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Amelioration by glucose-6-phosphate and NADP of potato glycoalkaloid inhibition in cell, enzyme and liposome assays

James G. Roddick*, Anna L. Leonard

School of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, UK

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Abstract

Lysis of human erythrocytes by 20 μM chaconine was reduced by 0.5 mM glucose-6-phosphate (G6P) and NADP. Both compounds caused ~50% inhibition of haemolysis at 1 mM. Glucose, glucose-1-phosphate, rhamnose, galactose and galactose-6-phosphate were ineffective; NAD was effective, although not to the extent of NADP. Of the tested sugars, only G6P reduced solanine-induced haemolysis. G6P also reduced the synergistic haemolytic action of solanine and chaconine in combination. G6P and NADP at or above 5 mM antagonised chaconine-induced betanin loss from excised red beet root discs; NADP was more effective than G6P. Disruption of PC/cholesterol liposomes by chaconine and inhibition of acetylcholinesterase by chaconine or solanine, were unaffected by up to 10 mM NADP or 50 mM G6P.

Keywords: Solanum tuberosum; Solanaceae; Potato; Amelioration; Glycoalkaloids; glucose-6-phosphate; NADP

1. Introduction

Glycoalkaloids are saponin-like, nitrogen-containing steroidal glycosides mainly found in the genus *Solanum* (Roddick, 1996). Questionably the most active and certainly the most studied are the glycoalkaloids of potato, *S. tuberosum*, which occur throughout the plant, with the possible exception of the inner flesh of the tuber (Friedman & McDonald, 1997). Potato glycoalkaloids are toxic or inhibitory to organisms across the biological spectrum, including humans (Roddick, 1996; Smith, Roddick, & Jones, 1996). Like many saponins, they have powerful membrane-disruptive properties but, in addition, can inhibit the enzyme, acetylcholinesterase. At higher concentrations, they also impart a bitter/burning taste. As a result, they are widely considered to contribute to the plant's defences against herbivores (especially insects) and pathogenic microoganisms, although a number of counteradapted pests or pathogens are able to avoid or overcome their repellent action.

The glycoalkaloid profile in potato plants is dominated by two compounds, α-chaconine and α-solanine. α-Chaconine is normally the more abundant and more active of the two glycoalkaloids. Considerable evidence now exists that these compounds interact synergistically with each other in disrupting synthetic membranes (Roddick & Rijnenberg, 1987; Keukens et al., 1995; Keukens et al., 1996) and in impairing a number of biological systems or processes, e.g. plant, animal and fungal cells/mycelium (Roddick, Rijnenberg, & Osman, 1988; Fewell & Roddick, 1993, 1997), rat gut ion transport (Gee et al., 1996), frog embryo development (Rayburn, Friedman, & Bantle, 1995) and snail feeding (Smith, 1997). These synergisms have important implications not only for the defensive role of glycoalkaloids but also for the safety and acceptability of potatoes and potato products (Roddick, 1996; Smith et al., 1996).

Recently, antagonism of glycoalkaloid toxicity and teratogenicity towards frog embryos by glucose-6-phosphate (G6P) and NADP was reported (Rayburn, Bantle, Qualls, & Friedman, 1995), with G6P being the more active compound. This observation originated from earlier findings that an in vitro metabolic activating system comprising these (and other) compounds reduced the developmental toxicity of chaconine and solanine in the frog embryo teratogenesis assay (FETAX) (Friedman, Rayburn, & Bantle, 1991), but that deactivation was not entirely due to mixed function oxidase activity.

The present study aimed to determine whether the glycoalkaloid-ameliorating effects of G6P and NADP were restricted to a complex developing animal system or were effective in other simpler chaconine-sensitive systems, such as relatively undifferentiated animal and

* Corresponding author. Tel.: +44-1392 264 606; fax: +44-1392 264 6668; e-mail: j.g.roddick@exeter.ac.uk.

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plant cells/tissues, synthetic lipid vesicles (liposomes) and the enzyme, acetylcholinesterase.

2. Results and discussion

Erythrocytes were chosen as an animal cell test system. Confirming previous findings, these were lysed (~90%) by low concentrations (20 μM) of chaconine. Addition of G6P and NADP at 0.5 mM or greater caused a significant and progressive reduction in chaconine-induced haemolysis (Figs 1 and 2). The shapes of the curves differed, but the activities of G6P and NADP were similar in that the EC_{50} for both compounds was ca. 1 mM.

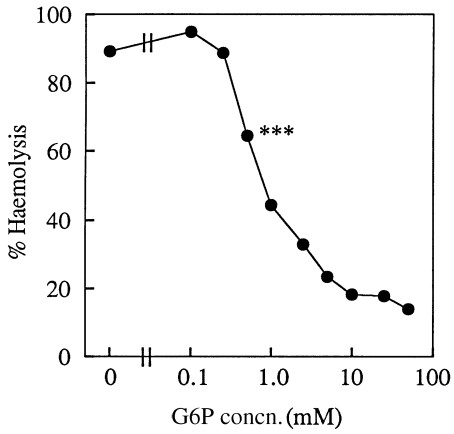

Fig. 1. Effect of G6P on chaconine-induced haemolysis of human erythrocytes. % Haemolysis was calculated with reference to untreated controls. All treatments contained 20 μM chaconine. Values are means of five counts on each of three replicate samples after a 2 h incubation at 37°C. **, *** = significantly different from chaconine-only treatment at $p = 0.01$ and 0.001 respectively.

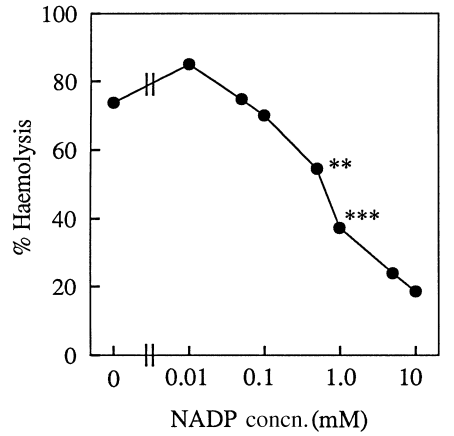

Fig. 2. Effect of NADP on chaconine-induced haemolysis of human erythrocytes. % Haemolysis was calculated with reference to untreated controls. All treatments contained 20 μM chaconine. Values are means of five counts on each of three replicate samples after a 2 h incubation at 37°C. *** = significantly different from chaconine-only treatment at $p = 0.001$.

Table 1
Comparison between the ameliorating activity of various sugar and nucleotide compounds on chaconine-induced haemolysis of human erythrocytes

Treatment	% Haemolysis
20 μM chaconine	84.1
20 μM chaconine + 5 mM glucose	73.5
20 μM chaconine + 5 mM galactose	80.3
20 μM chaconine + 5 mM rhamnose	74.0
20 μM chaconine + 5 mM glucose-1-phosphate	85.8
20 μM chaconine + 5 mM G6P	9.9***
20 μM chaconine + 5 mM galactose-6-phosphate	74.2
20 μM chaconine + 1 mM NADP	15.8***
20 μM chaconine + 1 mM NAD	42.1***

% Haemolysis was calculated with reference to untreated controls. Values are means of five counts on each of three replicate samples after a 2 h incubation at 37°C. ***Significantly different from chaconine-only treatment at $p = 0.001$.

Neither G6P nor NADP alone affected erythrocytes adversely at the concentrations tested.

Comparison between the effects of 5 mM G6P and 1 mM NADP with related compounds is shown in Table 1. The antagonistic effect of G6P was confirmed, with haemolysis reducing from 84 to ca. 10%, but glucose-1-phosphate and glucose (one of the two sugars in the chacotriose moiety of chaconine) showed no ameliorating activity. Rhamnose (the other sugar of the chacotriose moiety of chaconine), galactose (which is present in the solatriose moiety of solanine) and galactose-6-phosphate were also ineffective. NAD caused significant amelioration, although the effect was much less marked than that of NADP.

The weaker haemolytic action of solanine than chaconine was confirmed (Figure 3). The haemolytic effect of solanine was reduced by 5 mM G6P (ca. 86%, similar to

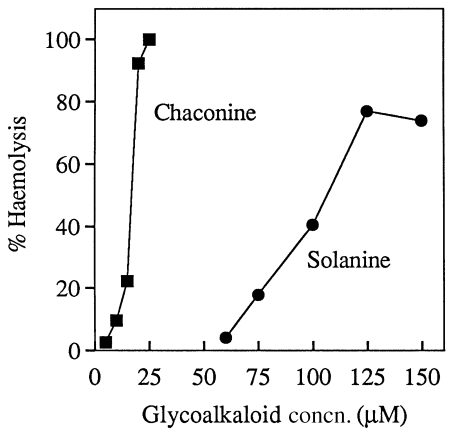

Fig. 3. Different haemolytic action of chaconine and solanine on human erythrocytes. % Haemolysis was calculated with reference to untreated controls. Values are means of five counts on each of three replicate samples after a 2 h incubation at 37°C.

Table 2
Effect of G6P on haemolytic action of chaconine and solanine, individually and in combination

Treatment	% Haemolysis
20 μM chaconine	96.9
20 μM chaconine + 10 mM G6P	0
120 μM solanine	90.6
120 μM solanine + 10 mM G6P	8.6
10 μM chaconine	0
60 μM solanine	6.0
10 μM chaconine + 60 μM solanine	100.0
10 μM chaconine + 60 μM solanine + 10 mM G6P	56.1

% Haemolysis was calculated with reference to untreated controls. Values are means of five counts on each of three replicate samples after a 2 h incubation at 37°C.

the effect with chaconine Table 1), but not by any of the other tested sugars (data not shown). The well-established synergism between solanine and chaconine was reduced by 44% by 10 mM G6P, although this reduction was proportionately much less than the reduction in the action of chaconine and solanine individually (100 and 91%, respectively) (Table 2).

The ameliorating action of G6P and NADP on chaconine-induced cell disruption was also apparent in plant cells from the root of red beet. Membrane damage in this tissue was monitored by the loss of the vacuolar pigment, betanin. At the lowest tested concentration (1 mM), both G6P and NADP were inactive (Fig. 4). At 5 mM, both compounds caused small but significant reductions in chaconine-induced pigment loss, with NADP appearing slightly more active. This trend was confirmed by the

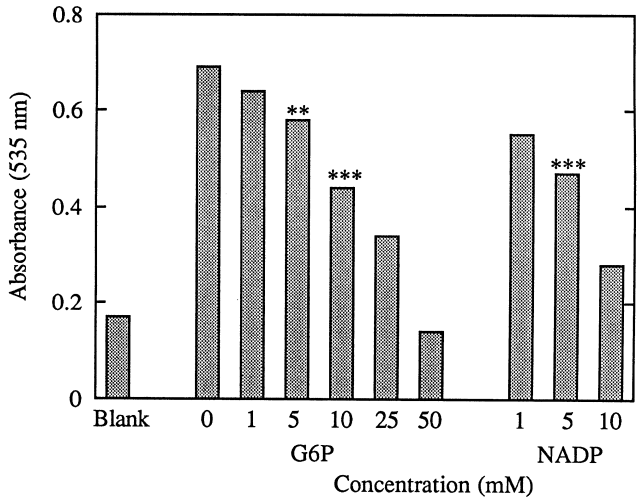

Fig. 4. Effect of G6P and NADP on chaconine-induced betanin leakage from red beet root discs. Pigment leakage was assessed as absorbance at 535 nm after incubating on an orbital shaker for 4 h at 25°C. With the exception of the blank, all treatments contained 100 μM chaconine. Values are means of five replicates. **, *** = significantly different from chaconine-only treatment at $p = 0.01$ and 0.001 respectively.

EC_{50} for NADP being ca. 8 mM compared with ca. 25 mM for G6P. Higher concentrations of both compounds progressively increased amelioration which was total, i.e. pigment loss was the same as in chaconine-free controls, at 50 mM G6P and, by extrapolation, 20 mM NADP. Neither G6P nor NADP alone affected the beet cells adversely at the concentrations tested.

Artificial membranes in the form of PC/cholesterol liposomes, which are lysed by 150 μM chaconine, did not show reduced disruption with up to 10 mM G6P or NADP (data not shown). Increasing G6P to 50 mM did not affect chaconine-induced lysis. In beet and liposomes, solanine was inactive at 100 μM and was not tested further.

As well as disrupting eukaryotic membrane systems, chaconine also inhibits the enzyme, acetylcholinesterase (Bushway, Savage, & Ferguson, 1987; Roddick, 1989). Although α-solanine has reduced membrane-lytic activity compared with chaconine, against acetylcholinesterase, it is as active or even more so than chaconine. This activity was confirmed for both chaconine and solanine. A concentration of 100 μM reduced enzyme activity by 65 and 79%, respectively, but in neither case was inhibition diminished to any significant extent by up to 50 mM G6P or up to 10 mM NADP (data not shown).

3. Discussion

The reduction in chaconine toxicity by G6P and NADP, which was observed previously in frog embryos, has been confirmed in mammalian and plant cells. As yet, however, no information is available on the mechanism of action of these compounds. The inability of these compounds to reduce chaconine-induced disruption of PC/cholesterol liposomes or acetylcholinesterase suggests that a direct chemical interaction between G6P/NADP and chaconine (e.g. between their phosphate groups and the tertiary nitrogen of chaconine), as speculated by Rayburn et al. (1995), is not involved and that the effect is highly system dependent. This tends to be supported by the differential activity of G6P and NADP on frog embryos, blood and plant cells and liposomes. Further evidence against binding by phosphate groups is the lack of ameliorative activity of glucose-1-phosphate and galactose-6-phosphate. Rayburn et al. (1995) further suggested that G6P might compete with the carbohydrate groups of chaconine for receptor sites on the cell membranes of frog embryos, in which case nonphosphorylated glucose and also rhamnose, which together comprise the chacotriose moiety of chaconine, might also be expected to show activity. Also, rhamnose has been reported to minimise the cytotoxic activity of a mixture of solasodine-based glycoalkaloids to sarcoma 180 in mice (Cham & Daunter, 1990). However, neither hexose proved active. The quite different effects of

glucose, galactose and their phosphates point to both the phosphorylation site and the identity of the sugar as being crucial. This is further supported by the reduced amelioration by NAD. Rayburn et al. (1995) found NADPH to be ineffective but did not test NAD. The effects of these nucleotides therefore require substantiation. In NADP, all phosphate groups are linked to ribose, suggesting that the effects of ribose phosphates should be examined.

Although solanine has a different triose moiety from chaconine and is less haemolytic, the various sugars tested here showed similar effects to those on chaconine. The fact that 10 mM G6P caused a smaller reduction in the combined action of solanine and chaconine compared with individual glycoalkaloids, may be due to the magnitude of the synergistic effects being masked by the assay method (% haemolysis).

Information on chemical moderation of glycoalkaloid action is still limited. Early work by McKee (1959) demonstrated that the toxicity of solanine to spores of *Fusarium caeruleum* was reduced by calcium ions but enhanced by sodium ions (not osmotically) and established the pH dependency of glycoalkaloid action. Solanine inhibition of mycelial growth in vitro was also reduced by the incorporation of 2% glucose into the culture medium. The antifeedant activity of glycoalkaloids against Colorado potato beetle is influenced by the protein content of the diet (Hare, 1987), whilst the toxicity and teratogenicity of chaconine to *Xenopus* embryos can be largely overcome by additions of folic acid (Friedman, Burns, Butchko, & Blankemeyer, 1997) The fact that glycoalkaloid activity can be enhanced or reduced by cooccurring metabolites and by the chemical environment, emphasises the need for a more holistic approach to allelochemical studies of glycoalkaloids generally, as well as for a 'whole food approach' when assessing the hazards associated with these natural toxicants in potatoes.

4. Experimental

4.1. Chemicals and materials

Unless otherwise stated, all fine biochemicals were purchased from Sigma Chemicals, UK. α-Solanine was a gift from Professor Dr K. Schreiber, Institute of Plant Biochemistry, Halle, Germany and was recrystallised from EtOH before use. Erythrocytes were prepd from certified human blood (type O, Rhesus-positive) obtained from the Royal Devon and Exeter Hospital Transfusion Unit, Exeter, UK. The blood supplied (in sealed containers) was just past its expiry date for transfusion but the erythrocytes appeared normal when viewed under the microscope and responded to glycoalkaloids in a similar way to that found previously. Roots of red beet were purchased fresh from a local supplier.

4.2. Preparation and treatment of erythrocytes

Methods were as previously described (Roddick et al., 1988). Blood was stored at 4°C. Blood was washed 2× with 9× its vol. Pi-buffered saline, pH 7.4. Erythrocytes were resuspended in the original blood vol. of buffered saline and dild to 2% with buffered saline. Dild erythrocyte suspension (3 ml) was mixed with 1 ml of glycoalkaloid soln prepd in buffered saline and the mixt. incubated at 37° for 2 h. Erythrocyte density was counted using a haemocytometer at ×40 and % haemolysis determined by ref. to erythrocyte counts in glycoalkaloid-free controls.

4.3. Preparation and treatment of beet root discs

Methods are described in Roddick et al. (1988). Discs (10 mm diam, 2 mm thick) were cut from excised cylinders and washed in running H_2O for 16 h. Ten discs were placed in 10 ml of glycoalkaloid soln prepd in phosphate-citrate buffer, pH 7.2, in 50 ml conical flasks and incubated at 25°C on an orbital shaker (100 rpm) for 4 h, after which the liquid was decanted and its absorbance measured at 535 nm.

4.4. Preparation and treatment of liposomes

Details of methods have been reported previously (Roddick & Rijnenberg, 1987). PC/cholesterol liposomes were prepd by sonication of the lipid mixt. in the presence of horseradish peroxidase in Pi buffer. Liposomes were washed 2× then suspended in Tris–HCl buffer, pH 7.2. Glycoalkaloids were prepd in HCl and 0.3 ml of a 2 mM soln used to treat 50 μl of liposome suspension in a total vol. of 4 ml for 1 h at 25°C. After centrifuging to pellet intact liposomes, the extent of liposome disruption was assessed by determining the peroxidase activity in the pellet and supernatant. Assay of peroxidase was based on oxidation of pyrogallol by H_2O_2-derived oxygen and measurement of ΔA_{420}.

4.5. Estimation of acetylcholinesterase inhibition

A spectrophotometric method (Roddick, 1989) was employed. The acetylcholinesterase used was from bovine erythrocytes (Sigma type XII-S) and was the last addition to the cuvette. Glycoalkaloids were dissolved in 2 mM HCl. Absorbance was measured at 412 nm over a period of 3 min at room temp. and enzyme activity expressed as ΔA_{412}/min.

4.6. Replication and statistical analyses

All treatments comprised between three and five replicates as indicated and all experiments were repeated at least once. Statistical significance was determined using a

one-way analysis of variance and t-test on untransformed means.

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