Characterization of D-[3H]cis-diltiazem binding to membrane fractions and specific binding of calcium channel blockers to isolated flagellar membranes of *Chlamydomonas reinhardtii*

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Summary

Plasma membranes were separated from the intracellular membranes by using an aqueous two-polymer phase system. D-[3H]cis-diltiazem was employed to characterize benzothiazepine-selective receptors in these different membrane fractions of *Chlamydomonas reinhardtii*. The separation revealed that one type of binding site with higher affinity (K_D = 33 nM) can be attributed to the intracellular membrane fraction and a second type with lower affinity (K_D = 313 nM) to the plasma membrane fraction. The apparent dissociation constants determined from the association and dissociation rate constants in kinetic experiments are comparable to those determined by saturation experiments. The maximum numbers of binding sites of the intracellular membrane fraction and the plasma membrane fraction are B_max = 6.4 pmol mg⁻¹ protein and B_max = 19 pmol mg⁻¹ protein, respectively. D-[3H]cis-diltiazem binding is inhibited by (±)-verapamil and calcium chloride in both fractions. Moreover, nifedipine stimulates D-[3H]cis-diltiazem binding by the intracellular membrane fraction, but shows no effect on the plasma membrane fraction.

Ligand displacement binding studies with isolated flagella revealed the occurrence of a D-cis-diltiazem binding site with about the same affinity to this drug (K_D = 400 nM) as the one found in the plasma membrane fraction. The maximum number of binding sites is 4.5 pmol mg⁻¹ protein. The apparent dissociation constants for specific [3H]nimodipine and [3H]verapamil binding to the flagella were calculated to be 8 nM and 38 nM, respectively. The corresponding B_max values are 345 fmol mg⁻¹ protein and 1.3 pmol mg⁻¹ protein, respectively.

Key words: *Chlamydomonas reinhardtii*, phototaxis, calcium channels, calcium channel blockers, calcium channel blocker binding sites.

Introduction

The role of calcium ions in the control of swimming and photobehavioural responses of *Chlamydomonas* has been investigated by several authors (Stavis & Hirschberg, 1973; Schmidt & Eckert, 1976; Nultsch, 1979; Morel-Laurens, 1987; Dolle et al., 1987). Moreover, Nultsch et al. (1986) found that phototaxis is specifically inhibited by organic calcium channel blockers, such as verapamil, diltiazem and nimodipine. The galvanotactic response of *C. reinhardtii* in an externally applied electric field is also strongly calcium-dependent and is inhibited by the same drugs (Dolle & Nultsch, 1988a). Bessen et al. (1980) have demonstrated that the beat pattern of isolated flagella of *Chlamydomonas* lacking both the flagellar membrane and the basal body depends on the external calcium concentrations. Hyams & Borisy (1975, 1978), who worked with isolated, reactivated flagellar apparatuses of a cell wall-less mutant of *C. reinhardtii*, observed similar phenomena. They suggested that the depolarization of the flagellar membrane in response to a stimulus causes an influx of calcium ions, thus inducing a change in the flagellar beat.

On the basis of these results Nultsch (1983) has proposed a hypothetical model of the phototactic...
reaction chain of *C. reinhardtii* according to which voltage-operated calcium channels in the plasma membrane and in the flagellar membrane are involved in the sensory transduction. Since the primary mechanism of the action of calcium antagonists is the binding to calcium channels (Lee & Tsien, 1983), the use of radiolabelled calcium channel blockers has helped to identify calcium channel proteins (Borsotto et al. 1984). The physiological significance of calcium channel blocker binding sites in plants is not as clear as in animal cells. Andrejauskas et al. (1985) reported on a specific [³H]verapamil binding site in plant membrane fractions. Moreover, Andrejauskas et al. (1986) have demonstrated the similarity of [³H]verapamil binding to plant and animal membranes by showing the stimulatory effect of 3,4,5-triiodobenzoic acid on [³H]verapamil binding to membrane fractions of both zucchini cells and rabbit skeletal muscle.

Recently, Dolle & Nultsch (1988b) have demonstrated the occurrence of specific [³H]verapamil binding sites in both plasma membrane and intracellular membrane fractions of *C. reinhardtii*, which were separated by using the two-polymer phase system described by Widell et al. (1982) and Kjellbom & Larsson (1984). They found a high-affinity binding site in the plasma membrane fraction and a second one with lower [³H]verapamil affinity in the intracellular membrane fraction. Similar results were obtained with specific [³H]nimodipine binding sites (Dolle, 1988).

In contrast to verapamil, which inhibited both motility (by about 20%) and phototaxis, D-cis-diltiazem showed a very specific effect by inhibiting only the phototactic response without impairing motility (Nultsch et al. 1986). As this specific effect of diltiazem on phototaxis indicated the involvement of calcium channels in the photosensory transduction (Nultsch et al. 1986), it was necessary to demonstrate the existence of specific D-[³H]cis-diltiazem binding sites in purified plasma membranes. Provided that a calcium channel blocker binding site is a part of the calcium channel, as suggested by Glossmann et al. (1983) and Ferry & Glossmann (1983), these findings would be indicative of calcium channel. Moreover, the photosensory transduction model suggested by Nultsch (1983) postulates also the occurrence of calcium channels in the flagellar membranes. Since D-cis-diltiazem and nimodipine did not inhibit motility, it was of great interest to look for specific calcium channel blocker binding sites in the flagellar membrane also.

**Materials and methods**

**Growth conditions and preparation of a microsomal fraction**

The strain Göttingen 11-32(+) of *C. reinhardtii* Dangeard was grown at 4500 lux and 22°C in 41 of a nutrient medium described by Nultsch (1979). The culture was aerated with air + 2.5 % CO₂, and after 12–15 days the cells were harvested for the experiments. The preparation of the microsomal fraction has been described in detail by Dolle & Nultsch (1988b).

**The aqueous two-polymer phase system**

The microsomal pellet of 20–23 g wet weight suspended to a volume of 10 ml in 0.33 M sucrose, 60 mM NaCl, 10 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM potassium phosphate (pH 7.8) was applied to a 60 g (final weight) two-phase system containing 6.5 % (w/w) dextran T500, 6.5 % (w/w) polyethylene glycol (PEG) M, 3350. The system was mixed by 20 inversions of the tube and centrifuged in a swing-out rotor (Labofuge II, Heraeus Christ, Osterode, FRG) at 1500 g for 3 min to decrease the time for phase settling. The upper phase was removed carefully with a pipette without disturbing the interface. Material left on the interface was included in the lower phase. Upper (U) and lower (L) phases were repartitioned twice with freshly prepared lower and upper phases, respectively, to obtain washed upper and lower phases (U₁ and L₁) before diluting and pelleting (Dolle & Nultsch, 1988).

**Marker enzyme, protein and chlorophyll assays**

Vanadate-sensitive, nitrate- and molybdate-insensitive K⁺-stimulated, Mg²⁺-dependent ATPase was used as a marker for the plasma membrane, cytochrome c oxidase as a marker for mitochondria, antimycin A-insensitive NADPH-cytochrome c reductase as a marker for endoplasmic reticulum and chlorophyll as a marker for thylakoid fragments. The marker assays have been described by Dolle & Nultsch (1988b). Protein was determined after precipitation with 0.3 M-trichloroacetic acid by the Lowry procedure with bovine serum albumin (BSA) as the standard; 0.01 % Triton X-100 was included in each assay tube to solubilize membrane proteins.

**Isolation of the flagella**

A 20–23 g wet wt sample of cells was harvested and suspended in 44–51 ml of an extraction medium as described by Dolle & Nultsch (1988b). The piperazone derivative flunazine (10⁻⁵ M) was added, which has been shown to cause immediate detachment of the flagella from the cell bodies (Nultsch et al. 1986). The suspension was stirred for 15 min in an ice-cold water bath and subsequently centrifuged at 4°C for 30 min at 1500 g (4000 revs min⁻¹), MSE high-speed 18 centrifuge, rotor angle head no. 69180. The flagella-containing supernatant was stored at 4°C and the pellet was resuspended in extraction medium once more. A second centrifugation of this suspension followed, and the resulting supernatant was combined with the first one. This flagella-containing suspension was pelleted at 28 000 g (15 000 revs min⁻¹), MSE high-speed 18 centrifuge, rotor angle head no. 69180) for 60 min and resuspended in 2–3 ml of 'assy medium' for the radioligand binding studies, consisting of 50 mM-Tris-Mes (pH 7.5), 0.33 M sucrose, 10 mM-DTT and 1 mM-PMSF. The protein yield amounted to about 0.02 % of the wet weight.
Radioligand binding studies

The binding of the tritiated calcium channel blockers to the membrane fractions (20–50 μg protein) was determined at 22°C in 250 μl of 50 mM-Tris-HCl buffer (pH 7.5) containing 0.1 mM-PMSF in the presence or absence of unlabelled drugs. In standard equilibrium binding experiments incubations (120 min) were stopped by adding 4 ml of cold dilution buffer (50 mM-Tris-HCl, pH 7.5) and rapid filtration of the samples through Whatman GF/B filters. The filters, which had been pretreated during 2 h with 5% polyethyleneimine, were washed twice with 4 ml of cold dilution buffer. The vacuum-dried filters were counted for retained radioactivity after adding 8 ml of a suitable liquid scintillation cocktail.

Non-specific binding of a tritiated calcium channel blocker was determined in the presence of 10⁻⁴ M of the respective unlabelled drug and was subtracted from total binding to obtain the specific binding. Experimental work and incubation using nimodipine was performed in dim light and in darkness, respectively, because of the light sensitivity of dihydropyridine derivatives.

Chemicals

[3H]verapamil (80 Ci mmol⁻¹) and [3H]nimodipine (159 Ci mmol⁻¹) were purchased from New England Nuclear Corporation, Dreieich, FRG, d-[3H]cis-diltiazem (167 Ci mmol⁻¹) from Amersham Buchler, Braunschweig, FRG. Verapamil, d-cis-diltiazem, nifedipine, dextran T500 (approx. M r 500,000) and PEG 3350 (approx. M r 3350) were obtained from Sigma Chemie, Deisenhofen, FRG. The liquid scintillation cocktail ‘Aqualuma plus’ was purchased from J. T. Baker Chemicals, Deventer, Netherlands. Nimodipine was a gift from Professor Glossmann, Institut für Biochemische Pharmakologie, Innsbruck, Austria.

Results

Scatchard analysis of equilibrium binding of d-[3H]cis-diltiazem to a microsomal fraction of C. reinhardtii revealed two specific binding sites with apparent dissociation constants of KD = 21 nM and KD = 311 nM. The maximal numbers of binding sites (Bmax) were calculated to be 1.5 and 8.8 pmol mg⁻¹ protein, respectively (data not shown).

By preparing a chlorophyll-free plasma membrane fraction (Dolle & Nultsch, 1988b) it could be shown that the specific binding site with lower affinity to d-[3H]cis-diltiazem is to be attributed to the plasma membrane fraction, whereas the second one is found in the intracellular membrane fraction. Figs 1A, B and 2A, B show the saturation isotherms (panel A) and Scatchard plots (panel B) of d-[3H]cis-diltiazem binding to the plasma membrane fraction (U3) and intracellular membrane fraction (L3), respectively. The KD values of 313 and 33 mM are comparable to those found in the crude microsomal fraction. The increase of the Bmax values from 8.8 to 19 pmol mg⁻¹ protein indicates a 2.2-fold and from 1.5 to 6.4 pmol mg⁻¹ protein a 4.3-fold enrichment of the plasma membrane- and intracellular membrane-associated binding sites, as a result of the two-polymer phase partition method.

Since the equilibrium binding constant is a ratio of the binding reaction's forward and reverse rate constants (Bennett, 1978), it is desirable to estimate the rate constants for association (k1) and dissociation (k₋₁) so that the binding constants derived from saturation data (Figs 1B, 2B) may be compared with the binding constants resulting from the ratio of rate constants.

Figs 3 and 4 represent the association and dissociation kinetics of d-[3H]cis-diltiazem binding to the membranes of the U3 and L3 fractions, respectively. At 22°C association-equilibrium is reached after 100–120 min (U3 fraction) and after 80 min (L3 frac-
Fig. 2. A. Equilibrium binding of D-[^3H]cis-diltiazem to intracellular membranes (L3 fraction) of C. reinhardtii at 22°C. The range of free-ligand concentrations was 2-30 nM, the concentration of membrane protein 0.2 mg ml⁻¹. Direct representation of the data: (O) total binding, (●) specific binding and (□) non-specific binding measured in the presence of 10⁻⁴ M of the unlabelled drug. B. See Fig. 1B.

Among the calcium antagonists tested, D-cis-diltiazem and (±)verapamil were able to displace D-[^3H]cis-diltiazem specifically from its binding sites in the membranes of both the U3 and L3 fractions (Figs 5 and 6). In contrast, nifedipine at 22°C stimulates high-affinity binding of D-[^3H]cis-diltiazem in the L3 frac-
Fig. 5. Effect of unlabelled Ca$^{2+}$ antagonists and CaCl$_2$ on D-[3H]m-diltiazem binding (45 nM) to membranes of the U3 fraction. At pH 7.5 and 22°C membrane protein (20 µg/assay) was incubated for 120 min with increasing concentrations of (±)verapamil (▲), nifedipine (●), D-[3H]cis-diltiazem (○) and CaCl$_2$ (■). The control value for total D-[3H]cis-diltiazem binding (100%) was 7 fmol.

Fig. 6. Effect of Ca$^{2+}$ antagonists and CaCl$_2$ on D-[3H]cis-diltiazem binding (3 nM) to membranes of the L3 fraction. At pH 7.5 and 22°C membrane protein (20 µg/assay) was incubated for 120 min with increasing concentrations of (±)verapamil (▲), nifedipine (●), D-[3H]cis-diltiazem (○) and CaCl$_2$ (■). The control value for total D-[3H]cis-diltiazem binding (100%) was 13 fmol.

Discussion

The present biochemical study shows that two specific D-[3H]cis-diltiazem binding sites exist in a crude microsomal fraction of *C. reinhardtii*. The separation of plasma membranes from intracellular membranes revealed that the high-affinity binding site is located in intracellular membranes, whereas the one with lower D-[3H]cis-diltiazem affinity belongs to the plasma membrane. This finding is contrary to the results of [3H]verapamil and [3H]nimodipine binding studies, which showed that the high-affinity binding site is located in intracellular membranes, whereas the one with lower D-[3H]cis-diltiazem affinity belongs to the plasma membrane. This finding is contrary to the results of [3H]verapamil and [3H]nimodipine binding studies, which showed that the high-affinity binding site is located in the plasma membrane (Dolle & Nultsch, 1988); for nimodipine, Dolle, 1988).

The two binding sites have been characterized according to their ligand binding properties. From Scatchard plot analysis in saturation experiments (Figs 1 and 2) equilibrium dissociation constants of $K_D = 313$ nM for the plasma membrane fraction and $K_D = 33$ nM for the intracellular membrane fraction were determined, which are close to the dissociation constants calculated from the kinetic data (350 and 26 nM). The affinity of D-[3H]cis-diltiazem for the intracellular membranes is comparable to that found in rabbit skeletal muscle t-tubule membranes (Barhanin et al. 1987), cardiac sarcolemma from dog heart (Balwierczak & Schwartz, 1985) and partially purified t-tubule membranes from guinea-pig skeletal muscle (Glossmann et al. 1984b).

Nifedipine stimulates D-[3H]cis-diltiazem binding in the L3 fraction at 22°C (Fig. 5); 50% stimulation was reached at a concentration of 1 µM. Glossmann (1983) found 50% stimulation of D-[3H]cis-diltiazem binding to the calcium channels in skeletal muscle by applying a 100-fold lower concentration of nimodipine, which also

belongs to the 1,4-dihydropyridines, at a temperature of 30°C. It may be concluded that 1,4-dihydropyridines also act in a positively allosteric fashion on the diltiazem binding sites in the intracellular membranes of C. reinhardtii at 22°C. In the U_3 fraction nifedipine had no effect (Fig. 6). Apart from unlabelled D-cis-diltiazem, (±)verapamil and calcium chloride were also able to displace D-[^3]H]cis-diltiazem specifically from its binding sites in both membrane fractions (Figs 5 and 6). These findings are consistent with the results reported by Glossmann et al. (1984a), who observed an inhibition of D-[^3]H]cis-diltiazem binding by phenylalkylamines and Ca^{2+}.

In summary, the two specific D-[^3]H]cis-diltiazem binding sites in the plasma membrane fraction and the intracellular membrane fraction, which show different affinities to this drug, have similar molecular properties with respect to (±)verapamil and Ca^{2+} interactions, but only the high-affinity binding site found in the L_3 fraction responds to a 1,4-dihydropyridine by increasing the ligand binding capacity. The fact that specific D-cis-diltiazem binding to plasma membranes and intracellular membranes is attenuated by Ca^{2+} suggests that the binding sites might be involved in some unknown way in the regulation of the cellular Ca^{2+} metabolism. Moreover, a possible involvement of calcium channels in the regulation of the cytoplasmic free calcium concentration must be assumed.

Ligand displacement experiments with isolated flagellar membranes using drugs that belong to the three main classes of calcium channel blockers, namely the benzothiazepine D-[^3]H]cis-diltiazem, the phenylalkylamine [^3]H]verapamil and the 1,4-dihydropyridine [^3]H]nimodipine give the first evidence that for each drug a specific binding site exists with about the same affinity as established for the U_3 fraction. Nevertheless, these binding sites differ from those of the U_3 fraction in so far as they have lower specific binding capacities, indicating that they are typical of and exclusively located in the flagellar membrane. Further studies are necessary to characterize the flagellar membrane drug receptors in respect to pharmacological profile, binding kinetics and Ca^{2+}-chelator interactions.

If we suppose that the channel blocker binding sites found in both the plasma membrane of the cell body and the flagellar membrane are part of a calcium channel (Glossmann et al. 1983; Ferry et al. 1983; Ferry & Glossmann, 1983), our results support the idea that calcium channels in the plasma membrane of the cell body are involved in the phototactic transduction chain. Moreover, corresponding calcium channels in the flagellar membrane may be involved in the response to the given signal in the steering mechanism of the flagella, thus supporting the observation by Hyams & Borisy (1975, 1978) and Bessen et al. (1980) that varying the external calcium concentration causes isolated flagella to change their beat pattern.

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References


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