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Copper-induced early responses involve the activation of Transient Receptor Potential (TRP) channels, release of amino acids, serotonin and adrenalin, and activation of homologs of glutamate, adrenalin and serotonin receptors in the marine alga *Ulva compressa*

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ABSTRACT

It has been previously shown that *U. compressa* cultivated with 10 μM copper showed the activation of Transient Receptor Potential (TRP) channels at 4, 8 and 13 min allowing copper ions entry and transient depolarizations. Here, the alga was incubated with copper and with inhibitors of human TRP channels that were incorporated just after copper addition (time 0), and after 1 and 2 min of copper exposure, and membrane depolarizations were detected at 4, 8 and 13 min. Copper induced the activation of a TRP A1/C5/M8 at time 0, a TRP A1/C4/M8/V1 after 1 min, and a TRP A1/C5/M8, after 2 min of copper exposure. In addition, EGTA, a specific calcium chelator, did not inhibit depolarization events when added at time 0, and after 1 and 2 min, whereas bathocuproine, a specific copper chelator, inhibited transient depolarizations at 4, 8 and 13 min. In addition, inhibitors of human GluR of NMDA type, added at time 0, and GluR of AMPA/KA types, added after 1 and 2 min as well as inhibitors of serotonin and adrenalin receptors added after 2 min inhibited depolarizations at 4, 8 and 13 min. Furthermore, amino acids, serotonin and adrenalin were release to culture medium in response to copper excess. Thus, copper ions induces the activation of TRP channels allowing extracellular copper ions entry leading to transient depolarizations, release of aminoacids, serotonin, and adrenalin, and activation of homologs of glutamate, adrenalin and serotonin receptors which are involved in the activation of TRPs and depolarizations observed at 4, 8 and 12 min of copper exposure.

1. Introduction

Transient Receptor Potentials (TRP) are ionotropic cation channels present in mammalian, insect and nematode and algae, but not in plants [1]. In mammals, TRP channels are activated by environmental stimuli such as temperature, pH, osmolarity, pressure, injury and pungent compounds, or by intracellular stimuli as inositol-phosphates, diacylglycerol, prostaglandines, cannabinoids and reactive oxygen species (ROS), among others [1]. In animals, there are 28 TRP members classified in six families: ankyrin (A), canonical (C), melastatin (M), mucolipin (ML), polycystin (P) and vainilloid (V). In nematodes, there is an extra member corresponding to the nompC (N). TRP channels exist as homo- and hetero-tetramers and each monomer presents an intracellular N- and C-terminal domain [2,3]. In addition, TRP monomers show six trans-membrane domains (TMD) and the channel pore is located in TMD 5 and 6 [1]. Some TRP channels are highly selective for calcium, such as TRPC1/C5, TRPV5/V6 and TRPM2, but others are more permeable to sodium, potassium and magnesium ions, such as TRPA1 and TRPV1 [4]. TRPs can be homo- or hetero-tetrameric channels constituted by monomers of TRP A, C, M and/or V [2,3]. In humans, TRPs can be modulated by phosphorylation of the N- and/or C-terminal domains [5] and by metals such as zinc, copper, cadmium, lead, mercury and nickel ions [6,7,8,9,10].

Glutamate receptors (GluR) can be ionotropic (iGluR) or metabotropic (mGluR) and they are present in mammals, insects, nematodes and plants [11]. In mammals, there are 16 members of iGluR and eight members of mGluR [12]. iGluR are grouped into three families according to their agonists: *N*-methyl D-aspartate (NMDA), 3-hydroxy 5-methyl 4-isoxazolepropionate (AMPA) and kainate (KA) [11]. iGluRs present a long extracellular N-terminal domain, three TMD and an intracellular C-terminal domain [13]. iGluRs are hetero-tetrameric receptors that are activated by the simultaneous binding of L-glutamate

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(glu) to two subunits, and L-glycine (gly) to the other two subunits [11]. In addition, the aminoacid D-serine (D-ser) is a co-activator that binds to the gly site but only in the NMDA iGluR [12]. In addition, animal iGluR can be modulated by lead and nickel ions [14,15].

In plants, homologs to human iGluR have been found and they appeared to be more closely related to NMDA type iGluR [16]. *Arabidosis thaliana* iGluRs are composed by 20 members classified in three families: GluR1, GluR2 and GluR3 [17]. GluR3.3 is activated by six amino acids corresponding to glu, gly, L-alanine (ala), L-serine (ser), L-asparagine (asn) and L-cysteine (cys) [18]. In addition, GluR1.4 is activated by seven amino acids, mainly hydrophobic, such as L-methionine (met), L-tryptophan (trp), L-phenylalanine (phe), L-leucine (leu), L-tyrosine (tyr), asn and L-threonine (thr) [19]. Moreover, D-ser can activate plant iGluRs, allowing calcium entry, and the latter is required for pollen tube growth [20]. Phylogenetic analyses suggest that GluR originated in algae, since they are present in microalgae and mosses, arranged as non-tandem genes, and in higher plants, in contrast, they are arranged mainly as tandem genes [21].

Indoleamine (serotonin, melatonin) and catecholamine (dopamine, adrenalin and noradrenalin) receptors are present in neurons in the human central nervous system (CNS) and their principal role is to modulate the activity of glutamatergic neurons [22,23]. Indoleamines are synthesized from L-trp and catecholamines from L-tyr and; in turn, L-tyr is synthesized from L-phe by the enzyme phenylalanine hydroxylase [24]. For a long time, it was assumed that these neurotransmitters were exclusively released in the CNS of vertebrates but recently it fit has been found that plants also synthesize indoleamines and catecholamines [25]. Indeed, rice accumulates serotonin in the vascular parenchyma and this accumulation delays senescence [26,27]. Additionally, Fabaceae plants contain significant amounts of dopamine [28] as well as bananas, coffee, green tea, tomatoes and walnuts [29]. In addition, it has been demonstrated that the marine macroalga *Ulvaria obscura* contains dopamine, apparently involved in defence against herbivores [30]. Dopamine is released during rehydratation after desiccation induced by low tide [31], and has been postulated to inhibit growth of other macroalgae and invertebrate larvae [32].

Concerning TRP channels in microalgae, it has been shown that the green microalga *Chlamydomonas reinhardtii* exhibits at least eight TRP channels, and two of them have been observed to be functional [33,34]. Particularly, Cr-TRP1 is a mosaic TRP since it contains domains having homology with human TRPM and TRPC, and with nematode TRPN [34]. Moreover, Cr-TRPV1 channel was cloned and expressed in human HEK-293T; it was observed to permeate monovalent cations and be inhibited by BCTC, an inhibitor of TRPM8 and TRPV1 [34]. In addition, Cr-TRP11 was shown as a mechano-sensory channel located in the flagellum that participates in the avoidance reaction [33].

Concerning TRPs and macroalgae, it has been shown that the marine macroalga *Ulva compressa* exposed to 10 μM of copper showed the activation of potential TRP channels which have been inhibited by different specific inhibitors of human TRPs [35,36]. Copper-induced activation of these TRPs leads to copper ions entry and transient depolarizations at 4, 8, 13, 80 and 86 min, and at 5 and 9 h of metal exposure [35,36]. In addition, it was shown that the latter depolarizations mediate the activation of Voltage-Dependent Calcium channels (VDCC) at 2, 3 and 12 h of copper exposure leading to extracellular calcium entry and intracellular calcium release from endoplasmic reticulum (ER) [36,37].

In this work, we analyze early events occurring before TRP activation at 4, 8 and 13 min in *U. compressa*, specifically related to the activation of additional TRPs and homologs of glutamate, indoleamine and catecholamine receptors. In addition, the eventual release of amino acids, indoleamines and catecholamines to the culture medium was also analyzed as well as the possible involvement of protein kinases such as CaMK, PKA, PKC and PKG in the activation of TRP channels and homologs of glutamate, serotonin and adrenalin receptors.

2. Methods

2.1. Algae and seawater collection and pre-experimental treatments

Ulva compressa was collected in Cachagua (32° 34′S), a site in central Chile with no history of metal pollution [38]. Sampling was performed during autumn, winter and spring of 2016. The algae were transported to the laboratory inside a cooler with ice, in sealed plastic bags. Algae were rinsed three times with filtered seawater, cleaned manually and sonicated twice for 2 min using an ultrasound bath Branson 3200 (Danbury, CT, USA) in order to aid removing epiphytic bacteria and epibionts. Seawater was obtained from Quintay (33° 12′S), a pristine site in central Chile, filtered through 0.45 and then 0.2 μm pore size membranes and stored in darkness at 4 °C until experiments.

2.2. Inhibitors

Inhibitors were purchased from Sigma-Aldrich (St Louis, MI, USA). Specific inhibitors of human TRP channels were: HC-030031, an inhibitor of TRPA1 [39]; ML204, an inhibitor of TRPC4 and TRPC5 [40]; SKF96365, an inhibitor of TRPC5 and TRPM8 [41]; M8B, an inhibitor of TRPM8 [46], and capsazepin (CPZ), an inhibitor of TRPV1 [42]. Specific inhibitors of human glutamate, indoleamine and catecholamine receptors were: kynurenic acid (KYNA), an inhibitor of glutamate receptors of NMDA/AMPA/KA types [43]; CNQX, an inhibitor of glutamate receptor of AMPA/KA types [44]; DNQX, an inhibitor of glutamate receptor of AMPA/KA type [45]; propranolol, an inhibitor of adrenalin receptor [46]; ketanserin, an inhibitor of serotonin receptor [47]. Chelating agents were: EGTA, a specific chelator of calcium ions, and bathocuproine, a specific chelator of copper ions [48]. Inhibitors of protein kinases were: K5720, an inhibitor of cAMP-dependent protein kinase A (PKA) [49]; chelerytrin, an inhibitor of calcium and diacylglycerol-dependent protein kinase C (PKC) [50]; KT5823, an inhibitor of cGMP-dependent protein kinase G (PKG) [51]; and KN62, an inhibitor of calcium/calmodulin-dependent protein kinases (CaMK) [52].

2.3. Treatment with inhibitors and detection of transient depolarizations

Depolarization events were detected by confocal microscopy as described in Gómez et al. [35,36]. Briefly, each treatment consisted in three algal laminae that were first incubated for 10 min with the fluorofor DiOC2, to detect depolarization events, or with Fluo-3 AM, to detect intracellular calcium increases. After, the algae were exposed to 10 μM copper, or 20 nM of each inhibitor and 10 μM copper; inhibitors were incorporated after copper addition (time 0), or applied after 1 and 2 min of copper exposure. For treatments with copper- and calcium-chelating agents, 10 μM copper were incorporated; then, EGTA or bathocuproine sulphonate were added just after copper addition (time 0) or after 1 and 2 min of copper addition. In each ach treatment, algae were visualized using a confocal microscope LSM 510 (Carl Zeiss, Oberkochen, Germany) for 15 min. The green fluorescence of DiOC2 was detected with an emission of 488 nm produced by an argon laser with a filter of 505–530 nm. The intensity of green and red fluorescence of chloroplasts was quantified in each lamina using LSM510 software. The fluorescence intensity in each simple was normalized using chloroplast autofluorescence.

2.4. Detection of aminoacids, indoleamines and catecholamines

The alga (250 mg) was incubated in 1 mL of seawater in triplicates, and copper was added at final concentration of 10 μM. An aliquot of the supernatant was recovered after 0 of copper exposure or after 0.5 min until 5 min of copper exposure. Aminoacids, indoleamines and catecholamines were detected by HPLC as described by Bartolomeo and Maisano [53]. The supernatant (20 μL) was added to 10 μL of a reaction mixture containing 25 mM sodium borate buffer pH 9.3, 17.5 mM β-mercaptoethanol, 0.75 mg mL^{-1} O-phtaldehyde (OPA) and 75% v/v methanol. An aliquot of