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Research article

Characteristic early membrane effects induced by tryptophan in pulvinar motor cells

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ABSTRACT

Tryptophan at concentrations higher than 0.1 mM, triggered characteristic early physiological effects such as rapid (within 5 min) dose-dependent membrane hyperpolarization in *Mimosa pudica* motor cells and modification of the time course of the spontaneous proton efflux monitored in the incubation medium of pulvinar tissues. The rapid modifications of the leaf turgor-mediated movements seen on the primary pulvini of *M. pudica* following a shock and on *Cassia fasciculata* leaflets during a transition from light to darkness indicate that tryptophan disturbed the ionic migrations involved in the electrophysiological events and in the osmocontractile reaction of the motor cells. These reactions were specific to tryptophan compared to those induced by serine and 5-hydroxytryptophan. The tryptophan mode of action cannot be linked to a direct modification of the plasma membrane H^+-ATPase activity as monitored on purified pulvinar plasma membrane vesicles. The tryptophan metabolism-linked products tryptamine and indole also inhibited the motile reactions, activated in a continuous manner the H^+ secretion of pulvinar tissues and showed properties of a protonophore and an ATPase activity inhibitor on plasma membrane vesicles, respectively. The specific behavior of tryptophan in the reaction studies here is discussed in light of the previously reported action of phytohormones.

1. Introduction

The amino acid tryptophan (Trp) plays a primary role in all living organisms, from bacteria and fungi to plants and animals, by being an essential amino acid required for protein synthesis. In addition, Trp intervenes in the synthesis of many pivotal compounds. In animals which are incapable of synthesizing this amino acid, Trp after being ingested as residues within proteins is used for the synthesis of serotonin, melatonin and vitamin nicotinic acid (Moffett and Namboodiri, 2003). In plants, Trp plays a direct role in regulating plant development by providing precursors for the multiple pathways of synthesis of the hormone indole-3-acetic acid (IAA) that controls numerous physiological processes (Woodward and Bartel, 2005). Furthermore, Trp has been shown to assist in plant tolerance of heavy metals. Thus, the Trp content increased in *Noccaea caerulescens* treated with increasing concentrations of Cd (Zemanova et al., 2014), resulting in reduced Cd availability to the plants, decreased Cd transport and reduced Cd accumulation (Sanjaya et al., 2008). In addition, enzymes involved in Trp synthesis were induced under different kinds of stresses such as amino acid starvation, the oxidative stress-inducing herbicide acifluorfen and the abiotic elicitor α-amino butyric acid (Zhao et al., 1998). Plants also utilize Trp as a precursor in the synthesis of a large variety of secondary metabolites, known to intervene in pathogen defense responses such as serotonin (Ishihara et al., 2008), phytoalexins (particularly camalexin), glucosinolates and indole-derived alkaloids (Radwanski and Last, 1995; Frerigmann et al., 2016). However, Trp has been shown to affect *per se* various long-term physiological processes in plants as shown by its effects observed on leaf growth and cell expansion in *Arabidopsis* (Jing et al., 2009).

In addition to its action on these types of processes involving gene regulation, Trp, similar to other amino acids, induces short-term cellular events, as evidenced by its triggering of rapid ionic fluxes during their uptake through the plasma membrane mediated by proton-coupled symports (Delrot et al., 2001 and references therein).

The aim of this work was to show whether, compared to other natural amino acids, Trp triggers characteristic early events at the membrane level, and in particular, noteworthy modifications of the bioelectrical transmembrane potential and disturbances of proton

Abbreviations: Ind, indole; OHTrp, 5-hydroxytryptophan; PMVs, plasma membrane vesicles; Ser, serine; Try, tryptamine; Trp, tryptophan

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fluxes. For this purpose, the motor cells of pulvini of *Mimosa pudica* and *Cassia fasciculata* were chosen because the kinetics, energetics and some of the reaction steps of the stress-induced turgor driven leaf movements are now well documented (Samejima and Sibaoka, 1980; Moran, 2007). The reaction of the primary pulvini of *M. pudica* following a shock consists of a rapid spectacular drooping movement of the leaf in approximately 2 s, and the recovery takes 15–20 min (Volkov et al., 2010). Leaflet movements can be induced in *M. pudica* and *C. fasciculata* by transferring plants from light to darkness or from darkness to light. As the result of the coordinated contraction of the pulvinar cells, the motor reactions are driven by rapid turgor changes mainly associated with K^+ and Cl^- fluxes. In plant cells, K^+ fluxes have been shown to be coupled to reverse H^+ fluxes driven by the regulation of the activity of a proton-pumping ATPase located at the plasma membrane (Serrano, 1989). Thus, attention has been paid on the way Trp may have a direct effect on the H^+-ATPase using purified plasma membrane vesicles (PMVs) isolated from pulvinar tissues of *M. pudica*, which allow such an experimental approach.

The results obtained regarding Trp action on these different biological events are discussed in light of the comparative effects observed following treatments with 5-hydroxytryptophan (OHTrp) and by compounds linked either to Trp synthesis, namely, serine (Ser) and indole (Ind) (Kriechbaumer et al., 2008) or to Trp catabolism, namely, tryptamine (Try) (Kang et al., 2008). It can be concluded that, among amino acids, Trp triggers specific actions on early ionic exchanges at the plasma membrane level and may serve as a signal modulating more long-lasting physiological processes triggered in plants by environmental changes.

2. Materials and methods

2.1. Plant growth conditions

Seeds of *Mimosa pudica* L. and *Cassia fasciculata* Michx. were germinated in an organic compost. Seedlings and older plants were grown in the compost watered daily and were kept in climate-controlled chambers at 27 ± 0.5 °C and 65 ± 5% relative humidity. Illumination was regulated to give 16 h of light (photophase 06:00–22:00 h) provided by fluorescent tubes (mixing Osram fluora and Osram day-light types) giving a photon flux density (400–800 nm) of 80 μmol m^{-2} s^{-1} at the plant apex.

2.2. Electrophysiological measurements

The particular anatomy of the primary pulvinus of *M. pu*dica characterized by many layers of parenchyma cells surrounding the central cylinder allows impalement of a microelectrode in a well-defined kind of cell. The transmembrane potential was measured by the classical electrophysiological method using glass microelectrodes (tip diameter < 1 μm, tip resistance from 5 to 30 MΩ) drawn from capillaries provided with an internal microfibre (GC 150F15; Clark Electromedical Instruments). Briefly, a leaf was excised from the stem of 2-month-old plants and the pulvinus fixed to the bottom of a 4 ml plexiglass chamber filled with a buffered medium (10 mM MES, pH 5.5) containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM $CaCl_2$. The microelectrode was impaled into a motor cell of the abaxial ("extensor") half of the organ. Under these conditions, the calculated value of the resting transmembrane potential (Ψ_0) from 33 assays was −118 ± 4 mV (SEM). For details, see Saeedi et al. (2013) and references therein.

2.3. Measurement of pH variations

To record H^+ excretion, primary pulvini (400 mg) of 2-month-old *M. pudica* plants generally bearing 10 leaves were excised at 10:00 h. The organs were divided in transverse sections and treated following the same procedure previously described (Saeedi et al., 2013): an incubation medium composed of 0.50 mM $CaCl_2$, 0.25 mM $MgCl_2$, and variations of pH read on a pH-meter provided with combined electrodes (Futura micro-combination, Beckman Coulter) and linked to a potentiometric recorder. Compounds were added 3 h after the beginning of the experiments when the pH decrease in the bathing medium reached a plateau. To quantify the amount of mobilized protons, titration was performed with NaOH or HCl at 5×10^{-3} M onto a 2 ml incubation medium aliquot collected 3 h after application of the various tested compounds. The experiments were repeated at least 3 times.

2.4. Preparation and use of plasma membrane vesicles (PMVs)

Purified PMVs were prepared by phase partitioning of microsomal fractions from *M. pudica* primary pulvini. PMVs were frozen in liquid nitrogen and stored at −80 °C. The PMVs were put in the inside-out configuration by adding 0.05% Brij in the assay medium. In the 4 batches of vesicles used in this work, treatment with the plasma membrane H^+-ATPase inhibitor sodium orthovanadate at 0.25 mM showed that 60% of the enzyme activity can be attributed to plasma membrane functioning. Vanadate-sensitive ATPase activity of the PMVs was measured in a medium buffered with 50 mM MES/Tris at pH 6.5. Proton pumping was followed by the decrease of 9-aminoacridine absorbance at 495 nm in a medium buffered with 10 mM MES/Tris at pH 6.5. The reaction was started by the addition of 3 mM $MgSO_4$ into the medium. For details, see Saeedi et al. (2013).

2.5. Experimental procedure for observation of pulvinar movements

Plantlets of *M. pudica* bearing the first fully developed leaf were excised at 10:00 h by cutting the hypocotyl 3 cm below the cotyledons and then were dipped in distilled water contained in small plastic tubes to allow for recovery from harvesting. Leaf position determined by the angle formed by the petiole and the line prolonging the hypocotyl was measured using a transparent protractor provided with a movable pointer, allowing angle measurement by homothety preventing therefore leaves from mechanical touch. At 14:00 h (middle of the photophase), the distilled water was replaced by the tested solution buffered at pH 5.5 with 2.5 mM MES. After measurement of the initial angle (αi), the pulvini were stimulated by a shock at 1-h intervals for 4 h. Motor reactions were monitored by measuring the variation of the angle (Δα) within 10 s after the stimulation. The experiments were repeated 3 times on ten seedlings.

Fully expanded leaves of *C. fasciculata* were excised at 09:00 h from 2-month-old plants bearing 12 leaves. The cut petioles were dipped in distilled water to recover. Leaves were then transferred to test solutions buffered with 2.5 mM MES at pH 5.5 (except in particular assays dealing with pH action) for durations extending from 5 to 180 min and were exposed to darkness in the middle of the photophase of the photoperiodic cycle or in the light in the middle of the nyctophase (in this case, see **additional data S2**). At the end of the incubation period, the petiole tips were rinsed with buffer solution and the leaves were transferred to 2.5 mM MES adjusted at pH 5.5. The pulvinar movements induced by the variation in the light regime were monitored by measuring the distance between the leaflet tips with a calliper square which prevents disturbing of the leaf position by a mechanical touch. This linear measurement was then converted into angular values according to the formula $d/2 = L/2 \sin \sigma/2$ in which σ was the calculated angle, d was the distance measured between the leaflet tips and L the maximum distance measured when the leaflets were fully opened ($\sigma = 180°$). The curves represent the mean values obtained from the sum of at least three separate experiments on 8 individual leaves.

2.6. Uptake and metabolism of tryptophan

The experiments dealing with 1 mM labelled Trp uptake were carried out on the excised plantlets of *M. pudica* as described above. The

uptake of [^{3}H] Trp (final specific activity of the solution 8 kBq ml^{-1}) was measured by sectioning primary pulvini from the leaves with a razor blade at regular intervals. The excised organs were weighed and digested in a cocktail of 30% hydrogen peroxide, 70% perchloric acid and Triton X100 (2:1:1, v/v/v) for 24 h at 55 °C. Radioactivity was then counted after addition of a scintillation medium (Ecolite, ICN) by liquid scintillation spectrometry (Packard Instruments). The experiments were repeated at least three times on 10 seedlings, so that each experimental point represents observations on 30 motor organs.

In a specific assay, a set of pulvini was harvested after 2 h of treatment in the labelled Trp, before being washed twice in 30 ml cold Trp-free buffered medium. The samples were ground by mortar and pestle with 1 g quartz in 1 ml distilled water. After centrifugation, the residue was extracted again with medium and the combined extracts were vacuum evaporated. Thin-layer chromatography was performed to identify the Trp on Merck silica gel 60F254 plates (2 mm thick) developed with butanol/acetic acid/H_2O (60:20:20, v/v/v). In these conditions, the R_F value of the Trp visualized under UV light (254 nm) was 0.66. A 50-µl volume of the labelled extract was deposited onto a plate. After migration, the plate was dried and 0.5-cm-high silica gel areas were scraped off along the lane and placed into scintillation vials with 4 ml Ecolyte for counting.

2.7. Data analysis

Data were statistically analyzed according to Student-Fisher *t*-test. Depending whether the number of sample (n) was above or below 20, data were respectively given as mean ± standard deviation (SD) and mean ± standard error (SE) in the figures and tables. A significant level of $P \leq 0.05$ was selected for determination of statistical significance.

2.8. Chemicals

All chemicals were purchased from Sigma-Aldrich Chimie (St-Quentin-Fallavier, France). Solutions of Trp, OHTrp, Try, and Ser were prepared at 10 mM final concentration and diluted in 2.5 mM HEPES (pKa 3.5 and 7.5) for experiments conducted at pH 4.5 and 7.5, and in 2.5 mM MES (pKa 6.1) for experiments conducted at pH 5.5 and 6.5. The pH of the solutions was adjusted with either 0.1 M KOH or 0.1 M HCl. Control assays have shown that buffering the media did not affect the pulvinar movements. Ind at 1 mM final concentration was dissolved in absolute ethanol, the final concentration of solvent being 0.3%. A stock solution of Fusicoccin (FC) prepared at 1 mM was dissolved in 7% final concentration of methanol. It has been verified that the respective solvent concentration assayed in controls did not affect the studied processes.

3. Results

3.1. Effect of tryptophan on the transmembrane potential of motor cells

The addition of Trp to the bathing medium of *M. pudica* motor cells at pH 5.5 induced a long-lasting hyperpolarization of the motor cell membrane in a concentration-dependent manner from 0.05 (threshold value) to 1 mM. Fig. 1A presents typical recordings of the time course of the bioelectrical modifications: the lag period lasted some minutes and the subsequent rise of potential reached a plateau within 20–30 min after application of the compound. Additional quantitative data are given in Table 1. The effect of Trp completely differed from that of OHTrp (Fig. 1A) and serine (Table 1) both of which induced a depolarization indicating that the effect of Trp is specific compared to that of the other tested amino acids. FC at 10 µM strongly hyperpolarized the cell membrane, ensuring the bioelectrical responsiveness of the impaled cell.

3.2. Effect of tryptophan on the H^+-ATPase activity

In a previous study, it was shown that slices of primary pulvini spontaneously acidified their incubation medium as a result of the FC-activated proton pump activity (Saeedi et al., 2013). Fig. 1B shows the time course of the pH variation monitored in the bathing medium of the motor organ tissues after addition of the tested compounds. In the presence of Trp, the time course monitored in the controls was modified and displayed a biphasic pattern. First, Trp promoted a transient concentration-dependent acidification of the medium after a latency of approximately 5 min in the range 0.1–1 mM, lasting from 0.5 h for 0.1 mM to 1.5 h for 1 mM. Then, in the next phase, it induced a dose-dependent rise in pH lasting for 2–3 h. By contrast, 1 mM OHTrp and 1 mM Ser both induced a sharp rise of pH observed some min after application which reached an optimum level after 2.5 h for OHTrp and 1 h for Ser, before decreasing back to the level of the control in about 5 h for OHTrp and 2 h for Ser. Another pattern was seen with 1 mM Try and 1 mM Ind as they both induced a continuous pH decrease in the bathing medium starting approximately 15 min after application of the products and lasting 3 h for Try and 5 h for Ind (Fig. 1B). Additional data quantified the amount of mobilized proton following the various treatments (Table 1).

We further investigated whether a close relationship could be drawn between the H^+ fluxes monitored previously and a direct effect on the functioning of the plasma membrane H^+-ATPase activity. In this goal, additional experiments were conducted on PMVs purified from pulvinar tissues in which proton pump assays and determination of ATPase catalytic activity were carried out. As seen in Table 2, Trp did not significantly modify both components of the proton motive force showing the same behavior as Ser and OHTrp. By contrast, Try strongly inhibited the proton pumping but did not modify the catalytic activity, presenting, therefore, the characteristics of a protonophore. Ind showed a vanadate-like behavior with particular characteristics. Indeed, Ind inhibited more strongly proton pumping (86% *vs.* 60%) but inhibited ATPase activity with a lower efficiency (37% *vs.* 66%).

3.3. Characteristics of the inhibition of the osmocontractile reaction by tryptophan

The seismonastic reaction of the *M. pudica* primary pulvinus was gradually inhibited in a dose-dependent manner for Trp concentrations higher than 1 mM (Fig. 1C). The effect was clearly seen 1 h after the Trp addition and developed over the course of the experiment. After 2 h of Trp action, the inhibition was 6% at 1 mM, 18% at 5 mM and 35% at 10 mM. The time course of Trp effect can be closely paralleled with the amount of the labelled product measured in the motor organs (Fig. 1C). The Trp uptake inhibition (77%) obtained following a treatment with 10 µM of the protonophore CCCP argues for an active Trp uptake mechanism driven by a H^+ gradient-dependent process and supports the hypothesis of a Trp-H^+ symport in motor cells. TLC analysis using [^{3}H] labelled Trp indicated that after 2 h of treatment, 97% of the radioactivity was recovered at the R_f of the Trp migration spot, indicating that Trp metabolism occurred at a very low extent in these experimental conditions. Furthermore, the fact that the Trp-induced decrease of angle variation ($\Delta\alpha$) resulted from an incomplete leaf sinking and not from an incomplete leaf recovery (since the pulvini returned to their initial position after the stimulation) argues for a restriction of the K^+ efflux, while the K^+ influx was not affected. OHTrp showed no significant effect whereas Try and Ind both strongly inhibited the reaction (25 and 64% after a 2 h treatment, respectively). Of note, Ind drastically hindered the recovery as measured by the increasing α_i (inset in Fig. 1D).

As shown in Fig. 2A, Trp disturbed the dark-induced motor reactions of *C. fasciculata* leaflets. The handling of this model is simpler than that of *M. pudica* because of its low seismonastic sensitivity. The induced closure was inhibited in a dose-dependent manner at

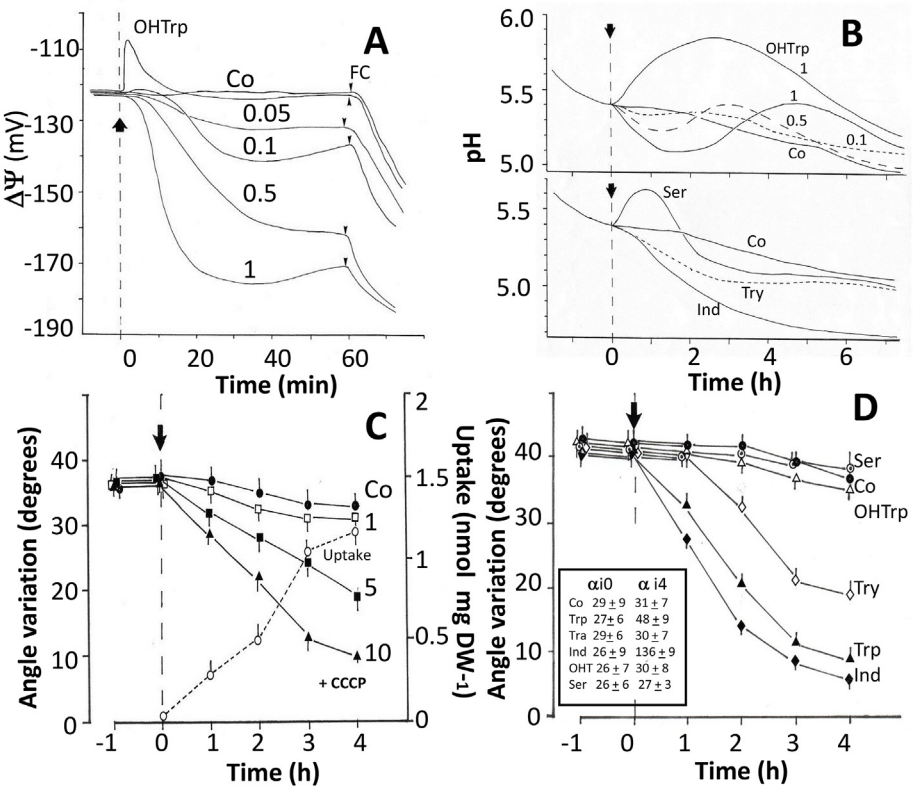

Fig. 1. Characteristics of the early events induced by tryptophan (Trp) in primary pulvini of *Mimosa pudica*. (A) Typical recordings of transmembrane potential variations in pulvinar parenchyma motor cells induced by tryptophan at various concentrations (0.05, 0.1, 0.5, 1 mM) and by 1 mM 5OH-tryptophan (OHTrp). Compounds were added at time 0 (black arrow) and 10 µM fusicoccin (FC) was added at 60 min (black arrowheads). The number of assays is indicated in Table 1 (B) Representative time courses of pH variations monitored in the bathing medium of pulvinar tissues induced by various compounds. In the upper part, effect of tryptophan at various concentrations (0.1, 0.5, 1 mM) and of 1 mM 5OH-tryptophan (OHTrp). In the lower part, effect of serine (Ser), tryptamine (Try) and indole (Ind) applied at 1 mM. The experiments were conducted at least three times with the same general result. (C) Time courses of the inhibition of the seismonastic pulvinar osmocontractile reaction following treatment with tryptophan at various concentrations expressed in mM (mean ± SE, n = 30) and time course of the amount of 1 mM labelled tryptophan absorbed by the pulvinar tissues from excised leaves (white symbols, dotted line) and effect of CCCP (△) at 10 µM on the uptake (mean ± SD, n = 4). (D) Comparative time courses of the inhibition of the seismonastic pulvinar osmocontractile reaction observed after application of serine, 5OH-tryptophan, tryptamine and indole (at 1 mM) (mean ± SE, n = 30). In inset, initial angle in degrees measured at the beginning of the treatment (αi_o) and after 4 h (αi_4) (mean ± SE, n = 30). Co: control.

concentrations higher than 0.1 mM. After 2 h of Trp action, the inhibition was 6% at 0.1 mM, 26% at 0.5 mM, 50% at 1 mM and 78% at 5 mM. It should be stressed that the *Cassia* model is more sensitive (approximately 5 times) than the primary pulvini of *M. pudica.* To determine the influence of treatment duration, 1 mM Trp was applied for 5, 15, 30, 60, and 180 min before the onset of darkness. Fig. 2B shows that a treatment as short as 5 min induced a significant hindering of the reaction. This inhibitory effect increased with treatment duration being complete after 30 min. Applied at different pH in buffered media, 1 mM Trp inhibited the dark-induced reaction at all the pH values tested but the inhibition was well-marked towards alkaline pH values (Fig. 2C). Nevertheless, the choice of pH 5.5 in 2.5 mM MES was thought to be the most suitable in our experiments as it was near the physiological apoplastic pH. Data in Fig. 2D show that the L- or D-configuration of the molecule did not influence the reaction. By contrast, a modification of the structure by the introduction of an OH-group abolished the efficiency. Try and Ind both showed an inhibitory effect in the same range of concentrations (from 0.1 to 1 mM).

As shown in the histograms of Fig. 2E, the Trp inhibition induced on dark-induced reactions was noteworthy compared to other amino acids. While most of the amino acids tested did not influence the leaflet closure, it must be noted, however, that arginine, cysteine, phenylalanine and serine induced a low but significant inhibition when applied for 3 h in this assay.

4. Discussion

4.1. Tryptophan as a multifunctional component in the plant cell life

Trp is an essential aromatic amino acid since it is one of the building

Table 1
Effect of tryptophan (Trp) at various concentrations, 5-hydroxytryptophan (OHTrp) at 1 mM and serine (Ser) at 1 mM on the characteristics of the transmembrane potential of pulvinar motor cells and on the characteristics of the proton fluxes measured in the bathing medium of excised pulvini of *Mimosa pudica*. Calculations of ΔΨ and of the amount of mobilized protons were respectively made 10 min and 90 min after application of the compounds. n: number of assays. Mean ± SD. Superscript letters indicate significant differences at the 5% probability by Student-Fisher *t*-test (*b-f*) or values not significantly different from the control (*a*). +, Hyperpolarization; -, depolarization.

Treatment	Membrane potential changes				Proton fluxes					
	[Conc]	Latency	ΔΨ	n	Latency	Acidification duration	H^+ excreted	pH rise duration	H^+ absorbed	n
	(mM)	(s)	(mV)		(s)	(min)	(nmole)	(min)	(nmole)	
Control	0	–	+1 ± 1*a*	(5)	—	—	—	—	—	(3)
Trp	0.05	480 ± 80*b*	+7 ± 7*b*	(5)						(3)
Trp	0.1	360 ± 60*c*	+24 ± 3*c*	(4)	370 ± 30*b*	60 ± 5*b*	15 ± 5*b*	80 ± 8*b*	24 ± 6*b*	(3)
Trp	0.5	200 ± 48*d*	+35 ± 8*d*	(4)	180 ± 12*c*	80 ± 5*c*	54 ± 8*c*	100 ± 10*c*	66 ± 8*c*	(3)
Trp	1	120 ± 36*e*	+48 ± 8*e*	(5)	138 ± 24*d*	96 ± 4*d*–	90 ± 8*d*–	150 ± 10*d*	105 ± 10*d*	(4)
OH-Trp	1	15 ± 1*f*	−28 ± 6*e*	(6)	240 ± 72*e*	–	–	180 ± 10*e*	135 ± 10*e*	(4)
Ser	1	19 ± 6*f*	−14 ± 5*f*	(4)	120 ± 20*f*			60 ± 5*e*	105 ± 8*d*	(3)

Table 2
Modifications of net proton movement (measured by absorbance variation from the control absorbance at 495 nm) and vanadate-sensitive ATPase activity in plasma membrane vesicles purified from *Mimosa pudica* pulvini treated with tryptophan, 5-hydroxytryptophan, tryptamine, indole, serine and vanadate. Values are mean ± SD. Into brackets, percentage of inhibition (−) or activation (+). Letters following bracket indicate significant differences at the 5% probability by Student-Fisher *t*-test (*b, c*) or values not significantly different from the control (*a*).

Product	Concentration (mM)	Absorbance variation (unit mg.prot^{-1}.min^{-1})	n	Vanadate-sensitive activity (nmol Pi.mg prot^{-1}.min^{-1})	n
Control	0	0.35 ± 0.03 *a*	8	433 ± 17 *a*	8
Tryptophan	1	0.34 ± 0.01 [-3] *a*	8	452 ± 28 [+4] *a*	8
Tryptamine	1	0.05 ± 0.03 [-86] *b*	6	444 ± 23 [+3] *a*	8
Indole	1	0.05 ± 0.02 [-86] *b*	8	272 ± 43 [-37] *b*	8
Vanadate	0.25	0.14 ± 0.06 [-60] *c*	8	147 ± 25 [-66] *c*	8
5OH Tryptophan	1	0.31 ± 0.02 [-11] *a*	4	421 ± 15 [-3] *a*	4
Serine	1	0.36 ± 0.05 [+3] *a*	3	441 ± 13 [+2] *a*	4

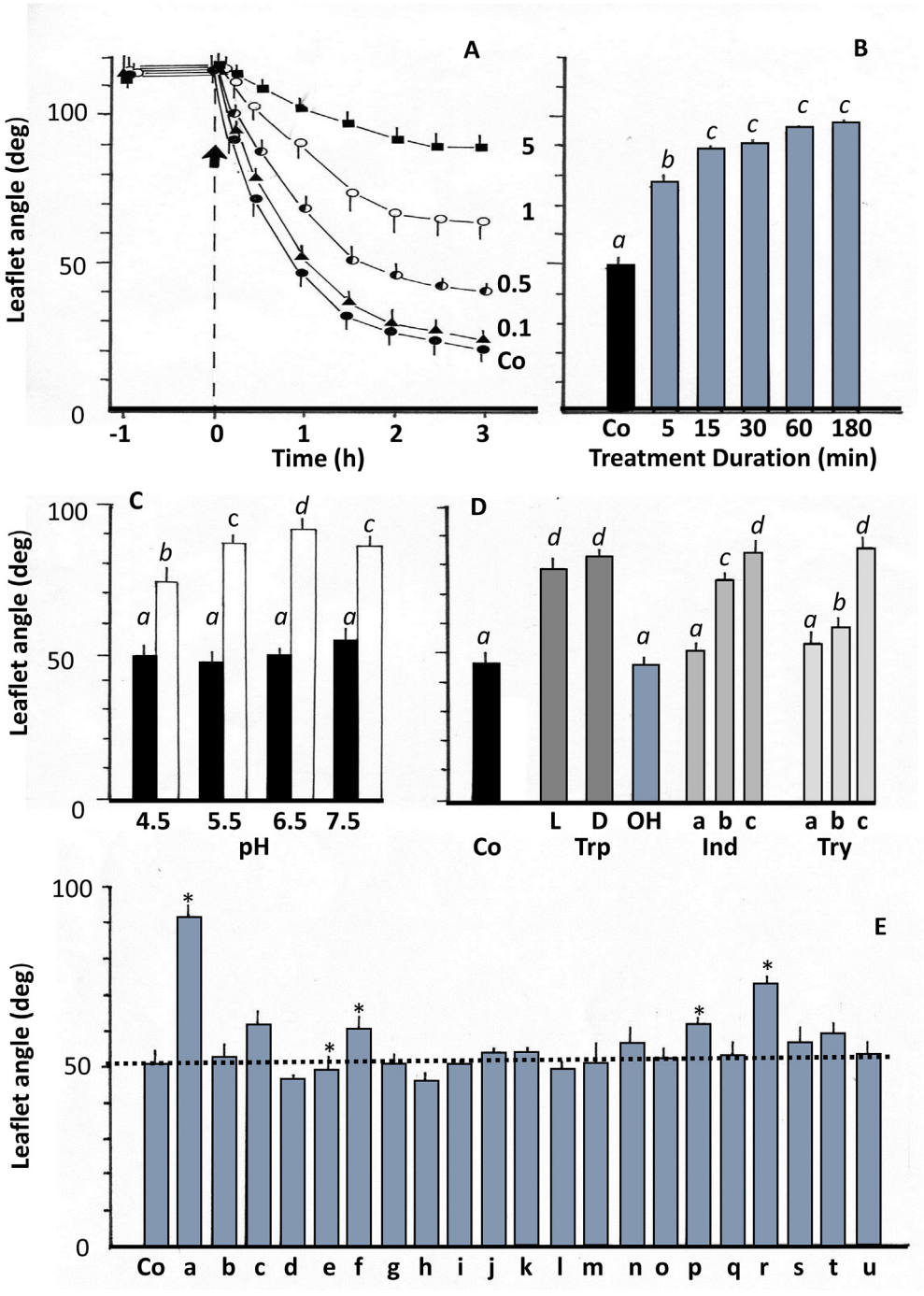

Fig. 2. Characteristics of the inhibitory effect induced by tryptophan on the dark-induced closure of *Cassia fasciculata* leaflets. (A) Modification in the time course of the closure as a function of concentration applied (from 0.1 to 5 mM) for 3 h before the darkness (black arrow). (B) Effect of 1 mM tryptophan treatment duration. (C) Effect of the pH of the incubation medium as indicated in controls (black columns) and in 1 mM tryptophan-treated leaves (white columns). (D) Effects of 1 mM, tryptophan in the L- and D-form, 1 mM 5OH-tryptophan (OH), and of indole (Ind) and tryptamine (Try) at various concentrations (a, b, c respectively 0.1, 0.5 and 1 mM). (E) Comparative effect of tryptophan with other natural amino acids (**Co**: control; **a**: tryptophan, **b**: alanine; **c**: arginine; **d**: asparagine; **e**: aspartic acid; **f**: cysteine; **g**: glutamine; **h**: glutamic acid; **i**: glycine; **j**: histidine; **k**: hydroxyproline, **l**: isoleucine**; m**: leucine; **n**: lysine; **o**: methionine; **p**: phenylalanine; **q**: proline; **r**: serine**; s**: threonine; **t**: tyrosine; **u**: valine). Otherwise indicated, the calculations were made 1 h after the start of darkness on sets of leaflets treated for 3 h with the compounds prepared in buffer at pH 5.5. Mean ± SE; n = 24. Superscript letters and asterisks in (E) indicate significant differences at 5% probability level by Student-Fisher *t*-test (*b-d*) or values not significantly different from the control (*a*).

blocks for protein synthesis and the source of many important compounds, particularly melatonin and nicotinamide adenine dinucleotide in animals (Moffett and Namboodiri, 2003) and IAA and phytoalexins in plants (Zhao et al., 1998). In plants, Trp has been shown to affect various long-term physiological processes such as organ growth (Jing et al., 2009), responses to stress and pathogen elicitors (Zhao et al., 1998; Bednarek et al., 2009) and tolerance of heavy metals (Sanjaya et al., 2008). Nevertheless, little has been reported about the mechanism by which Trp affects growth and development at the cellular and molecular level.

In this work, we focused our investigations on the early events triggered by Trp on plant cell functioning. Thus, we determined that Trp is rapidly recognized at the cell membrane level and induced characteristic and specific short-term cellular events by disturbing processes involved in rapid changes of membrane permeability to water and ions which are the early processes allowing osmocontractile pulvinar reactions.

4.2. Tryptophan as a putative signal molecule

The proposal that Trp is rapidly recognized at the plasma membrane level is greatly supported by the observation of the large concentration-dependent membrane hyperpolarization triggered a few minutes after application of the compound, indicating early modifications in ionic fluxes. Ion fluxes through the plasma membrane are common and early events reported as a prerequisite for subsequent cell reaction (Colcombet et al., 2009; and references therein). The early recognition of Trp at the membrane level can be analyzed according to three phases.

(a) The occurrence of a lag period indicates that the recognition of the compound at the membrane triggers a chain reaction requiring a lapse of time to express the bioelectrical response and suggests therefore that Trp does not act directly on particular membrane effectors. A complementary study should now be carried out to identify the steps triggered before the onset of the hyperpolarization with a particular focus on the possible involvement of a Ca^{2+} signaling process. Indeed, Ca^{2+} is considered to be a ubiquitous internal second messenger that regulates various cellular processes acting through intracellular [Ca^{2+}] increase in response to various environmental stimuli (Knight, 2000) or following recognition of elicitors (Lecourieux et al., 2002).

(b) The membrane hyperpolarization could result either from cation effluxes or anion influxes. However, close lag times in $\Delta\Psi$ and ΔpH variations (Table 1) and similar time courses of the bioelectrical and the pH variations noted in the bathing medium of pulvini strongly suggest that proton efflux may be the main process sustaining the hyperpolarization (Fig. 1). However, it can be concluded from experiments performed on PMVs that Trp did not increase the activity of the H^+-ATPase by directly acting on the pump (Table 2) when compared to FC that also hyperpolarized the pulvinar cells and activated the H^+-ATPase activity in PMVs (Moyen et al., 2007). This outcome clearly demonstrated different modes of action for these two compounds. Obviously, the membrane potential variations triggered by Trp strongly differed from those evoked by OHTrp (Fig. 1) and glycine (Roblin et al., 2018) on *M. pudica* motor cells and by various amino acids on *Lemna gibba* cells (Fisher and Lüttge, 1980) and on barley mesophyll cells (Felle and Zimmermann, 2007). In these latter cases, potential variations appear as depolarization spikes after a latency period reduced to seconds and followed by the return to the basal potential in approximately 30 min. Felle and Zimmermann (2007) monitored the temporal sequence of the ionic migrations showing that Ca^{2+} was the first ion to move and was required to trigger the subsequent Cl^- and K^+ fluxes. As shown in Fig. 1B, the time course of the proton translocation appears complex possibly because of a dual role of Trp. In the first minutes following its application, Trp induced a continuous H^+ efflux from cells reminding of an auxin-like effect, as observed with IAA (Bourbouloux et al., 1994) and, in a second step, the observed H^+ influx may be related to its uptake (Fig. 1C) *via* a H^+/substrate cotransport mechanism evidenced in amino acid transport by plant cells (Delrot et al., 2001). The recorded curve represents the resultant of the bidirectional proton migration (See additional data S1).

(c) By comparing the time course of bioelectrical variations and proton fluxes, it should be stressed that both the plateau and the repolarization phase did not coincide with the beginning of the pH increase recorded in the bathing medium, suggesting that H^+ influx into the cells may not be the only driving component involved in this latter process. Considering the ionic species (Cl^- and K^+) underlying the action potential (Samejima and Sibaoka, 1980), it can be expected that these pivotal ions may also contribute to the early bioelectrical modifications triggered by Trp. The time course of pH variations also evidenced that H^+ fluxes were disturbed in a long-lasting manner (for hours) and, could therefore be considered to be involved in the regulation of long-term processes. In this direction, H^+ fluxes were suggested to be involved in elicitation processes (Schaller and Oecking, 1999) and cytosolic acidification to act as a second messenger, mediating MAP kinase activation in the response of plant cells to various stresses (Tena and Renaudin, 1998).

By considering its electrogenic properties shown here, Trp may constitute an intermediary in long distance signaling by inducing an electrical stimulus through its transport over long distance in plant as other amino acids. Thus, Trp may create a direct cell-to-cell electrical conduction following its accumulation and therefore may be implicated in plant resistance processes. Indeed, action potentials or wound-induced potential variations are able to transmit information over long distances (Fromm and Lautner, 2007) and to induce the expression of pathogen response genes (Fisahn et al., 2004). However, Trp is unlikely to be directly involved in the Hypersensitive Response as revealed by the assay of Ueno et al. (2003) showing that following infection with *Magnaporthe grisea,* H_2O_2 formation and subsequent lesions on the leaves were due to an accumulation of Try resulting from increased Trp synthesis.

4.3. Tryptophan as an osmotic agent

The data obtained on the osmocontractile reaction of pulvinar cells (Figs. 1 and 2) indicated that the application of Trp modified bulk ionic migration and the associated water movement by considering the well documented mechanism driving motor cell shrinkage following the application of shock and darkness. Large K^+ and Cl^- effluxes (Samejima and Sibaoka, 1980; Moran, 2007) induced a rapid decrease in cell turgor. The ionic migrations are likely regulated by the activity of K^+ and Cl^- channels and the subsequent water movement allowed by the activity of aquaporins present in motor cell membranes (Fleurat-Lessard et al., 1997; Temmei et al., 2005; Moran, 2007). As seen in Fig. 1C, there is a close relationship between the extent of inhibition of the motor reaction of *M. pudica* pulvini and the amount of Trp available in the vicinity of the motor cells. The fact that the sole drooping movement was affected but not the recovery argues for a process involving a vectorial water influx, resulting from the hindering of the K^+ efflux. This hypothesis is corroborated by the Trp-induced inhibitory effect on the dark-induced leaflet movement of *C. fasciculata* (Fig. 2A), and by the Trp-promoting effect observed on the light-induced leaflet movement (See Supplementary data 2). However, it should be stressed that there is no direct link between motile reaction (and the underlying K^+ and water fluxes) and the process of Trp uptake, since the uptake of amino acids is stimulated at an acidic pH (Otsiogo-Oyabi and Roblin, 1985; and reference therein) whereas leaflet movement was promoted at neutral/alkaline pH levels (Fig. 2C). These remarks concerning an increase in water availability in the cells are in accordance with some data showing that Trp increased the water content of wheat and maize cells and reduced the effect of drought in maize (Rao et al., 2012; and references therein).

4.4. The effects induced by tryptophan are specific in several aspects

Many previous studies have claimed that the physiological responses seen after an exogenous application of Trp may result from the conversion of Trp into IAA (Abdel-Monem et al., 2010; and references therein). However, other works have also shown that Trp exerts a direct effect on plant cell physiology in processes as different as embryo germination in *Triticum aestivum* (Morris et al., 1988) and on cell expansion in *Arabidopsis* mutants (Jing et al., 2009). A major point to ascertain in this study was that Trp acted itself and that the effects may not be attributed to products of its metabolism, in particular to the possible synthesis of IAA. On the motor cell model, Trp induced similar physiological effects as IAA molecules regarding the induction of membrane hyperpolarization, the inhibitory effect on the shock-induced reaction (unpublished result), the inhibitory effect on the dark-induced reaction and the promoting effect on the light-induced reaction (Everat-Bourbouloux et al., 1990). It should be emphasized that Trp is less effective than IAA, since 1 mM of the amino acid was required to trigger reactions of similar amplitude as those obtained with 10 μM IAA. However, Trp triggers a complex biphasic change in H^+ fluxes as previously described (Fig. 1B) in contrast with IAA and FC which continuously acidified the bathing medium of motor organs (Bourbouloux et al., 1994). This pattern of Trp responses looks similar to those obtained with 2,4-D. Indeed, the herbicide also induced at first a pH acidification followed by a pH rise in relation to its phenolic structure (Moyen et al., 2007), indicating that the proton exchanges are modulated according to the effect triggered by each moiety of the molecular complex. The fact that Trp is the active component was first supported by the short latency (within min) occurring between the Trp application and the observation of the cell responses which tended to rule out a putative effect of a compound resulting from the metabolism of Trp by the motor cells. Such eventuality was disproven with the use of [^{3}H] labelled Trp combined with TLC analysis, which evidenced a very low rate of metabolism during the first 2 h of Trp uptake by the tissues.

We have also characterized the specific effect of Trp by including in our study the effects of compounds involved in its synthesis (Ind and Ser) and its metabolism (Try). Clearly, we have shown that these compounds acted through mechanisms which are fundamentally different from those underlying Trp effects, although they can result in the same final osmoregulated reaction of the pulvinar cells. Thus, we showed that Try acted on membrane functioning through a protonophore effect and that Ind acted directly on both processes of the H^+-ATPase activity as observed on PMVs. In previous reports, we showed that disturbing the H^+-ATPase functioning resulted in modification of the motile reactions (Saeedi et al., 2013). However, an additional effect has to be considered, since a sole protonophore action would induce a pH alkalinisation in the bathing medium of motor tissues as observed with dinitrophenol (Saeedi et al., 2013). In contrast, Try induced a continuous acidification of the medium, thus possibly meaning that Try induced a modification of the membrane permeability leading to a leakage of protons. This continuous H^+ efflux may be one step in a chain reaction leading to a necrotic effect linked to H_2O_2 formation as observed in rice infected with the fungus *Magnoporthe grisea* (Ueno et al., 2003). In the case of Ind, stronger ionic disorders have to be considered as indicated by the also unexpected medium acidification and by the observation of the large gradual increase in initial angle in course of the assay (see inset in Fig. 1D). These last data may indicate that Ind drastically disturbed cell functioning and led to a necrosis of the pulvinar cells upon increased treatment duration that requires further microscopic observations to identify the wounded cell compartments.

It can further be highlighted that, compared to many other amino acids, Trp affects in a specific way the electrophysiological events, the proton exchange status and more generally the ionic migrations as indicated by the data on the stimulus-induced motile reactions. Of note, the Trp-induced membrane potential changes strongly differed from those induced by the structurally related compound OHTrp which triggered a spiky depolarization of the motor cell membrane identical to that evoked by glycine application (Roblin et al., 2018). Furthermore, differences were also apparent in the shock- and dark-induced reactions: Trp strongly inhibited these reactions while glycine promoted them (Otsiogo-Oyabi and Roblin, 1984). Interestingly, arginine, cysteine, phenylalanine and Ser also inhibited the dark-induced reactions in *C. fasciculata* leaflets when observed after 3 h of action (Fig. 2E). The case of Ser is interesting since this amino acid combines with Ind to form Trp (Kriechbaumer et al., 2008), so that we can suppose that the effect observed 4 h after the application on the leaves could result from Trp synthesis. Altogether, these data prompted us to investigate further whether these particular amino acids may play particular roles in the plant cell functioning. In this way, a recent study concerning cysteine has confirmed that this amino acid also induced characteristic effects on the processes studied here (Roblin et al., 2018).

5. Conclusions

The data highlight that some amino acids may have specific roles outside of a simply metabolic role as demonstrated by the characteristic properties evoked by Trp in plant cell physiology. Further experiments should be devoted to the underlying mechanisms of these non-metabolic effects, since some actions of Trp did not correspond to a classical mode of action as compared to other amino acids. In particular, the mechanism sustaining the variation of membrane permeability to water and ions should be investigated further, as it does not result from an activation of the proton pump activity, raising the hypothesis of a direct action on ionic channels (K^+ channels in particular). Another pivotal line of research would be focused on the processes linked to the membrane sensing of Trp. The perception at the membrane level may be achieved through a specific receptor as shown for IAA (Woodward and Bartel, 2005) or may be induced through a transceptor (Dinkeloo et al., 2018) linking transport function and signaling in the cells.

Author contributions

Christelle Moyen performed the electrophysiological studies, Fabienne Dédaldéchamp participated to the assays on the metabolization experiments, Pierrette Fleurat-Lessard and Gabriel Roblin carried out the observations on leaf reactions and the analysis carried out on PMVs. All the authors participate in the writing of the manuscript and approved the final version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.006.

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