taylor (1980) the initiation of hatching may involve the uptake of Ca^{2+} and its transport through the eggshell. Calcium-chelating agents such as EDTA or EGTA inhibit cellular Ca^{2+}-transport systems. Complete inhibition can be obtained by decreasing the concentration of free Ca^{2+} to $< 10^{-8}$ M with EGTA or EDTA (see Reed & Bygrave, 1975). Fig. 3 shows a substantial

0.4M trehalose remained inert even when the medium contained 4mM $CaCl_2$.

Atkinson, Taylor and Ballantyne (1980) have commented on the uptake of Ca^{2+} by *G. rostochiensis* juveniles in eggs treated with PRE. We suggest the Ca^{2+} uptake may be incidental to the inferred change in permeability of the egg-shell (Clarke & Perry, 1977 ; Clarke, Perry & Hennessy, 1978) rather than indicative of the hatching mechanism. The Ca^{2+} uptake by juveniles coincides with the uptake of water by the unhatched juveniles (Ellenby & Perry, 1976) and may be part of the maintenance of the internal salt balance of the juvenile in response to water uptake.

This paper reports several experiments which suggest that the hatching mechanism of *G. rostochiensis* does not involve the transport of Ca^{2+} through the eggshell. We have shown that *G. rostochiensis* eggs hatch in test solutions in which there is a virtual absence of free Ca^{2+}. We have indicated that the addition of Ca^{2+} to DPRE probably does not increase its ability to initiate hatching, and shown that Ca^{2+} does not activate juveniles immobilised in 0.4 M trehalose.

As an alternative hypothesis for the initiation of hatching we suggest that the hatching factor may change eggshell permeability (Clarke & Perry, 1977 ; Perry & Clarke, 1981) by its effect on the bound Ca^{2+} of the eggshell.

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