Presence of sterol-binding sites in the cytosol of French-bean (*Phaseolus vulgaris*) roots

Manuel A. VEGA and Ricardo L. BOLAND*

Departamento de Biología, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina

Vitamin D₃ (cholecalciferol) and stigmasterol have been shown to stimulate Ca²⁺ uptake and to induce calmodulin synthesis in cultured French-bean (*Phaseolus vulgaris*) roots. In addition, the appearance of calmodulin in the cultures in response to vitamin D₃ could be prevented by RNA-synthesis inhibitors. To investigate the possibility that the sterols affect root DNA transcription through a receptor-mediated mechanism, the existence of sterol-binding sites in *P. vulgaris* roots was investigated. Specific binding of [³H]vitamin D₃ could be demonstrated with intact tissue and the cytosolic fraction obtained therefrom. Equilibrium in the binding reaction with cytosol was attained after 4 h of incubation at 0 °C. The [³H]vitamin D₃ was reversibly bound, since it could be displaced by an excess of unlabelled sterol. An equilibrium binding constant (K_D) of $(3.48 \pm 0.09) \times 10^{-9}$ M and a maximum binding-site concentration (n_{max}) of 32 ± 2.54 (3) pmol/mg of protein could be calculated by Scatchard [(1949) Ann. N.Y. Acad. Sci. 51, 660–672] analysis. In addition to vitamin D₃, stigmasterol and sitosterol were effectively able to compete with [³H]vitamin D₃ for binding to root cytosol. Cortisol, oestradiol and progesterone displaced bound labelled vitamin D₃ to a lesser extent, whereas 5 β -dihydrotestosterone, lanosterol and diosgenin were ineffective. The affinity and specificity of the root sterol-binding sites are in agreement with the characteristics of tissue responses to the sterols (Ca²⁺ uptake and calmodulin synthesis).

INTRODUCTION

Sterols are widely distributed in the plant kingdom (Goodwin, 1980; Grunwald, 1980). The presence of secosterols such as vitamin D₃ (cholecalciferol) and its metabolites has been demonstrated in several plant species (Boland, 1986). Various sterols of plant and animal origin have been shown to affect growth and tissue differentiation in plant systems *in vitro* (Geuns, 1978). However, the physiological role of these compounds in plant cells is still unknown. It has been reported that low concentrations (10^{-9} M) of vitamin D₃ affect the growth of French-bean (*Phaseolus vulgaris*) roots *in vitro*. Evidence indicating that the effects of the sterol on root growth were mediated by an increase in root Ca²⁺ uptake was also obtained. Moreover, cycloheximide and puromycin blocked vitamin D₃ stimulation of root Ca²⁺ uptake, suggesting that the process requires protein synthesis *de novo* (Vega *et al.*, 1985). In agreement with this observation, it has been shown that vitamin D₃ induces the synthesis of calmodulin in *P. vulgaris* roots growing *in vitro*. Moreover, the appearance of calmodulin in root cultures in response to vitamin D₃ could be prevented by RNA-synthesis inhibitors (Vega & Boland, 1986). Recent studies have shown that stigmasterol is also effectively able to stimulate root Ca²⁺ uptake and calmodulin synthesis *de novo* (Boland & Vega, 1986).

It is widely accepted that the selective action of sterols on gene transcription in animal target tissues is mediated by binding to specific and high-affinity soluble receptor proteins (Jensen *et al.*, 1982). The existence of sterol-binding proteins in fungi has also been reported (Loose *et al.*, 1981; Feldman *et al.*, 1982; Riehl & Toft, 1984).

To obtain information about the mechanism by which sterols act on plant tissues, we investigated the existence of sterol-binding sites in *P. vulgaris* roots.

MATERIALS AND METHODS

Materials

Vitamin D₃, stigmasterol, cholesterol, sitosterol, oestradiol, progesterone, cortisol, 5 β -dihydrotestosterone, lanosterol, diosgenin and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{1,2-³H}(n)]-vitamin D₃ (sp. radioactivity 22 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Methods

Sterilized French-bean (*Phaseolus vulgaris*, cv. Conchancha) seeds were germinated for 48 h at 28 °C in the dark as previously described (Vega *et al.*, 1985). The primary roots (approx. 1.5 cm long) of the seedlings obtained were homogenized in ice-cold 50 mM-sodium phosphate (pH 7.4)/250 mM-sucrose/10 mM- β -mercaptoethanol/0.5 mM-PMSF (1 ml per four roots) in an Ultra-Turrax homogenizer at maximum speed for 30 s. The homogenate was passed through a double layer of cheesecloth and the filtrate was centrifuged in a Beckman Ti50 rotor at 133 000 *g* for 90 min. The floating lipid layer was removed, and the supernatant was immediately used for binding studies. The protein content was analysed by the Bradford (1976) procedure. Binding analysis of the cytosol was performed in glass test tubes

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; n_{max} , maximum concentration of binding sites.

* To whom correspondence and reprint requests should be addressed.

containing 1 ml of cytosol (50 μ g of protein), 2 nM-[3 H]-vitamin D₃ and unlabelled sterols in the concentrations indicated for each experiment. Sterols were dissolved in propan-2-ol. The concentration of solvent in assays was 5%. [3 H]Vitamin D₃-specific binding was approx. 2-fold higher in the presence of 5% propan-2-ol than in 0.1% ethanol. The samples were incubated at 0 °C for 4 h in the dark. To separate bound from free sterol, 250 μ l of 0.5% dextran/5% charcoal in 50 mM-phosphate buffer, pH 7.4, were added (Haddad & Chyu, 1971). The samples were further incubated for 5 min and centrifuged at 1200 g for 5 min. Portions (750 μ l) of the supernatant were taken for radioactivity measurements in a Beckman liquid-scintillation spectrometer.

To estimate binding of [3 H]vitamin D₃ *in vivo*, roots were cultured in Shenk-Hildebrandt (1972) medium for 14 h at 28 °C in the dark. Each root was then incubated in 500 μ l of Shenk-Hildebrandt (1972) medium containing 4 nM-[3 H]vitamin D₃ at 28 °C for 3 h in the presence or absence of unlabelled vitamin D₃ (10⁻⁵ M). The roots were extensively washed with tap water, blotted on filter paper and dissolved in 0.1 M-NaOH at 100 °C. Portions were taken for protein and radioactivity measurements. Specific binding was calculated from the difference between the radioactivity retained by roots incubated in the absence (total binding) and that in the presence of unlabelled sterol (non-specific binding).

RESULTS

As shown in Table 1, specific binding of [3 H]vitamin D₃ by *P. vulgaris* roots *in vivo* could be demonstrated in several experiments. The binding of [3 H]vitamin D₃ by *P. vulgaris* root cytosol was then investigated. Fig. 1 shows total, specific and non-specific binding of labelled vitamin D₃ as a function of cytosol protein concentration. Unlike non-specific binding, specific binding of the sterol did not increase proportionally with the amounts of cytosolic protein in the assay at levels higher than 0.2 mg of protein/ml. A concentration of 0.05 mg of protein/ml was selected for further characterization of binding of [3 H]vitamin D₃ *in vitro* by root cytosol. The time course of the binding reaction between [3 H]vitamin D₃ (2 $\times$ 10⁻⁹ M) and root cytosol at 0 °C is shown in Fig. 2. Under these conditions equilibrium was attained after 4 h of incuba-

Fig. 1. Binding of 3 H-vitamin D₃ by *P. vulgaris* root cytosol as a function of cytosolic protein content

P. vulgaris root cytosol was isolated as described in the Materials and methods section. Binding assays were performed by incubating cytosol (0.1–0.6 mg of protein/ml of assay mixture) with 2 nM-[3 H]vitamin D₃ in the presence or absence of 500 nM unlabelled vitamin D₃ at 0 °C for 20 h. Total (●), specific (△) and non-specific (○) binding were determined by the dextran-coated-charcoal procedure (Haddad & Chyu, 1971). Each point represents the average of triplicate determinations $\pm$ S.D.

Fig. 2. Time course of specific [3 H]vitamin D₃ binding by *P. vulgaris* root cytosol

Root cytosol (50 μ g of protein/ml of assay mixture) was incubated with 2 nM-[3 H]vitamin D₃ at 0 °C for various times in the absence or presence of 200 nM unlabelled vitamin D₃ as described for Fig. 1. After 11 h incubation, unlabelled vitamin D₃ (400 nM) was then added (broken line). Each point represents the average of triplicate determinations.

Table 1. Binding *in vivo* of [3 H]vitamin D₃ by *P. vulgaris* roots

P. vulgaris roots obtained as described in the text were cultured for 14 h in Shenk-Hildebrandt (1972) medium. The tissue was then incubated with 4 nM-[3 H]vitamin D₃ for 3 h at 28 °C in the presence or absence of 10⁻⁵ M unlabelled vitamin D₃. Total, specific and non-specific binding were determined as described in the Materials and methods section. Values represent means $\pm$ S.D. (number of roots).

Expt.	Binding (d.p.m. of [3 H]vitamin D ₃ /mg of protein)		
	Total	Non-specific	Specific
I	928.8 $\pm$ 182.0 (9)	693.6 $\pm$ 114.0 (10)	235.2
II	491.2 $\pm$ 120.8 (11)	396.0 $\pm$ 148.0 (8)	95.2
III	1009.6 $\pm$ 90.0 (3)	608.4 $\pm$ 53.4 (3)	401.2
IV	443.2 $\pm$ 82.0 (6)	306.0 $\pm$ 80.0 (6)	137.2

tion. It is also seen that the addition of 400 nM unlabelled vitamin D₃ decreased bound [3 H]vitamin D₃ to 30% by 90 min.

The binding of [3 H]vitamin D₃ by *P. vulgaris* root cytosol was analysed as described by Scatchard (1949). Over the concentration range employed, a linear relationship was obtained by plotting bound/free versus bound vitamin (Fig. 3). However, a lack of data points at low sterol concentrations, attributable to the rather low specific radioactivity of the [3 H]vitamin D₃ employed, does not exclude the possibility of deviations from

Fig. 3. Scatchard (1949) analysis of specific [^3H]vitamin D_3 binding by *P. vulgaris* root cytosol

Cytosol (50 μg of protein/ml) was incubated with 2 nM [^3H]vitamin D_3 and increasing concentrations (0.2–20 nM) of unlabelled vitamin D_3 at 0 $^\circ\text{C}$ for 8 h. Specific binding was determined as described in Fig. 1.

linearity at these concentrations. Calculations of the equilibrium dissociation constant (K_D) from the slope of the curve yielded a value of $(3.48 \pm 0.09) \times 10^{-9}$ M (average for three independent experiments). The maximum concentration of binding sites (n_{max}) was calculated to be 32 ± 2.54 pmol/mg of protein (three experiments). In

agreement with the Scatchard (1949) analysis, determination of the equilibrium binding constant by the ratio of the dissociation rate constant to the association rate constant, using the data from Fig. 2, gave a value of 3.26×10^{-9} M.

To obtain information about the specificity of vitamin D_3 binding by *P. vulgaris* root cytosol, the ability of various steroids of differing structure to displace bound [^3H]vitamin D_3 was evaluated. The results obtained are shown in Fig. 4. In addition to vitamin D_3 , stigmasterol and sitosterol were effectively able to compete with [^3H]vitamin D_3 for binding to root cytosol. Cortisol, oestradiol and progesterone displaced bound labelled vitamin D_3 to a lesser extent. 5β -Dihydrotestosterone, lanosterol and diosgenin were ineffective in competing with [^3H]vitamin D_3 for binding to root cytosol. Cholesterol also exhibited no displacement ability up to a concentration of 2 nM. However, at higher concentrations, the ability of cholesterol to displace bound vitamin D_3 from cytosol was comparable with that of vitamin D_3 , stigmasterol and sitosterol.

DISCUSSION

In the present study, specific binding of [^3H]vitamin D_3 by *P. vulgaris* roots could be demonstrated both *in vivo* and *in vitro*. In the assays *in vitro*, specific binding by the root cytosolic fraction was demonstrated. This is in

Fig. 4. Competition of a variety of steroids for [^3H]vitamin D_3 -binding sites in *P. vulgaris* root cytosol

Cytosol (50 μg of protein/ml) was incubated with 2 nM [^3H]vitamin D_3 in the absence or presence of different concentrations of: vitamin D_3 (1, ●), stigmasterol (2, ○) and sitosterol (3, △) or cholesterol (▼) (panel a); cortisol (4, ○), oestradiol (5, △) and progesterone (6, ▽) (panel b); 5β -dihydrotestosterone (7, ○), lanosterol (8, △) and diosgenin (9, ▽) (panel c). Specific [^3H]vitamin D_3 binding was determined as described in Fig. 1. Relative potency was calculated as the capacity of steroids (2 nM) to displace bound [^3H]vitamin D_3 relative to that of 2 nM-vitamin D_3 .

Fig. 5. Structures of steroids used to evaluate the specificity of vitamin D₃ binding of *P. vulgaris* root cytosol

general agreement with several previous studies, which have shown the presence of sterol-binding macromolecules in the cytosol of animal, yeast and fungi cells (Loose *et al.*, 1981; Feldman *et al.*, 1982; Jensen *et al.*, 1982; Riehl & Toft, 1984).

Characterization of the time-dependence of the binding of vitamin D₃ to root cytosol at 0 °C demonstrated that, at the ligand and cytosol concentrations used, a relatively short time was required for the binding reaction to reach equilibrium. In addition, the binding of sterol to cytosol was also shown to be a readily reversible process (Fig. 2). The results of the Scatchard (1949) and kinetic analysis revealed high-affinity binding of the sterol by root cytosol preparations (K_D approx. 10^{-9} M). The affinity of the *P. vulgaris* root cytosol binder for [³H]vitamin D₃ was in the range of the affinity reported for steroid-hormone-binding proteins for their ligands in mammals, fungi and yeast. However, the binding capacity was considerably

higher in root cytosol than in cytosol of both animals and the more primitive eukaryotic organisms (Franceschi *et al.*, 1981; Loose *et al.*, 1981; Feldman *et al.*, 1982; Riehl & Toft, 1984; Maruyama & Fukai, 1985).

Sitosterol, stigmasterol and cholesterol at slightly higher concentrations effectively competed with [³H]-vitamin D₃ binding by root cytosol, indicating that relatively minor modifications in the C₁₇ side chain as well as the presence of an intact B-ring do not play a significant role in the interaction between the sterol and the cytosolic binding molecule (Fig. 5). On the other hand, lanosterol and diosgenin produced no displacement of bound [³H]vitamin D₃, suggesting that the 3-hydroxy group and the C₁₇ side chain, sterically hindered in lanosterol by two vicinal methyl groups and drastically modified in diosgenin respectively, may be essential for the binding reaction to take place. The requirement of a side chain in C₁₇ and a hydroxy group in position 3 was

additionally suggested by the fact that substitution of the C₁₇ side chain by a hydroxy group and of the 3-hydroxy group by a carbonyl group in 5 β -dihydrotestosterone resulted in low displacement ability. However, maintaining the carbonyl group and adding a more bulky R-group to the C₁₇ position, as in progesterone and cortisol, partially restored the relative potency of the sterol. However, a larger number of steroids should be tested in order to establish clearly structure-binding relationships.

Interestingly, there is evidence which suggests that the biological activity of sterols in root tissue may be related to their ability to bind to root cytosol. Other studies have demonstrated that stigmasterol affects growth, Ca²⁺ uptake and calmodulin synthesis in *P. vulgaris* roots to the same extent as vitamin D₃, whereas lanosterol has no effects (M. A. Vega & R. L. Boland, unpublished work). In addition, the value obtained for the equilibrium dissociation constant is in the same range as the concentration at which vitamin D₃ and stigmasterol are active. However, the possibility that the root cytosol sterol-binding sites may represent a sterol carrier or storage molecule in root tissue cannot be excluded, in view of their high concentration.

This research was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina.

REFERENCES

- Boland, R. L. (1986) *Nutr. Rev.* **44**, 1–8
 Boland, R. L. & Vega, M. A. (1986) *Int. Conf. Calcium Regul. Horm. Bone Metab.* 9th, Nice, France, abstr. p. 370
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 Feldman, D., Do, Y., Burshell, A., Stathis, P. & Loose, D. S. (1982) *Science* **218**, 197–198
 Franceschi, R. T., Simpson, R. V. & De Luca, H. F. (1981) *Arch. Biochem. Biophys.* **210**, 1–13
 Geuns, J. M. C. (1978) *Phytochemistry* **17**, 1–14
 Goodwin, T. W. (1980) in *The Biochemistry of Plants: vol. 4: Lipids: Structure and Function* (Stumpf, P. K. & Conn, E. E., eds.), pp. 485–507, Academic Press, New York
 Grunwald, C. (1980) *Encycl. Plant Physiol.* **8**, 221–225
 Haddad, J. G. & Chyu, K. J. (1971) *Biochim. Biophys. Acta* **248**, 471–481
 Jensen, E. V., Greene, G. L., Closs, L. E., De Sombre, E. R. & Nadji, M. (1982) *Recent Prog. Horm. Res.* **38**, 1–35
 Loose, D. S., Schurman, D. J. & Feldman, D. (1981) *Nature (London)* **293**, 477–479
 Maruyama, A. & Fukai, F. (1985) *J. Biochem. (Tokyo)* **98**, 1083–1090
 Riehl, R. M. & Toft, D. O. (1984) *J. Biol. Chem.* **259**, 15324–15330
 Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
 Shenk, R. U. & Hildebrandt, A. C. (1972) *Can. J. Bot.* **50**, 199–204
 Vega, M. A. & Boland, R. L. (1986) *Biochim. Biophys. Acta* **881**, 364–374
 Vega, M. A., Santamaria, E. C., Morales, A. & Boland, R. L. (1985) *Physiol. Plant.* **65**, 423–426

Received 5 May 1987/29 September 1987; accepted 4 November 1987