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Phytochrome modulation of calcium fluxes in wheat (*Triticum aestivum* L.) protoplasts

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Abstract

Employing the metallochromic dye murexide and by monitoring the uptake of radiolabelled calcium, photoreversible calcium fluxes were measured in wheat leaf protoplast suspensions. Results obtained by both methods were identical – red light promoted and subsequent far-red irradiation reversed an influx of Ca^{++} ions into the protoplasts. These findings imply phytochrome regulation of Ca^{++} fluxes across the plasma membrane. The influx of Ca^{++} stimulated by 2 min red irradiation could be maintained in total darkness for the initial 16–18 min after illumination, after which a 6–8 min efflux process was triggered and the basal Ca^{++} level restored. Verapamil, a calcium channel blocker, inhibited the red-promoted influx, whereas the far-red mediated efflux could be checked by the use of the ATPase inhibitor vanadate, and also by the calmodulin antagonist chlorpromazine, thus suggesting a role of ion channels and pumps in phytochrome-controlled Ca^{++} fluxes. The possible involvement of phosphoinositides in phytochrome-modulated calcium fluxes was also investigated.

Abbreviations: Δ A = difference in absorbance; CPZ = chlorpromazine; FR = far-red (light); MX = murexide; PI = phosphatidylinositol; PIP_2 = phosphatidylinositol 4, 5-bisphosphate; PIPES = piperazine-N,N′-bis[2-ethanesulfonic acid]; POPOP = 1, 4-bis [2-(5-phenyl-1, 3-oxazolyl)]-benzene; PPO = 2, 5-diphenyl-1, 3-oxazole; R = red (light); SOV = sodium orthovanadate

Introduction

Phytochrome is ubiquitously present in higher plants and is vital for controlling developmental events such as seed germination, chloroplast development, induction of flowering and senescence [14]. The molecular mechanism by which phytochrome regulates all these diverse phenomena is largely unknown. With regard to the early events that are triggered following phototransformation of phytochrome, at present the most widely accepted hypothesis is that phytochrome stimulates phosphatidylinositol metabolism and alters Ca^{++} fluxes across the membrane [2]. That phytochrome may mediate physiological responses by enhancing the intracellular Ca^{++} levels was first suggested by Haupt and Weisenseel [11]; their hypothesis was based upon the fact that an external signal, such as light, is able to stimulate ion movement in plants. Subsequently, a direct coupling between the phototransformation of phytochrome and change in Ca^{++} flux has been demonstrated in the green algae *Mougeotia* and *Mesotaenium* [7, 32] and in at least three higher plants, *Avena sativa* [5, 10, 26],

Zea mays [6] and *Vallisneria* [24, 25]. The change in Ca^{++} flux has also been inferred from the studies on several phytochrome-mediated responses [29], including depolarization of the internodal cell membranes of *Nitella* [33], photoreversible electrical potential changes in oat coleoptiles [16], unrolling of barley [30] and wheat [27] leaf sections, germination of dormant turions of *Spirodela polyrhiza* [1] and movement of *Cassia fasciculata* leaflets [19]. Germination of *Onoclea* spores is phytochrome-stimulated and an influx of Ca^{++} upon irradiation with R light has also been shown in hydrated spores of *Onoclea*, employing atomic absorption spectroscopy [31].

In most of the investigations, the radiotracer ($^{45}Ca^{++}$) technique [6, 7, 26], the spectrophotometric method using Ca^{++}-sensitive dyes, murexide [10] and Quin-2 [5], and the Ca^{++}-sensitive electrode [32], have been employed to demonstrate phytochrome-modulated Ca^{++} fluxes. The results from all these laboratories favour in general the hypothesis that phytochrome modulates the transport of Ca^{++} across the plasma membrane via regulation of calcium-channel activity [3, 26, 29] but, apparently, they differ in the direction of the Pfr-mediated Ca^{++} movement. The results on *Mougeotia* [7, 32] and *Zea mays* [6] and the work of Chae et al. [5] and Tretyn [26] on *Avena sativa* indicated that R light-promoted net *influx* of Ca^{++}, but the data obtained by Hale and Roux [10] with the coleoptile tip cells of *Avena sativa*, and an aquatic plant, *Vallisneria* [24], advocate red light-induced net *efflux* of Ca^{++}. In an attempt to resolve this contradiction, Roux [21] suggested that the murexide technique detected a net Ca^{++} efflux subsequent to an initial influx of Ca^{++} upon stimulation with R light, whereas, under identical conditions, a net Ca^{++} influx would be recorded if $^{45}Ca^{++}$ uptake were monitored; however, no experimental evidence was provided in support of this assumption.

In the present study, employing the metallochromic dye murexide, and also by monitoring the uptake of radiolabelled calcium, the effect of R and FR irradiation on the direction of Ca^{++} fluxes in protoplasts isolated from the primary leaves of dark grown wheat seedlings was investigated. Kinetics of Ca^{++} influx/efflux in continuous darkness, following R irradiation, was monitored by murexide method. An attempt was also made to study the mechanism of Ca^{++} movement.

Materials and methods

Plant material

Seeds of *Triticum aestivum* var. CPAN 1676 (obtained from the Indian Agricultural Research Institute, New Delhi), presoaked in water (25 °C) overnight, were sown in trays layered with 1" thick absorbant cotton and placed in the dark in a growth chamber at 27° ± 1 °C. The 4-day-old dark grown seedlings were harvested for protoplast isolation. All operations were performed under dim green safe light.

Isolation of protoplasts

Protoplasts were isolated from unexposed primary leaves ensheathed in 4-day-old dark grown seedling coleoptiles, according to the procedure of Hale and Roux [10], with certain modifications. The peeled leaf tissue was incubated in an enzyme solution consisting of 2% cellulase R-10, 1% macerozyme R-10 (Yakult Honsa Co., Japan) in Buffer A (0.6 M mannitol and 10 mM PIPES; pH 6.2). The incubation was carried out at 27° ± 1 °C in complete darkness for 4 h, at the end of which the protoplasts were sieved through a 42 μm mesh and subjected to two low speed (600 rpm) centrifugation washes for 3 min each. The final pellet was resuspended in the required amount of Buffer A to adjust the final yield to $2–5 \times 10^5$ protoplasts/ml. Protoplast quantitation was performed using a haemocytometer.

Measurement of Ca^{++} fluxes

Murexide method

Purified protoplasts ($\sim 2 \times 10^5$) suspended in 0.5 ml of 25 μM murexide solution in Buffer A were placed over 10% Ficoll pad in a 1 cm pathlength quartz cuvette. The protoplasts were allowed to settle, concentrating just below the path of the measuring beam, and rest of the cuvette being filled with the bathing solution (25 μM MX and 2.5 mM $CaCl_2$, dissolved in Buffer A). The system was allowed to equilibrate in darkness and record-

ings taken employing a Hitachi 557 dual wavelength spectrophotometer after $\Delta A(502-540\,nm)$ stabilization. During irradiation of the sample with R or FR light, the lid of the sample chamber was maintained open in such a manner that the shutter of the photomultiplier remained closed. Temperature of the sample chamber was $25° \pm 1\,°C$ throughout. All experiments were repeated at least twice with qualitatively similar results but data of only single representative experiment are presented.

$^{45}Ca^{++}$ uptake method

The procedure of Das and Sopory [6] was followed. About 10^5 protoplasts were taken for each set of experiments and uptake was initiated by adding $1\,\mu Ci\,^{45}Ca^{++}$ (specific activity $77.4\,mCi/g\,Ca^{++}$; BARC, Bombay) in a final concentration of $2.5\,mM\,Ca^{++}$. The protoplast suspension was loaded over $800\,\mu l$ of 5% Ficoll prepared in Buffer A, before different R and FR treatments were given. The uptake reaction was terminated by centrifuging the protoplasts (in a microcentrifuge) through the Ficoll layer. The protoplasts pelleted at the bottom of the Eppendorf tube were lysed by adding $100\,\mu l$ of 1% SDS, and the lysed suspension was transferred to a scintillation vial containing 10 ml scintillation fluid [0.5 mg POPOP, 5 g PPO and 160 ml Biosolv (Beckman), made up to 1 l using scintillation grade toluene]. The radioactivity was counted using Beckman LS-1800 liquid scintillation counter. The values presented represent mean ± S.E. of three to five independent experiments.

Light sources and irradiation

Monochromatic light was obtained using interference filters with λ max 655 nm (Carl Zeiss, Jena) and λ max 720 nm (Shimadzu, Japan), both having a half-bandwidth of 10 nm. For radioactive method, a custom-built projector with a 250 W lamp (Wotan, Germany) was employed. The incident energy reaching inside a Eppendorf tube (as estimated by transmission through a longitudinally cut tube interspersed between the light source and held close to the sensor) was $9.6\,W/m^2$ for R and $5.8\,W/m^2$ for FR. For MX method, the light irradiations were given with the help of a 120 W lamp installed in the standard light accessory of Hitachi 557 spectrophotometer. The incident energy reaching the surface of the cuvette for R and FR was $21.6\,W/m^2$ and $15.6\,W/m^2$, respectively. Light energy measurements were made using LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska).

Results

The protoplasts isolated from the primary leaves of 4-day-old dark grown wheat seedlings were treated with the vital stain fluorescein diacetate [34] and approximately 90% protoplasts were found to fluoresce under UV illumination, indicating their viability. Photoreversible phytochrome changes, as measured by dual-wavelength spectroscopy, could also be demonstrated in these protoplasts (details not presented), thus, indicating that the protoplasts isolated by subjecting the leaf pieces to enzymatic treatments were suitable for monitoring phytochrome-modulated Ca^{++} fluxes.

Photoreversible Ca^{++} fluxes in protoplasts

A typical recording of the photoreversible Ca^{++} fluxes in wheat protoplasts as observed by MX method is illustrated in Figure 1. When the protoplasts bathed in a solution containing murexide were irradiated with R light, a decrease in $\Delta A(502-540\,nm)$ was observed, thus indicating an influx of Ca^{++} ions into the protoplasts from the bathing solution. FR irradiation reversed this effect; the increased $\Delta A(502-540\,nm)$ indicates efflux of Ca^{++} from the protoplasts. The $\Delta(\Delta A)$ was *ca* 0.0004–0.0005 absorbance units/2×10^5 protoplasts, corresponding to a change in Ca^{++} concentration of about 4 to $5\,\mu M$ in the bathing solution.

In experiments with $^{45}Ca^{++}$, 120 s R irradiation stimulated $^{45}Ca^{++}$ uptake by protoplasts and 60 s FR given immediately following R irradiation reversed this effect (Fig. 2); FR alone caused no change in $^{45}Ca^{++}$ uptake. A second R irradiation of 120 s, following FR irradiation, stimulated an influx of $^{45}Ca^{++}$ equal to that obtained with the initial R irradiation (data not shown).

Kinetics of $^{45}Ca^{++}$ uptake

For monitoring uptake of $^{45}Ca^{++}$ by protoplasts as a function of time, the duration of R irradiation

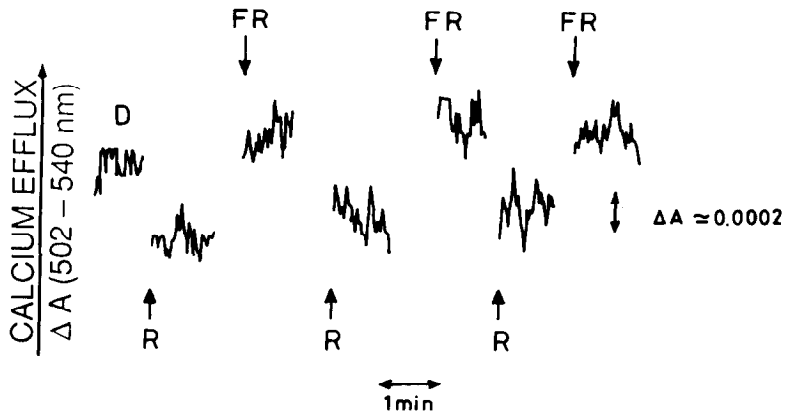

Fig. 1. Photoreversible calcium fluxes in protoplasts isolated from primary leaves of 4-day-old dark grown wheat seedlings. About 2×10^5 protoplasts were layerd over 10% Ficoll pad placed at the bottom of 10 mm pathlength cuvette. Care was taken that the protoplasts did not interfere in the path of the measuring beam. 2.5 ml of Buffer A (0.6 M mannitol, 10 mM PIPES, pH 6.2) containing 25 μM MX and 2.5 mM $CaCl_2$ was gently layered over the protoplasts. Δ A(502–540 nm) was allowed to stabilize in darkness. R or FR irradiations (for 2 min each) were given from one side of the cuvette and Δ A(502–540 nm) recorded for 1 min each.

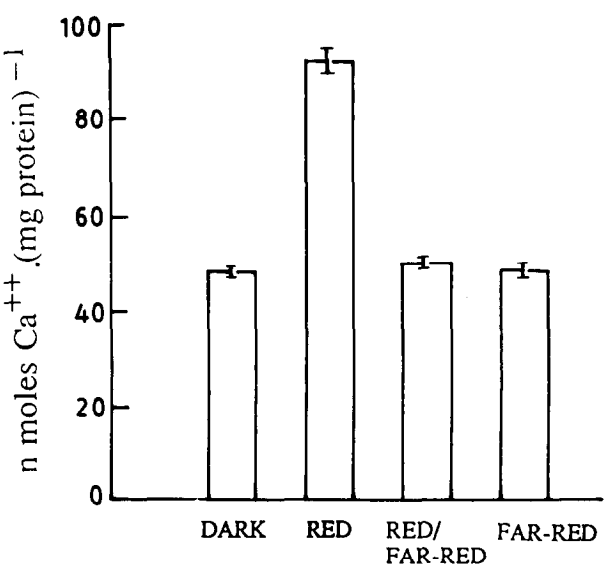

Fig. 2. Effect of R and FR irradiation on $^{45}Ca^{++}$ uptake in protoplasts isolated from primary leaves of 4-day-old etiolated wheat seedlings. An aliquot of 200 μl of protoplast suspension containing about 10^5 protoplasts was loaded over 800 μl of 5% Ficoll, in an Eppendorf tube, before irradiation with R (2 min) and/or FR (1 min) light. The protoplast bathing solution consisted of 2.5 mM $CaCl_2$ (1 μCi of $^{45}Ca^{++}$) in Buffer A, pH 6.2. The protoplasts, after light treatments, were centrifuged through the Ficoll layer and the pelleted protoplasts lysed by adding 100 μl of 1% SDS. Scintillation cocktail was added to this lysed mixture and radioactivity counted.

was varied from 30 to 300 s. No significant increase in $^{45}Ca^{++}$ uptake, over the dark control, was observed till 60 s of R irradiation (Fig. 3). However, in protoplasts irradiated with 120 s of R, the amount of radioactivity incorporated almost doubled. Longer durations of R irradiation, i.e. up to 300 s, had only marginal effect.

Essentially similar results (details not presented) on the kinetics of Ca^{++} uptake were obtained when estimated by MX method. Employing MX technique, that allows measurement of actual

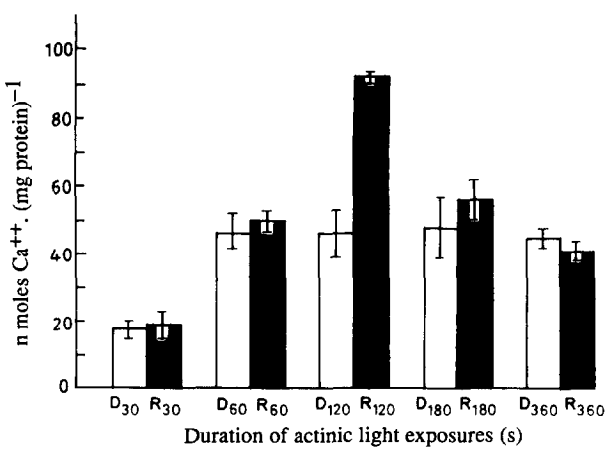

Fig. 3. Kinetics of $^{45}Ca^{++}$ uptake by the protoplasts in dark and under different duration of R light. Irradiation was applied for 30 to 300 s. The experimental procedure was similar to that described in legend to Figure 2.

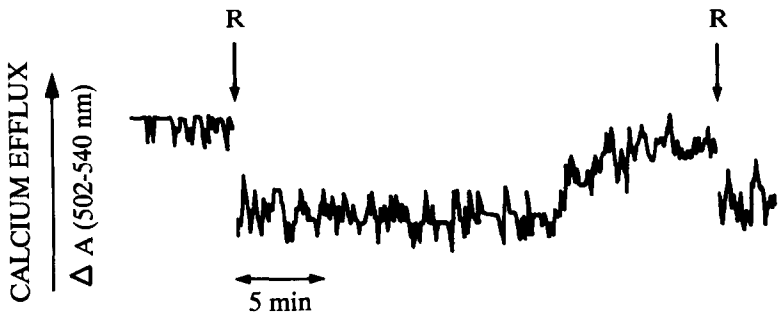

Fig. 4. Kinetics of calcium movement in protoplasts isolated from leaves of 4-day-old etiolated wheat seedlings. The protoplasts were irradiated with R light for 2 min and left in continuous darkness. Once the basal level of Ca^{++} was attained in response to the first R irradiation, a second 2 min R irradiation was given; the change in Ca^{++} levels was recorded only for ~3 min.

kinetics of Ca^{++} movement with time, calcium transport was also monitored for extended durations in dark following R irradiation. An initial irradiation of the protoplasts with 2 min R stimulated Ca^{++} influx and no further change was recorded in darkness for the subsequent 16–18 min. Thereafter an efflux of Ca^{++} was registered and the basal level was restored in the next 6–8 min (Fig. 4). A second pulse of 2 min R also stimulated Ca^{++} influx but the amplitude of the response was somewhat reduced.

Effect of ion channel and pump inhibitors

Verapamil, a Ca^{++} channel blocker, completely inhibited the R mediated influx of Ca^{++} ions into the protoplasts as investigated by MX method (Fig. 5). The data in Figure 6 demonstrate a similar situation as observed by $^{45}Ca^{++}$ uptake method; verapamil completely abolished radiolabelled calcium uptake.

SOV is a general ATPase inhibitor and has been shown to inhibit Ca^{++}-ATPase in many plant systems [13, 24]. In the present study, 5–15 μM SOV significantly inhibited Ca^{++} movement as observed by both MX and $^{45}Ca^{++}$ uptake methods. The results of the experiment performed only with radiolabelled Ca^{++} are presented in Figure 7 and clearly demonstrate that SOV affects net $^{45}Ca^{++}$ movement.

Effect of chlorpromazine

To examine whether Ca^{++}-ATPase associated with phytochrome-mediated Ca^{++} flux in wheat protoplasts is calmodulin-dependent, the effect of CPZ, a well-known calmodulin antagonist, was investigated. The data in Figure 8 show that treatment of the wheat protoplasts with 50 μM CPZ

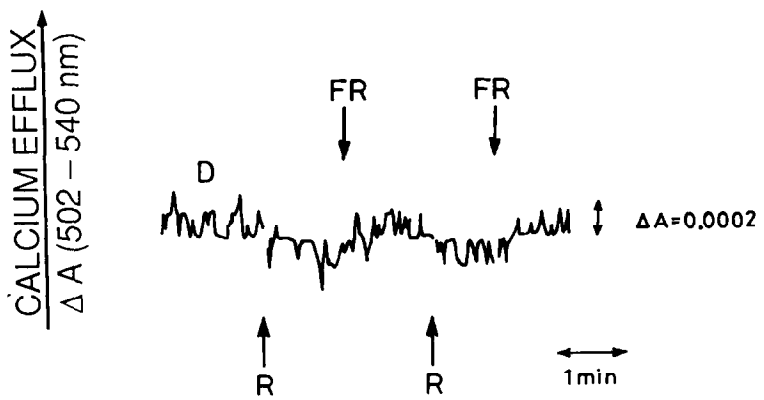

Fig. 5. Effect of verapamil (10 μM) on calcium movement in protoplasts as observed by MX method. The experimental protocol followed was similar to that described in legend to Figure 1.

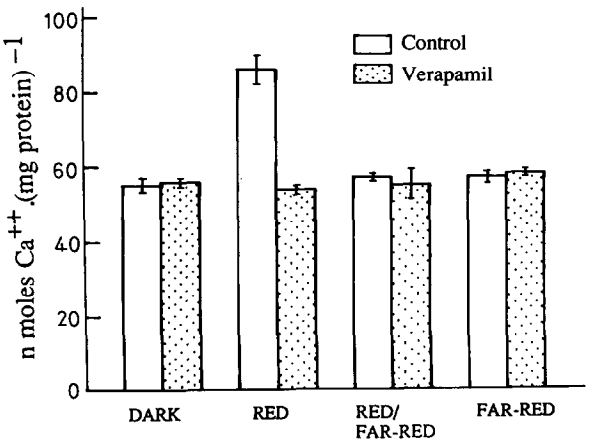

Fig. 6. Effect of verapamil (10 μM) on $^{45}Ca^{++}$ uptake by protoplasts. The protoplasts suspended in the incubation medium were subjected to different irradiation treatments and $^{45}Ca^{++}$ uptake monitored according to the protocol described in legend to Figure 2.

does indeed inhibit net $^{45}Ca^{++}$ movement in the protoplasts irradiated with FR light immediately following R irradiation. In a separate experiment effect of CPZ was also examined on the FR irradiated protoplasts and virtually no effect was observed on uptake of $^{45}Ca^{++}$ (data not presented); the level of Ca^{++} was essentially similar to that of dark control (*see also* Fig. 2).

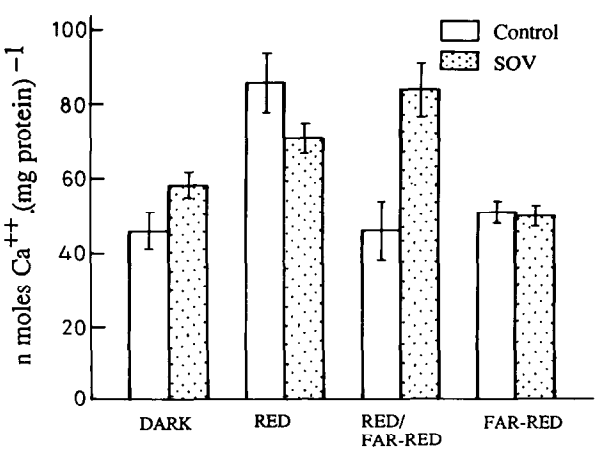

Fig. 7. Effect of sodium orthovanadate on $^{45}Ca^{++}$ uptake by protoplasts. An aliquot containing about 10^5 protoplasts was preincubated in 5 μM SOV (added to the incubation medium) for 10 min, and then subjected to R or FR irradiations. $^{45}Ca^{++}$ uptake was monitored as described in legend to Figure 2.

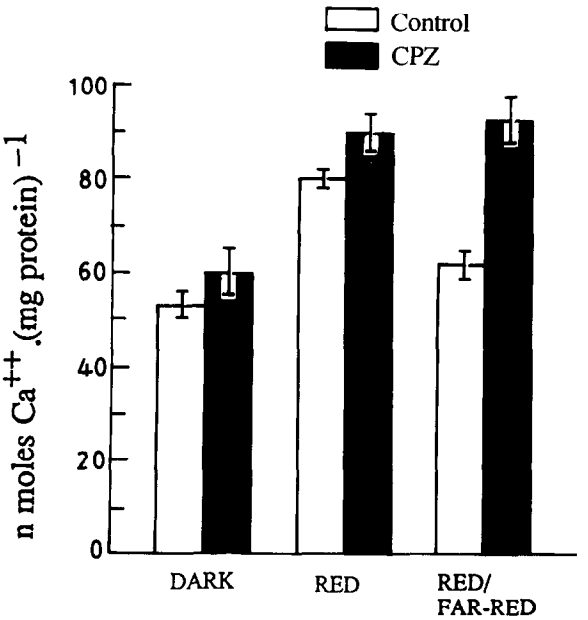

Fig. 8. Effect of chlorpromazine on $^{45}Ca^{++}$ uptake by protoplasts. The protoplasts were preincubated with 50 μM CPZ (added to the incubation medium) for 5 min before they were irradiated with R and/or FR light. For details, *see* legend to Figure 2.

Effect of neomycin

To explore any possible involvement of PIP_2 metabolism, the effect of neomycin, which is known to prevent hydrolysis of PIP_2 [8], was examined on R-mediated influx of Ca^{++} in wheat protoplasts. As is evident from the data presented in Figure 9, addition of 1 mM neomycin sulfate caused a complete reversal of R effect, i.e. the level of Ca^{++} ions moving in due to R irradiation was equal to that in the dark control.

Discussion

The data obtained in the present investigation, using the Ca^{++} sensitive dye MX or by monitoring uptake of radiolabelled calcium, clearly indicate that R irradiation stimulates influx of Ca^{++} ions into etiolated wheat leaf protoplasts, while FR reverses this effect. The experiments were also performed with dark-grown wheat coleoptiles containing and devoid of leaf sections, mesocotyl segments and leaves, and their results further substantiate the contention that R light causes an influx of Ca^{++}

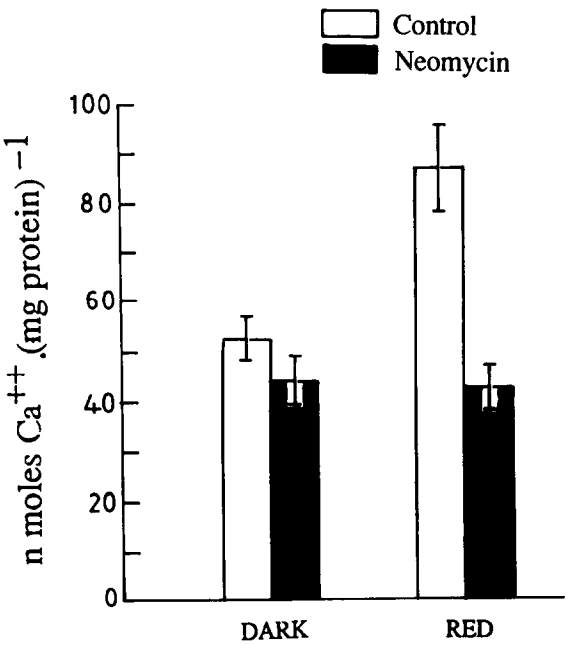

Fig. 9. Effect of neomycin on $^{45}Ca^{++}$ uptake by wheat protoplasts. About 10^5 protoplasts were preincubated with 1 mM neomycin sulfate (added to the incubation medium) for 15 min, and irradiated with 2 min R light. For details, *see* legend to Figure 2.

ions into the tissue [15]. Our findings provide support to the work of Bossen et al. [3] and Tretyn et al. [28] who reported the obligate Ca^{++} requirement for R light-induced swelling of etiolated wheat protoplasts. It was inferred from these studies that R irradiation probably facilitates Ca^{++} entry into the protoplasts *via* phytochrome phototransformation. It may be added here that protoplasts isolated from green wheat leaf tissue did not produce any change in Ca^{++} levels in the bathing solution when irradiated with R or FR light [15]; this is consistent with the observation that green leaf protoplasts did not have spectrophotometrically detectable levels of type I (etiolated-tissue abundant) phytochrome.

That phototransformation of phytochrome regulates Ca^{++} fluxes in plant cells has been demonstrated earlier only with few other plant species (see Introduction) but apparently there is no consensus on the direction of calcium flux stimulated by R/FR light, particularly amongst higher plants. Hale and Roux [10], using a Ca^{++}-sensitive dye MX, showed that R light caused net *efflux* of Ca^{++} ions in the cells and protoplasts of *Avena* and reported that its effect could be reversed by FR light. Similar results were obtained with *Vallisneria* [24]. In contrast, an influx rather than efflux of $^{45}Ca^{++}$ was observed in protoplasts of *Zea mays* in response to R irradiation [6]. Similarly, Chae et al. [5], using the flourescent dye Quin-2, demonstrated a R light-mediated net influx in oat protoplasts. They further resolved that Pfr form of phytochrome not only functions to stimulate Ca^{++} influx from extracellular sources, but also mobilizes Ca^{++} from intracellular stores, thus supporting an earlier report [23], where red light was shown to promote the release of calcium from purified oat mitochondria. Unpublished observations from our laboratory with a cultivar of oat (*Avena sativa* cv. HAU 11) also indicate that R light causes an influx of Ca^{++} ions into the coleoptiles and this effect being reversed by FR [15].

The discrepancy mentioned above, i.e. whether R light causes an influx or efflux, could possibly be attributed to the different techniques employed in various laboratories. Clearly, by the MX method, *net flux* is monitored. As Roux [21] stated there could be an initial influx by R irradiation which leads to a transient increase of Ca^{++} concentration. This increase could result in activation of Ca^{++}-pumps in the plasma membrane and push the Ca^{++} out to restore the low cytosolic Ca^{++} concentration. Thus, if Ca^{++}-ATPase pumps out Ca^{++} at a greater rate than the rate of R-mediated influx of Ca^{++}, then a net efflux would be recorded. However, Roux and co-workers did not observe any transient influx of Ca^{++} in their earlier experiments using MX technique [10], which is generally sensitive and fast enough to follow kinetics of a particular process [see also Grolig and Wagner, 9]. In 1983, Roux et al. [22] monitored changes using a flow-through cell and demonstrated the occurrence of cyclic rhythm in the influx/efflux pattern of Ca^{++} in continuous darkness; the absolute period of these cyclic fluxes varied between 22 to 38 min and depending upon the experiment the amplitude varied from 10 to 25 μM changes. Red light caused shortening of the normal period of cyclic calcium movement and also reduced the amplitude; a 5 min R exposure reduced the period in the second cycle to almost half, presumably due to increased rate of cytosolic Ca^{++} accumulation. However, there was little effect on the first cycle of Ca^{++} movement,

and the slope of the curve was not significantly affected following 5 min R irradiation. Since the protocol adopted in these experiments by Roux et al. [22] was entirely different, it is not clear whether this effect is related to the type observed in studies by other workers, including the present work, where a brief exposure to R stimulates changes in pattern of Ca^{++} flux quite rapidly. In the present investigation, kinetics of Ca^{++} influx/efflux in continuous darkness, following 2 min R irradiation, has been monitored by MX technique. Red light did indeed stimulate influx of Ca^{++} and the level was maintained in darkness for 16–18 min, following which the efflux process was triggered (Fig. 4); the basal level was restored in the next 6–8 min. A similar pattern of influx/efflux of Ca^{++} in continuous darkness was observed following 3 or 5 min R irradiation but the amplitude of the initial influx was reduced (details not presented), which is consistent with the data presented in Fig. 3. Similarly, in studies on $^{45}Ca^{++}$ uptake by etiolated maize leaf protoplasts [6] and changes in protein phosphorylation profile of oat protoplasts [18], 2–3 min R light caused maximum change (and its effect was FR reversible), but longer R irradiation brought about an abrupt decrease in the response. There is no ready explanation for the decrease in amplitude of initial influx triggered by R irradiation longer than 2 min (Fig. 3) and the occurrence of spontaneous reversal of Ca^{++} flux during prolonged darkness (Fig. 4). However, the possibility that the activation of Ca^{++}-pump, that is primarily responsible for regulating intracellular Ca^{++} homeostasis, follows a different time kinetics in prolonged darkness (following 2 min R pulse) and prolonged R illumination, may partly account for these observations.

Quantitative estimates of change in molar levels of Ca^{++} upon R/FR irradiation have been made only sparsely. In a study on *Vallisneria*, a net change (efflux) of 250 fmoles Ca^{++} per protoplast was observed upon R irradiation [24], as estimated by MX method. Utilizing fluorescent dye Quin-2/AM and based upon the estimated endogenous level of Ca^{++} in oat protoplasts, 5 min R irradiation was found to increase the cytosolic Ca^{++} concentration from 30 to 193 nM and a subsequent FR irradiation of 5 min decreased it by about 48 nM [5]. In the present work on wheat, an average influx of 50 fmoles Ca^{++} per protoplast was recorded by both the protocols adopted for measuring Ca^{++} fluxes (calculations were based on the data presented in Fig. 1 and 2; see Mehta, 15). However, a precise comparison of these reports on the estimated change in net Ca^{++} level upon stimulation by R or FR irradiation is difficult because of the differences in the methods employed to monitor Ca^{++} flux in different plants.

Regarding mechanism of Ca^{++} movement, the studies with various inhibitors support a role for ion channels and pumps. Verapamil, a calcium channel blocker, which is known to inhibit Ca^{++} movement in both animal and plant systems [12, 26, 29], completely inhibited the R light-mediated influx of Ca^{++} ions, thus implying that phytochrome phototransformation does stimulate the opening of Ca^{++} channels. In contrast, verapamil had no significant effect on Ca^{++} flux in the protoplasts irradiated with FR, either following R irradiation or alone. In the presence of SOV, protoplasts irradiated with FR following R irradiation showed a decreased efflux of Ca^{++} ions (*see* Fig. 7), thus suggesting that a pump is activated directly or indirectly by FR irradiation. In case of R irradiated protoplasts, SOV treatment decreased magnitude of Ca^{++} influx. A precise explanation for this effect of SOV is difficult to provide but it could well be that the inhibitor treatment leads to accumulation of so much Ca^{++} that mechanisms are set in motion to close the Ca^{++} channels. The effect of SOV on Ca^{++} movement was also demonstrated in *Vallisneria* protoplasts, though an inhibition of R-mediated efflux of Ca^{++} ions was observed as measured by MX method [24].

In the present study, CPZ, a calmodulin antagonist [17], was found to effectively abolish the effect of FR light on Ca^{++} efflux at a relatively low concentration of 50 μM; the maximum inhibitory effect of CPZ on Ca^{++} flux was observed in protoplasts irradiated with FR following R irradiation. This observation provides evidence, though indirect, for a probable involvement of CaM-dependent Ca^{++}-ATPase in FR-mediated Ca^{++} efflux and a general regulatory role of CaM in cellular homeostasis. However, since phenothiazines like CPZ are known to exert pleiotropic effects on cellular activity [20], therefore, the possibility exists that the effect of CPZ on FR-mediated Ca^{++} efflux is not through

interaction with calmodulin but is rather due to some non-specific effect on membrane integrity.

The possible involvement of PI metabolism in phytochrome regulation of Ca^{++} fluxes was indicated by Das and Sopory [6] who showed that 5-hydroxytryptamine (5-HT), an activator of phospholipase C (PLC), can mimic the R effect. However, in the present study on wheat protoplasts, we failed to observe any effect of 5-HT on R light-mediated influx of Ca^{++} ions (data not shown). Notwithstanding the negative results of our study with 5-HT, preliminary work with neomycin does indicate the involvement of PI metabolism in regulation of Ca^{++} flux. Neomycin, a polyanionic aminoglycoside antibiotic, is known to bind strongly and specifically to PIP_2 [8]. The binding of neomycin to PIP_2 inhibits in turn its hydrolysis by PLC and thus affects PI metabolism. In experiments with wheat protoplasts, neomycin was observed to block the R light-mediated influx, thereby suggesting a role of PI metabolism in the phytochrome-mediated Ca^{++} influx into the protoplasts. This observation is consistent with an earlier report [4] where neomycin was found to inhibit R light-induced swelling of wheat protoplasts, which is also Ca^{++}-dependent.

From the foregoing discussion it can thus be surmised that phototransformation of phytochrome to Pfr form results either directly or indirectly (i.e. with the involvement of PI metabolism) in opening of Ca^{++} channels and consequential influx of Ca^{++} ions. The irradiation of protoplasts with FR stimulates the calmodulin-sensitive Ca^{++}-ATPase activity and results in efflux of Ca^{++} ions. The results obtained with both MX method and $^{45}Ca^{++}$ uptake studies make us conclude that R irradiation causes influx of Ca^{++} ions. However, the two methods employed in the present investigation to monitor Ca^{++} fluxes have the limitation in that they can only provide estimates of Ca^{++} ions moving across the cell membrane and do not account for the changes occuring in cytosolic free calcium because of movement of ions across the organelle/membranes. Experiments are now underway in our laboratory to measure changes in cytosolic Ca^{++} using the flourescent dye Quin-2, which is more sensitive probe than MX to determine transient changes in cytosolic Ca^{++} level.

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