Studies on ion channel antagonist-binding sites in sunflower protoplasts

N. Vallée, C. Brière, M. Petitprez, H. Barthou, A. Souvré, G. Alibert*

Laboratoire de Biotechnologie et Amélioration des Plantes (BAP), INP-ENSAT/UA INRA, 145 Avenue de Muret, 31076 Toulouse cedex, France

Received 22 April 1997; revised version received 16 May 1997

Abstract The cytological location of ion channel antagonist-binding sites was studied in sunflower protoplasts using the fluorescent probes DM–Bodipy–PAA and DM–Bodipy–DHP. The binding specificity of the probes was established by competition experiments with Bepridil, phenylalkylamine (Verapamil) and dihydropyridine (Nifedipine) which are known as calcium and potassium channel antagonists. Quantitative image analysis of the fluorescence emitted by the protoplasts showed the existence of interactions between PAA- and DHP-binding sites. Moreover, studies on the cytolocalization of the PAA receptors by confocal imaging showed that in freshly isolated protoplasts, DM–Bodipy–PAA binds exclusively at sites located in the cortical region of the cell.

Key words: Ion channel; Phenylalkylamine; Dihydropyridine; Sunflower protoplast

1. Introduction

Like in animals, plant membranes have several types of ion channels [1,2] among which calcium and potassium channels have been intensively studied [3–9]. Several pharmacological drugs of the phenylalkylamine (PAA) and dihydropyridine (DHP) families, which are known as calcium channel antagonists in animal cells, are able to inhibit calcium fluxes in plant cells [3]. These substances can also inhibit potassium channels in plant [10,11] and animal cells [12]. Most of the studies on these channels have involved flux control experiments [3,7], patch-clamp analysis [11,13,14] or isolation, from membrane preparations, of polypeptides carrying the permeability property of ion channels [5,15–17]. Recently Knaus et al. [18] have introduced the use of fluorescent probes, namely DM–Bodipy–PAA, to study the interactions between phenylalkylamine and dihydropyridine receptors of the L-type Ca^{2+} channels, purified from rabbit skeletal muscle microsomes. In subsequent experiments they showed that ST–Bodipy–DHP can be used also for in vivo labeling of calcium channel antagonist-binding sites on GH3 cells [19]. Leclerc et al. [20] used the same probe to correlate the evolution of L-type calcium channels to the acquisition of competence in ectoderm cells of Pleurodels embryos and more recently Shaw and Quatrano [21] showed the involvement of putative calcium channels in the polarisation of the *Fucus* zygote by cytolocalization, using DM–Bodipy–DHP and ST–Bodipy–DHP. In this paper, using similar ion channel antagonist fluorescent probes, we demonstrate that this technique is also applicable to plant protoplasts for in vivo cytolocalization and quantitative image analysis. Moreover we show the presence of both PAA- and DHP-binding sites on or near the plasma membrane of sunflower hypocotyl protoplasts and the existence of possible molecular interactions between the binding sites of these two types of drugs.

*Corresponding author. Fax: (33) 5-61-42-30-29.
E-mail: alibert@ensat.fr

2. Materials and methods

2.1. Chemicals

DM–Bodipy–PAA and (−)DM–Bodipy–DHP (Molecular Probes, Eugene, OR) were prepared as 250 μM stock solutions in DMSO. Verapamil and Nifedipine were from Sigma (St. Louis, MO). (−)Bepridil was a gift from Dr. Ranjeva (CNRS/UPS, Toulouse, France). All the inhibitors were prepared as stock solutions in 95% ethanol at the following concentrations: 20 mM Verapamil, 3.5 mM Nifedipine and 5 mM (−)Bepridil.

2.2. Plant material

Seeds of *Helianthus annuus* L. hybrid Emil (Pioneer France-Maïs, Aussonne, France) were decoated and sterilized for 10 min in 0.01% $HgCl_2$ (w/v), 20 min in 5% $Ca(ClO)_2$ (w/v) and rinsed 3 times in sterile water. The seeds were germinated for 7 days in Podor pots on a MS basal medium [22] containing 2% sucrose and 0.8% Bacto-Agar (Difco), pH 5.8 in 16/8 h light/dark cycles at 26°C.

Protoplasts were isolated from 7-day-old hypocotyls as described by Chanabé et al. [23] and suspended at a final concentration of 5.10^4 protoplasts·ml^{-1} in TLD medium: L4 medium of Lenee and Chupeau [24] containing 5 g·l^{-1} sucrose and 16 μM naphthaleneacetic acid, 4.4 μM 6-benzylaminopurine and 0.45 μM 2,4-dichlorophenoxyacetic acid [25].

2.3. Ligand-binding studies

In chase experiments, the protoplasts were first incubated for 30 min with the fluorescent probe (1 μM) and then either with Verapamil (100, 250 or 500 μM), (−)Bepridil (500 μM), Nifedipine (50 or 500 μM) for a further 30 min. Conversely, in some experiments, the drugs were added to the protoplast suspension 30 min before the addition of the probes. Fluorescence was recorded 30 min later.

2.4. Video recording and image analysis of fluorescence

A Leitz orthoplan microscope (Weitzlar, Germany), equipped with an 100 W HBO lamp (Osram, Germany) and a Silicon Intensified Target (SIT) Video Camera (Lhesa, Cergy-Pontoise, France) were used. Image recording, treatment and measurement were performed with image processing software, Imagenia, (Biocom, Les Ulis, France). The fluorescence was recorded using an 25/0.55 NPL Fluotar objective (Leitz Weitzlar) and a filter cube with excitation wavelength of 450–490 nm, a dichroic mirror at 510 nm, and an emission filter between 515 and 525 nm. For each protoplast a 200 frame summation was recorded in order to increase the sensitivity of the video system. Background fluorescence was assessed by measuring the fluorescence level in the neighborhood of the protoplast and subtracted from the raw fluorescence of the protoplast. The fluorescence levels of at least 25 protoplasts were recorded in each set of experiments and the mean and standard errors were calculated.

2.5. Confocal laser scanning microscopy

A confocal laser scanning microscope (Zeiss LSM Micro system, Germany) equipped with an argon-ion laser scanner (488 nm), a dichroic splitter at 510 nm and a 510–525 nm emission filter was used. Image acquisition was achieved with a 40×/1.3 oil Plan Neofluar Zeiss objective lens in scan mode and image averaging of 4 frames (total scanning time = 8 s). Photographs were taken with Kodak Ektachrome Elite ISO 100/21° films.

PII S0014-5793(97)00675-3

2.6. Spectrum analysis

Uncorrected steady-state fluorescence spectra were recorded with an SLM-Aminco spectrofluorometer model 500 equipped with a 100 W xenon lamp. The pass band was 2 nm in the excitation and emission modes.

3. Results

3.1. Spectral characteristics of DM–Bodipy–PAA

In order to determine the influence of the medium on the emission spectrum of the probe, spectra of DM–Bodipy–PAA were taken in different solvents. Fig. 1 shows that in 100% methanol the emission maximum was at 511 nm. In pure water no signal was recorded. Extinction was almost 100% in 25% methanol and 39% in 50% methanol. The addition of a detergent such as digitonin (0.1%) to water, induced a dual effect: a shift of the maximum emission wavelength from 511 to 517 nm and a rise of the emission intensity from 0 to 46% of the maximum emission in pure methanol. Moreover, when DM–Bodipy–PAA was dissolved in the TLD medium (the protoplast suspension medium), the light emission shifted to 515 nm and was only 18% of the emission of the molecule in solution in pure methanol. Thus, the fluorescence emission was highly dependent on the nature of the solvent. Because the fluorescence of DM–Bodipy–PAA is almost completely quenched in aqueous media, analysis can be made without eliminating the unbound probe from the suspension medium.

3.2. Fluorescent microscopy examination of protoplasts in the presence of DM–Bodipy–PAA

A protoplast suspension containing 1 μM DM–Bodipy–PAA was examined under white light (Fig. 2Ia) or the excitation wavelength (Fig. 2Ib). When illuminated by the excitation light the protoplasts emit a bright green/yellow fluorescence while the background remains uniformly dark green. When they were previously treated with 500 μM (−)Bepridil before the addition of 1 μM DM–Bodipy–PAA, protoplasts did not appear at all fluorescent (Fig. 2IIa,b). Thus, the fluorescent probe seems to bind to the same sites as the ion channel antagonist Bepridil.

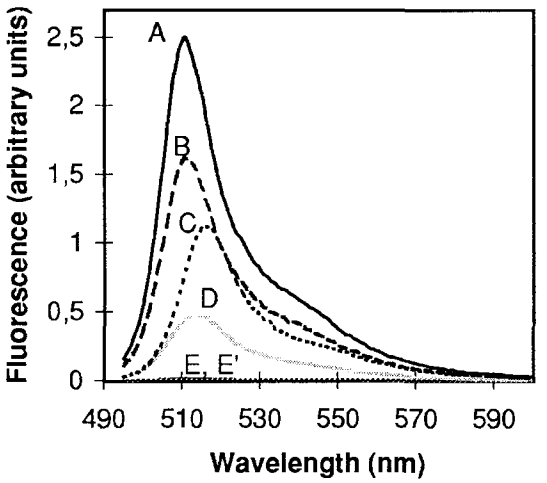

Fig. 1. Emission spectra of DM–Bodipy–PAA in solution in different solvents. DM–Bodipy–PAA (final concentration 250 nM) was dissolved either in pure methanol (A), 50% methanol (B), water+0.1% digitonin (C), TLD medium (D), 25% methanol (E) or in pure water (E'). Excitation wavelength 488 nm, band pass 2 nm.

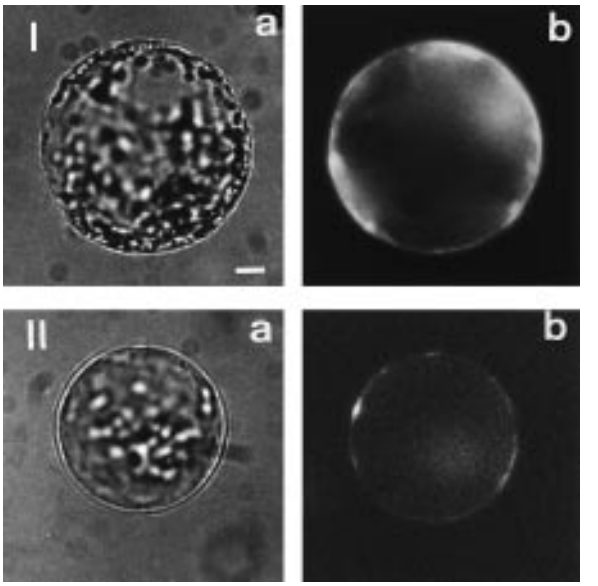

Fig. 2. Microscopic examination of sunflower hypocotyl protoplasts. Medium containing: (I) 1 μM DM–Bodipy–PAA, or (II) 500 μM (−)Bepridil during 30 min and then 1 μM DM–Bodipy–PAA. (a) Light microscopy and (b) fluorescence microscopy (excitation wavelength 450–490 nm, emission filter 515–525 nm). Photographs were taken with Fujichrome 1600, daylight film. Exposure time in photograph IIb was 5-fold that in photograph Ib. (Bar = 5 μm).

3.3. Confocal imaging of DM–Bodipy–PAA fluorescence

Freshly isolated sunflower protoplasts were examined under a confocal microscope in order to locate the cellular-binding sites of DM–Bodipy–PAA. Optical sections were made at different levels of the protoplasts. Fig. 3 presents an example of several parallel sections of a protoplast showing a fluorescence restricted to the plasmalemma or to the neighborhood of the plasmalemma.

3.4. Binding specificity of the fluorescent probes

Quantitative image analysis of the light emitted by the flu-

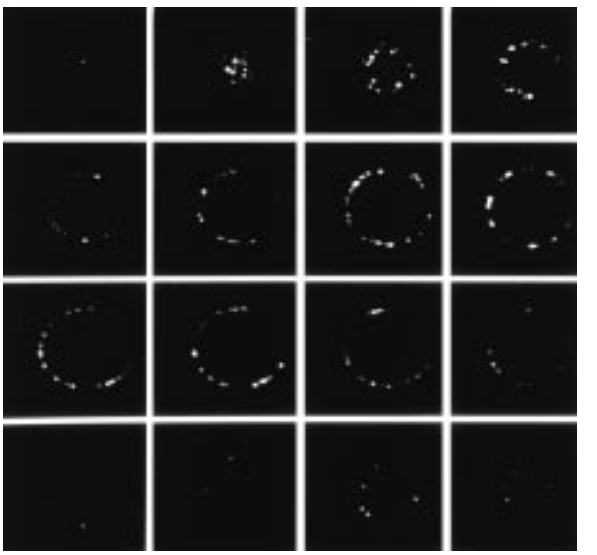

Fig. 3. Confocal images of 16 optical sections from the top to the bottom of a freshly isolated protoplast incubated with 1 μM DM–Bodipy–PAA. Excitation wavelength 488 nm, emission filter 510–525 nm.

orescent probes was made when they were incubated with a freshly isolated protoplast suspension in the absence or in the presence of various ion channel antagonist molecules.

Fig. 4 shows that the DM–Bodipy–PAA fixed on protoplasts was displaced by the specific PAA antagonist Verapamil and by (−)Bepridil. The displacement was dependent on the concentration of the channel antagonists. When the protoplasts were treated with Bepridil or Verapamil before being incubated with DM–Bodipy–PAA, the fluorescence was also strongly reduced. On the contrary, the addition of Nifedipine, a ligand of the dihydropyridine type, increased significantly the fluorescence level of the protoplasts (t test, $\alpha = 0.05$). Similar results (Fig. 5) were obtained with the (−)DM–Bodipy–DHP probe although, in this case, the fluorescence was decreased in a less extent by 500 μM Nifedipine, which specifically binds to the dihydropyridine sites.

4. Discussion

Until now most studies of ion channels have dealt with their identification, isolation and regulation at the molecular level. The aim of the present work was to study in vivo the binding of ion channel antagonists to sunflower protoplasts by using fluorescent probes. A careful study of the spectral properties of the fluorescent probe DM–Bodipy linked to a PAA radical showed that its fluorescence was largely quenched in aqueous media providing the opportunity to use it in suspensions of living cells as a marker of binding sites of the corresponding drug. These results were similar to those presented by Knaus et al. [18] and Leclerc et al. [20] on animal cells with ST–Bodipy–DHP, except that we demonstrate that it is not necessary to eliminate the probe before the analysis.

In sunflower protoplasts the fluorescence emitted by the fluorescently labeled phenylalkylamine DM–Bodipy–PAA was strongly reduced by adding to the suspension medium drugs of the PAA or Bepridil group. Thus, as demonstrated earlier in animal cells [26], we conclude that the DM–Bodipy–PAA probe fixed mainly on the same binding sites as these drugs. Moreover, confocal microscopy analysis shows that these sites are mainly located at the periphery of the protoplast on or near the plasmalemma. These results are in accordance with the isolation of ion channels from animal [19] or plant [15] membrane preparations. As compared to effective concentrations used in electrophysiological studies, rather high concentrations of the drugs (> 100 μM) were necessary to decrease significantly the fluorescence of labeled inhibitors. This has probably to do with the fact that the competition for binding sites has to occur on the protoplast surface.

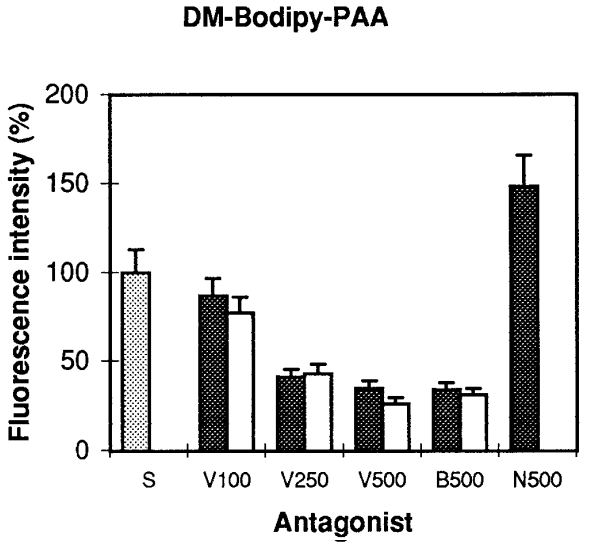

Fig. 4. Displacement of DM–Bodipy–PAA (1 μM) by ion channel antagonists. Base 100 refers to the standard condition (S) in which the fluorescent probe was added alone to the protoplast suspension. In the other assays ion channel antagonists were added 30 min after (black labels) or before (white labels) the fluorescent probe: Verapamil (V 100, 250, 500 μM), (−)Bepridil (B 500 μM), Nifedipine (N 500 μM).

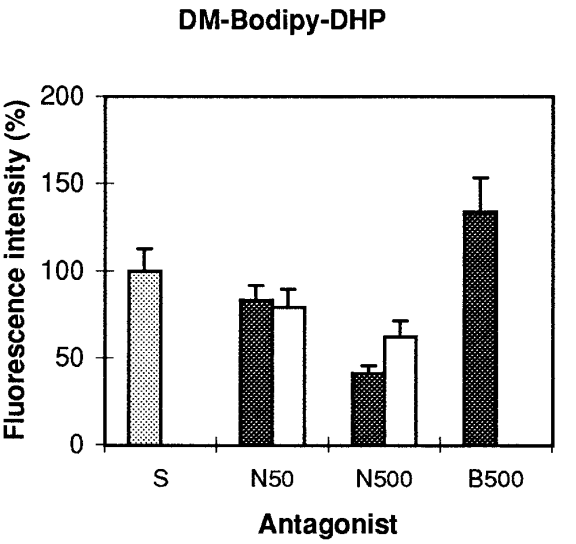

Fig. 5. Displacement of (−)DM–Bodipy–DHP (1 μM) by ion channel antagonists. Base 100 refers to the standard condition (S) as in Fig. 4. In the other assays ion channel antagonists were added 30 min after (black labels) or before (white labels) the fluorescent probe: Nifedipine (N 50, 500 μM), (−)Bepridil (B 500 μM).

In addition to PAA-binding sites, our results showed the presence of sites for the binding of molecules of the DHP group (DM–Bodipy–DHP) which were not generally found in previous works in higher plants [3,16,27]). The presence of such sites have been demonstrated in mosses and algae [28,29]. Sites for these drugs were transiently identified in developing corn by Ketchum and Pool [30] and also appeared to be present in carrot protoplasts freshly isolated from roots [31] and in protoplasts from *Nicotiana tabacum* cell cultures [11]. Recently, Shaw and Quatrano [21] observed the presence of dihydropyridine-binding sites on *Fucus* zygotes. Our results argue strongly in favour of the presence of DHP-binding sites on higher plant membranes.

In animal cells, Knaus et al. [19], working on purified membrane fractions from rabbit skeletal muscle, showed the existence of interactions between PAA and DHP antagonists-binding sites. In sunflower, protoplasts incubated with one type of fluorescent probe (PAA or DHP group) showed an increase of fluorescence after the addition of a drug belonging to the other group (DHP or PAA). This result suggests that interactions between PAA- and DHP-binding sites may also exist in plants.

Because both potassium and calcium channels seem to be targets for the pharmacological drugs PAA and DHP [10,11], we cannot make any firm statement as to the cellular structures which carry such binding sites. In *Fucus*, Shaw and

Quatrano [21] showed that the distribution of dihydropyridine receptors was asymmetrical during axis fixation of the egg and coincided with a local increase of intracellular calcium concentration, suggesting that calcium channels would be the target of the fluorescent probe. Therefore, efforts will be made in future experiments with sunflower protoplasts to try to correlate free cytoplasmic calcium and the presence of the specific plasma membrane structures which bind the fluorescently labeled phenylalkylamine and dihydropyridine.

Acknowledgements: We would like to thank Dr. R. Ranjeva, UMR 5546, CNRS/UPS and Dr. M. Moreau, UMR 9925, CNRS/UPS Toulouse, France, for helpful criticism and discussion. The technical assistance of Ph. Anson is also greatly acknowledged. This work was partly supported by a grant from the Conseil Regional Midi-Pyrénées (France) and by a Bonus Qualité Recherche (BQR) from INP Toulouse (France).

References

[1] Ward, J.M., Pei, Z.M. and Schroeder, J.I. (1995) Plant Cell 7, 833–844.
[2] Bush, D.S. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122.
[3] Graziana, A., Fosset, M., Ranjeva, R., Hetherington, A.M. and Lazdunski, M. (1988) Biochemistry 27, 764–768.
[4] Tester, M. (1990) New Physiol. 114, 305–340.
[5] Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.M., Gaymard, F. and Grignon, C. (1992) Science 256, 663–665.
[6] Obermeyer, G. (1993) Bot. Acta 106, 26–31.
[7] Huang, J.W., Grunes, D.L. and Kochian, L.V. (1994) Proc. Natl. Acad. Sci. USA 91, 3473–3477.
[8] White, P.J. (1994) Planta 193, 186–193.
[9] Klüsener, B., Boheim, G., Liß, H., Engelberth, J. and Weiler, E.W. (1995) EMBO J. 14, 2708–2714.
[10] Terry, B.R., Findlay, G.P. and Tyerman, S.D. (1992) J. Exp. Bot. 43, 1457–1473.
[11] Thomine, S., Zimmermann, S., van, D.B., Barbier-Brygoo, H. and Guern, J. (1994) FEBS Lett. 340, 45–50.
[12] Avdonin, V., Shibata, E.F. and Hoshi, T. (1997) J. Gen. Physiol. 109, 169–180.
[13] Thuleau, P., Ward, J.M., Ranjeva, R. and Schroeder, J.I. (1994) EMBO J. 13, 2970–2975.
[14] Marten, I., Gaymard, F., Lemaillet, G., Thibaud, J.B., Sentenac, H. and Hedrich, R. (1996) FEBS Lett. 380, 229–232.
[15] Thuleau, P., Graziana, A., Canut, H. and Ranjeva, R. (1990) Proc. Natl. Acad. Sci. USA 87, 10000–10004.
[16] Harvey, H.J., Venis, M.A. and Trewavas, A.J. (1989) Biochem. J. 257, 95–100.
[17] Piñeros, M. and Tester, M. (1995) Planta 195, 478–488.
[18] Knaus, H.G., Moshammer, T., Kang, H.C., Haugland, R.P. and Glossmann, H. (1992) J. Biol. Chem. 267, 2179–2189.
[19] Knaus, H.G., Moshammer, T., Friedrich, K., Kang, H.C., Haugland, R.P. and Glossmann, H. (1992) Proc. Natl. Acad. Sci. USA 89, 3586–3590.
[20] Leclerc, C., Duprat, A.M. and Moreau, M. (1995) Cell Calcium 17, 216–224.
[21] Shaw, S.L. and Quatrano, R.S. (1996) J. Cell Sci. 109, 335–342.
[22] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–497.
[23] Chanabé, C., Burrus, M. and Alibert, G. (1989) Plant Sci. 64, 125–132.
[24] Lenée, P. and Chupeau, Y. (1986) Plant Sci. 43, 69–75.
[25] Chanabé, C., Burrus, M., Bidney, D. and Alibert, G. (1991) Plant Cell Rep. 9, 635–638.
[26] Hosey, M. and Lazdunski, M. (1988) J. Memb. Biol. 104, 81–105.
[27] Andrejauskas, E., Hertel, R. and Marme, D. (1985) J. Biol. Chem. 260, 5411–5414.
[28] Conrad, P.A. and Hepler, P.K. (1988) Plant Physiol. 86, 684–687.
[29] Dolle, R. and Nultsch, W. (1988) Arch. Microbiol. 149, 451–458.
[30] Ketchum, K.A. and Poole, R.J. (1991) J. Memb. Biol. 119, 277–288.
[31] Antognoni, F., Pistocchi, R., Casali, P. and Bagni, N. (1995) Plant Physiol. Biochem. 33, 701–708.