Plant cell pH-static circuit mediated by fusicoccin-binding proteins

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Abstract On sugar beet protoplasts that carry two types of fusicoccin-binding sites, a pH downshift in a physiological range (7.0–6.6) markedly enhanced the efficiency of fusicoccin (FC) binding, mainly owing to increased avidity of low-affinity FC-binding sites. This may allow the FC-binding proteins to act as pH-sensitive modulators of cell activity, for instance, via plasma membrane H^+-ATPase or potassium channels.

Key words: Protoplast; Fusicoccin; Fusicoccin-binding protein; Cytoplasmic pH; Affinity

1. Introduction

One of the most known effects of fusicoccin (FC) on higher plant cells is the enhanced efflux of protons across the plasma membrane, which is attributed to activation of H^+-ATPase on the plasmalemma [1]. The latter carries specific FC-binding proteins (FCBP) which thus far appear ubiquitous among higher plants [2] and are believed to act as FC receptors [3]. Their interaction with FC causes H^+-ATPase activation [4–9], and they are abundant enough [10] to form 1:1 complexes therewith. Data are available in favor of the existence of such a complex [11] and direct interaction between H^+-ATPase and FCBP [10,12]. H^+-ATPase is one of the key enzymes in the plant cell, and one of its functions is to maintain the cytoplasmic pH. We supposed that not the H^+-ATPase as such but rather its complex with FCBP is a component of the cell pH-static machinery, and tried to find out how the changes in cytoplasmic pH may affect the FCBP performance as a FC receptor. The study was carried out on protoplasts derived from suspension-cultured sugar beet cells; we have previously shown that such protoplasts carry numerous FC-binding sites and, like most plant tissues, respond to FC by acidifying the medium [13].

2. Materials and methods

2.1. Object

Sugar beet (*Beta vulgaris* strain 2n) root cells were grown as a suspension culture in Shenk-Hildebrandt [14] medium in the dark at 25°C with constant shaking at 100 rpm in round-bottom flasks, with 21-day passaging. Protoplasts were derived as previously done [13] from mid-log cells (7–10 days).

2.2. Cytoplasmic pH assays

Cytoplasmic pH was monitored by measuring the changes in fluorescence at 530 nm excited at 490 versus 440 nm in fluorescein-loaded [13] protoplasts (10^5/ml). After 15 min preincubation with fluorescein diacetate, protoplasts were washed and exposed to isobutyrate or other agents altering the internal pH. After 5 min, half of the suspension was taken to record $F_{490(tot)}$ and $F_{440(tot)}$, and the other half was centrifuged to determine the corresponding values in the supernatant (SN). The cytoplasmic pH (pH_{cyt}) was determined from $[F_{490(tot)}-F_{490(sn)}]/[F_{440(tot)29}-F_{440(sn)}]$ using a calibration curve; the accuracy was 0.05 pH unit.

2.3. [^{3}H]DihydroFC binding

[^{3}H]DihydroFC (sp. act 3 Tbq/mmol) binding was assayed with 10^5 protoplasts in 100 μl of incubation medium. Nonbound label was removed by vacuum filtration [13].

3. Results

As evident from Fig. 1, [^{3}H]dihydoFC binding with protoplasts is virtually indifferent to the external but very sensitive to the cytoplasmic pH: acidification by 0.3 U causes a 6-fold increase in binding, with little effect of alkalinization.

To check whether cytoplasmic acidification affects the number of FC-binding sites on the protoplast surface or their affinity, the concentration dependence of [^{3}H]dihydroFC binding were determined (Fig. 2) and processed with the Enzfitter 1.05 (R.J. Leatherbarrow, Elsevier-Biosoft, Cambridge, UK) program (Table 1). The initial curves and Scatchard plots displayed in Fig. 2 for pH_{cyt} in control (7.0), isobutyrate-treated (6.7), and then washed (7.1) protoplasts show that normally the protoplast surface carries two types of FC-binding site: low and high affinity. The difference in affinity is

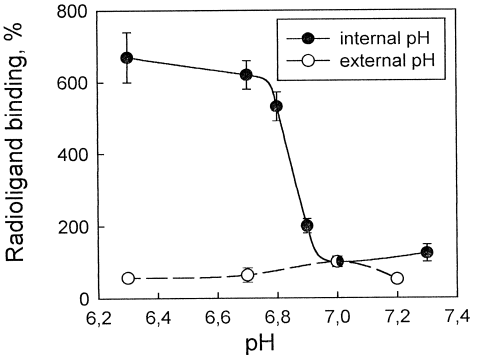

Fig. 1. Effect of internal and external pH on [^{3}H]dihydroFC binding with protoplasts. External pH was varied with nonpermeating buffer MES-BTP (whereby pH_{cyt} did not change). Internal pH was lowered by adding 1 M isobutyrate adjusted to pH 6.3 with BTP (final concn. 5, 10, 15, 20 mM), or increased with NH_4Cl (final concn. 20 mM, external pH 7.0). After establishing the pH_{cyt}, [^{3}H]dihydroFC was added to 10 nM and binding was measured in 15 min. The control pH_{cyt} was 6.95–7.0, and 15 fmol [^{3}H]dihydroFC bound per 10^5 protoplasts was taken as 100%. Data averaged for four independent triplicates.

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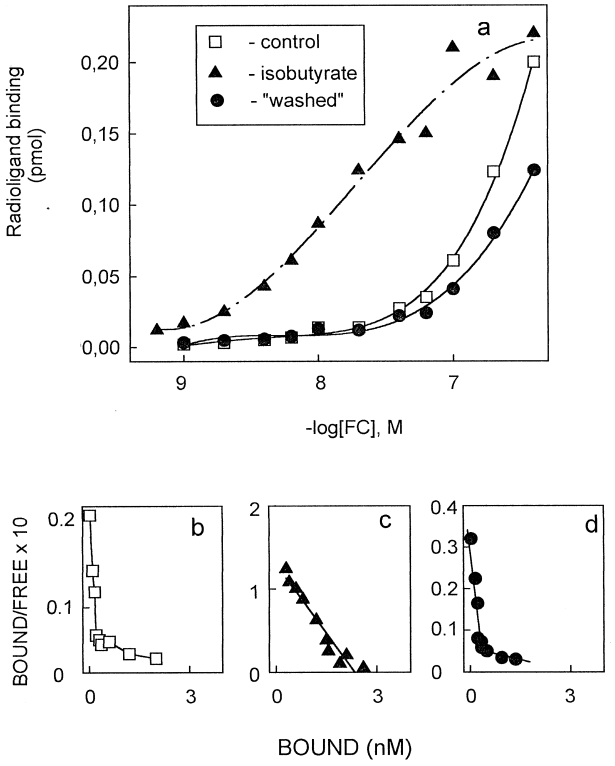

Fig. 2. Concentration dependences of [^{3}H]dihydroFC binding (a) and Scatchard plots for (b) control protoplasts, (c) those exposed to 10 mM isobutyrate, and (d) those washed afterwards.

about 2 orders of magnitude, and the low-affinity sites are 6–7 times more abundant (see Table 1). Upon acidification, the portion of bound FC increases, and the binding pattern fits a one- rather than two-site model, with intermediate affinity. The computation showed a decrease in the total number of binding sites (see Table 1), so enhanced binding can only be attributed to higher avidity. This effect is clearly reversible by simple washing. Because of the greater number of low-affinity sites, nothing definite can at present be said about the behaviour of the high-affinity ones at lowered internal pH.

Isobutyric acid is held to be a nonmetabolizable agent that only would decrease the cytoplasmic pH [15]. Nevertheless, to check for possible side effects, we tested other compounds that can cause internal acidification by permeabilizing the plasma membrane: dibucaine (altering the lipid–protein interaction [16] and EGTA (removing all surface-accessible calcium [17]. As evident from Fig. 3 and Table 1, all these agents act similarly to isobutyrate in that they abolish two-site binding and at the same time enhance overall binding. In separate experiments, neither of these three agents did affect [^{3}H]dihydroFC binding with osmotically ruptured protoplasts (not shown). Thus, intracellular acidification appears to be the only thing common as regards their influence on the FC-protoplast interaction.

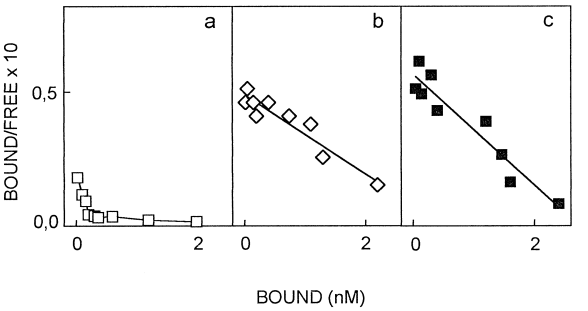

Fig. 3. Scatchard plots for [^{3}H]dihydroFC binding with (a) control protoplasts and those pretreated with (b) 1 mM dibucaine, and (c) EGTA (prewashed twice with Ca^{2+}-free medium containing 5 mM EGTA). Corresponding pH_{cyt} given in Table 1.

4. Discussion

Heretofore the effect of pH on FC binding has been tested on isolated microsomal or plasma membranes, and the binding was reported to be maximal at pH 6.0–6.5 and lower (by 20–50%) at pH 7.0 in the medium [5,11,18–20]. We believe that such a basic difference with our data stems primarily from the dissimilar approaches used. Indeed, FC-binding sites, especially the low-affinity ones most sensitive to pH_{cyt}, proved to be quite labile and readily lost during membrane isolation [13,21]. There may be still other unknown factors affect the FCBP sensitivity to pH in isolated membranes.

The effect of pHcyt on FC binding with protoplasts (i) takes place in a narrow range of physiological pH and in its steepness resembles a phase transition in FCBP; (ii) alters the affinity for the ligand rather than the number of binding sites; (iii) mainly affects the low-affinity sites; and (iv) is reversible. All this suggests a dynamic equilibrium between two FCBP conformational states with different affinity.

Starting this work, we suggested that the FC receptor forms a complex with H^+-ATPase, which operates as a component of the cell pH-static machinery. The involvement of H^+-ATPase proper in sustaining the cytoplasmic pH has been known for quite some while: lowering the pH_{cyt} with weak acids results in cell hyperpolarization [22] and enhanced proton efflux [23], being sensitive to H^+-ATPase inhibitors. Our results indicate that the FC receptor may act as a pH sensor regulating the activity of the H^+-ATPase pump. Furthermore,

Table 1
Fusicoccin binding with protoplasts

Parameter		Control	NH_4Cl (20 mM)	Isobutyrate (10 mM)	EGTA (5 mM)	Dibucaine (1 mM)
pH_{cyt}		7.0	7.3	6.7	6.5	5.8
B_{max} sites/protoplast $\times 10^{-5}$	high	2.8 ± 1.6	3 ± 0.2	18 ± 1	15 ± 0.7	36 ± 3
	low	23 ± 6	21 ± 2			
K_d, nM	high	1.9 ± 1	2.2 ± 0.4	19 ± 3	40 ± 3	60 ± 9
	low	240 ± 60	190 ± 40			

The sum number of binding sites corresponds to 120 pmol per mg membrane mass, assuming a mean protoplast diameter of 25 μM.

the abundance of FCBP in plasma membranes (over 100 pmol/mg, see Table 1) prompts one to think that H^+-ATPase is not their sole effector, and the scope of the latter may be much broader. For instance, it is also quite possible that this mechanism operates for the outward rectifying potassium channels of plant plasma membranes, which are both pH- and FC-dependent in conductivity [24,25].

By and large, we suppose that FCBP, apart from FC reception, perform an independent function in higher plants, acting as pH-dependent modulators: a shift in pH_{cyt} alters the FCBP conformation, which entails a change in the effector activity.

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