Copper-induced intracellular calcium release requires extracellular calcium entry and activation of L-type voltage-dependent calcium channels in *Ulva compressa*

Alberto González, María de los Ángeles Cabrera, Macarena Mellado, Susana Cabello, Sebastián Márquez, Bernardo Morales and Alejandra Moenne*

Universidad de Santiago de Chile; Departamento de Biología; Santiago, Chile

**Keywords:** copper, calcium, phospholipase C, voltage-dependent calcium channels, marine alga, *Ulva compressa*

**Abbreviations:** cADPR, cyclic ADP-ribose; IP$_3$, inositol 1, 4, 5 triphosphate; NAADP, nicotinic acid-adenine dinucleotide phosphate; CICR, calcium-induced calcium-release; VDCC, voltage-dependent calcium channels; CaMs, calmodulins; CDPKs, calcium-dependent protein kinases; MTP, mitochondrial transition pore; TRP, transient receptor potential

The marine alga *Ulva compressa* exposed to 10 $\mu$M copper showed a triphasic increase of intracellular calcium with maximal levels at 2, 3 and 12 h involving the activation of ryanodine-, Ins(1,4,5)P$_3$- and NAADP-sensitive calcium channels. In order to analyze the requirement of extracellular calcium entry for intracellular calcium release as well as the activation of voltage-dependent calcium channels (VDCC) and phospholipase C, *U. compressa* was treated with EGTA, a non-permeable calcium chelating agent, with verapamil, nifedipine and diltiazem, inhibitors of L-type VDCC, and with neomycin and U73122, inhibitors of phospholipase C. The release of intracellular calcium was partially inhibited with EGTA at 2 and 3 h and completely inhibited at 12 h of copper exposure and decreased with inhibitors of L-type VDCC and phospholipase C. Thus, copper-induced intracellular calcium release depends on calcium entry and activation of L-type VDCC and phospholipase C. An integrative model of copper-induced cellular responses in *U. compressa* is presented.

In animals and plants, physiological and pathological stimuli induced the release of intracellular calcium with characteristic spatiotemporal patterns which depends on the nature and intensity of the stimulus and the cell type. In animals, the release of intracellular calcium requires the activation of ryanodine-sensitive channels and inositol 1, 4, 5-triphosphate (IP$_3$)-sensitive channels located in endoplasmic reticulum (ER) as well as the activation of NAADP-sensitive calcium channels in lysosome-like acidic organelles. The activation of ryanodine-sensitive calcium channels depends on the synthesis of cyclic ADP-ribose (cADPR) derived from NAD and the entry of extracellular calcium. In addition, the activation of Ins(1,4,5)P$_3$-sensitive calcium channels depends on the release of Ins(1,4,5)P$_3$ from plasma membrane fatty acids by phospholipase C and the entry of extracellular calcium. The mechanism involving extracellular calcium entry to induce intracellular calcium release is designed as calcium-induced calcium release (CICR) mechanism and it governs the activation of ryanodine- and Ins(1,4,5)P$_3$-sensitive calcium channels. Moreover, the release calcium through Ins(1,4,5)P$_3$-sensitive calcium channels normally precedes the activation of ryanodine-sensitive canals and, thus, contributes to the activation of the latter. On the other hand, the activation of NAADP-sensitive calcium channels requires the synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) derived from NADP but their activation is regulated by luminal calcium and acidic pH in lysosome-like stores. In addition, the release of calcium through NAADP-sensitive channels contributes to the activation of ryanodine- and Ins(1,4,5)P$_3$-sensitive calcium channels. Furthermore, the increase in intracellular calcium activates phospholipase C located in the plasma membrane which is involved in the release of Ins(1,4,5)P$_3$ from fatty acids. Regarding the activation of calcium channels located in the plasma membrane, it has been shown that intracellular calcium released through NAADP-sensitive channels can activate non-selective cation channels in the plasma membrane favoring its depolarization. In addition, the increase in intracellular calcium can activate voltage-dependent calcium channels of the plasma membrane via calmodulins (CaMs). In animals, the entry of extracellular calcium requires the activation of voltage-dependent calcium channels (VDCC) and/or...
In this sense, VDCC are classified as L-, N-, P-, Q-, R- and T-types and they are mainly ionotropic. In particular, L-type VDCC are inhibited by dihydropyridines such as as nifedipine, phenytoin and verapamil, neomycin and diltiazem and with 10 μM copper for 2, 3 and 12 h. In addition, the non-permeable calcium chelating agent EGTA was added to control medium 30 min before each intracellular calcium increase. The level of intracellular calcium decreased with EGTA in 64, 73 and 100% at 2, 3 and 12 h, respectively. Thus, copper-induced intracellular calcium increases depend on extracellular calcium entry.

Furthermore, the alga was cultivated in control condition, with 10 μM copper or with the inhibitors of L-type VDCC nifedipine, verapamil and diltiazem and with 10 μM copper for 2, 3 and 12 h (Fig. 2). The level of intracellular calcium increased in 47, 77 and 85% with nifedipine, in 64, 82 and 98% with verapamil, and in 86, 100 and 97% with diltiazem, respectively. Thus, copper-induced calcium increases depend on activation of L-type VDCC.

In marine algae, it has been shown that Ulva compressa Chlorophyta displays a copper-induced triphasic release of intracellular calcium that involves the activation of ryanodine-, Ins(1,4,5)P3- and NAADP-sensitive calcium channels. In addition, the increase in intracellular calcium that involves the activation of ryanodine-, Ins(1,4,5)P3- and NAADP-sensitive calcium channels was located in ER, as in terrestrial plants. Moreover, the increase in intracellular calcium induced the synthesis of hydrogen peroxide and nitric oxide (NO) levels in mitochondria and chloroplasts and, in turn, hydrogen peroxide and NO activate calcium release from ER indicating a mutual influence (cross-talk) among these intracellular signals. Furthermore, the increase in intracellular calcium activates gene expression via CaM signals and calcium-dependent protein kinases (CaDPKs). In this work, we analyzed whether intracellular calcium release depends on intracellular calcium entry as well as on activation of L-type voltage-dependent calcium channels and phospholipase C. In addition, an integrative model of copper-induced cellular responses in Ulva compressa is presented.

The algae was cultivated in seawater without copper addition (control condition) or with 10 μM copper for 2, 3 and 12 h. In addition, the non-permeable calcium chelating agent EGTA was added to culture medium 30 min before each intracellular calcium release. In this sense, it is important to point out that intracellular calcium release was only partially inhibited by EGTA at 2 and 3 h of copper exposure whereas it was completely inhibited at 12 h. This suggests that the last increase in intracellular calcium is mainly dependent on calcium released through ryanodine- and IP3-sensitive calcium channels since these calcium channels require extracellular calcium entry for their activation. In addition, the partial inhibition of copper-induced calcium release at 2 and 3 h suggests an important contribution of NAADP-sensitive calcium channels since this type of calcium channels does not require extracellular calcium entry. Regarding the activation of ryanodine- and NAADP-sensitive calcium channels in Ulva compressa Chlorophyta and in terrestrial plants.

Figure 1. Level of intracellular calcium in Ulva compressa cultivated in control condition and with 10 μM copper for 2, 3 and 12 h or treated with 1 mM EGTA added 30 min before 2, 3 and 12 h and with 10 μM copper. Calcium level is expressed as the ratio of fluorescence of Fluo 3 and autofluorescence of chloroplasts. Bars represent mean values of three independent experiments ± SD. Different letters indicate a significant difference (p < 0.05).
U. compressa, it will be interesting to determine whether their activation requires the synthesis of cADPR and NAADP, as it has been shown in animals.

On the other hand, copper-induced intracellular calcium release requires the activation of L-type VDCC located in the plasma membrane. In this sense, there are only few examples of direct activation of calcium channels in the plasma membrane by copper or other heavy metals. An example is the activation of the transient receptor potential (TRP)A1, a damage-sensing receptor present in somatosensory neurons, by copper, cadmium and zinc. In this case, zinc enters into the cell through constitutively active TRP A1 channels and binds to an intracellular domain rich in cysteines and histidines activating the channel and increasing calcium entry. Thus, it is possible that L-type VDCC located in the plasma membrane of U. compressa may have a similar intracellular or extracellular domain that could bind copper leading to its activation. However, this kind of mechanism should induce a rapid activation of the channel which contrasts with the slow release of intracellular calcium detected in U. compressa at 2, 3 and 12 h of copper exposure. In this sense, it is possible that the synthesis of cADPR and/or NAADP is required for the activation of ryanodine- and NAADP-sensitive calcium channels allowing the intracellular calcium increase which, in turn, may promote the activation of VDCC located in the plasma membrane via CaMs, as it has been observed in neurons where the increase in intracellular calcium promoted the activation of non-selective cation channels in the plasma membrane and in cardiac cells where the increase in intracellular calcium activates of L-type VDCC in the plasma membrane via CaMs.

Based on present results and previous work, an integrative model of copper-induced cellular responses is presented (Fig. 4). The model shows a potential direct activation of plasma membrane VDCC by copper and the activation of phospholipase C by calcium entry. The activation of phospholipase C may promote Ins(1,4,5)P₃ release from plasma membrane fatty acids which, in turn, activates Ins(1,4,5)P₃-sensitive calcium channels in ER. In addition, extracellular calcium entry participates in activation of Ins(1,4,5)P₃- and ryanodine-sensitive calcium channels and a copper-induced synthesis of NAADP may occur leading to the activation of NAADP-sensitive channels. The activation of ryanodine-, Ins(1,4,5)P₃- and NAADP-sensitive calcium channels determines the increase of intracellular calcium which may favor its entry into mitochondria and chloroplasts. The increase of calcium concentration in organelles stimulates the synthesis of...
hydrogen peroxide and nitric oxide (NO) which may favor their exit to the cytosol. It is important to mention that hydrogen peroxide leaves mitochondria through mitochondrial transition pore (MTP) (A. González, unpublished results). Once in the cytosol, hydrogen peroxide and NO activate calcium channels in ER and the increase in intracellular calcium is transduced by calmodulins (CAMs) and CDPKs to activate gene expression in the nucleus. In conclusion, calcium orchestrates the synthesis of hydrogen peroxide and NO in organelles which, in turn, promotes calcium release from ER inducing the calcium-dependent activation of nuclear gene expression via CAMs and CDPKs.

**Materials and Methods**

**Algal and water sampling.** *Ulva compressa* was collected in Cachagua (32° 34'S), a non-impacted site in central Chile, during spring 2011 and transported to the laboratory in sealed plastic bags in a cooler at 4°C. Algal samples were rinsed three times in sterile filtered seawater and cleaned manually. Ultrasound was applied to remove epiphytic bacteria and organic debris, twice for 1 min using a Branson 3000 (Danbury) ultrasound bath. Seawater was obtained from Quintay (53° 12'S), a non-impacted site in central Chile, filtered through 0.45 and 0.2 μm pore size membrane filters and stored in darkness at 4°C.

**Treatment with inhibitors.** *U. compressa* (0.05 g) was incubated in seawater without copper addition (control), with 10 μM copper, and with inhibitors of voltage-dependent calcium channels, 100 μM nifedipine (Sigma), 100 μM verapamil (Sigma) and 100 μM diltiazem (Enzo Life Sciences), or with inhibitors of phospholipase C, 100 μM neomycin (Sigma) and 30 μM U73122 (Sigma) and with 10 μM copper for 2, 3 and 12 h. In addition, the non-permeable calcium chelating agent ethylene glycol-O, O'-bis (2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA) was added to seawater supplemented with 10 μM copper at a final concentration of 1 mM, 30 min before each intracellular calcium increase at 2, 3 and 12 h.

**Detection of intracellular calcium.** Three lamina of *U. compressa* were gently removed from culture media and incubated in seawater containing 20 μM Fluo-3AM (Molecular Probes, Invitrogen) during 40 min at room temperature. The laminae were washed three times in filtered seawater to remove fluorophor excess. The green fluorescence of Fluo 3 was visualized by confocal microscopy using an Axiovert 100 confocal microscope (Carl Zeiss), an emission wavelength of 488 nm produced by an argon laser and a filter of 505–530 nm. The intensity of green fluorescence of Fluo 3 and the red fluorescence of chloroplasts was quantified in each lamina using LSM510 software of the confocal microscope. The intensity of green fluorescence in each sample was normalized using chloroplast autofluorescence (relative fluorescence).

**Statistical analysis.** Significant differences were determined by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (T). Differences between mean values were considered to be significant at a probability of 5% (p < 0.05).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

This work was financed by Fondecyt 1085041 to A.M.
References
1. Galione A, Churchill GC. Interactions between calcium release pathways: multiple messengers and multiple stores. Cell Calcium 2001; 30:349-54; PMID:11548854.

2. Galione A, Brailoiu GC, Churchill GC, Brailoiu E, Rietdorf K. A non-NAADP-mediated channel targeted to the plasma membrane. Biochim Biophys Acta 2009; 1793:1627-33; PMID:19598706; http://dx.doi.org/10.1016/j.bbamcr.2009.04.005.

3. Pitt SJ, Funnell TM, Chatterton D, Piele, DL, Brailoiu GC, Hooper R, et al. A NAADP-gated channel targeted to the plasma membrane. Biochim Biophys Acta 2009; 1793:1627-33; PMID:19598706; http://dx.doi.org/10.1016/j.bbamcr.2009.04.005.

4. Brailoiu GC, Brailoiu E, Parkesh R, Galione A, Morgan AJ, Arredouani A, Davis LC, Churchill GC. Interactions between calcium and modulating the phosphoinositide balance. Prog Lipid Res 2010; 49:429-37; PMID:20553968; http://dx.doi.org/10.1016/j.plipres.2010.06.001.

5. Fukami K, Inanobe S, Kanemaru K, Nakamura Y, Brailoiu GC, Hooper R, et al. NAADP-mediated channel, operating as a dual sensor of luminal pH and Ca2+. J Biol Chem 2010; 285:30611-6; http://dx.doi.org/10.1074/jbc.M110.162073.

6. Yamasaki M, Churchill GC, Galione A. Calcium signalling by nicotinic acid adenine dinucleotide phosphate (NAADP). FEBS J 2005; 272:4598-606; PMID:16060480; http://dx.doi.org/10.1111/j.1742-4658.2005.05860.x.

7. Michard E, Lima PT, Borges F, Silva AC, Portes MT, Ruas M, et al. TPC2 is a novel NAADP-sensitive Ca2+ release channel, operating as a dual sensor of luminal pH and Ca2+. J Biol Chem 2010; 286:30599-60; http://dx.doi.org/10.1074/jbc.M110.162073.

8. Chatterton D, Hooper R, et al. An NAADP-gated two-pore channel targeted to the plasma membrane. Biochim Biophys Acta 2009; 1793:1627-33; PMID:19598706; http://dx.doi.org/10.1016/j.bbamcr.2009.04.005.

9. Christel C, Lee A. Ca2+-dependent modulation of phosphatidylinositol turnover in the plasma membrane of tobacco suspension cultured cells. Biochim Biophys Acta 2005; 1721:45-51; PMID:15728063; http://dx.doi.org/10.1016/j.bbamcr.2004.05.033.

10. Ollere S, Misko P. [Regulation of N-methyl-D-aspartate receptors by serotonin, dopamine, and norepinephrine]. Neurosci Lett 2000; 284:127-8; PMID:10999979.

11. Bliss GMW. Molecular anatomy of major neurotransmitter receptor systems in the mammalian central nervous system: neuropeptides, dopamine, serotonin, acetylcholine, and glutamate. J Child Neurol 2001; 16:271-80; discussion 281; PMID:11332462.

12. Rietdorf K, Ruas M, et al. NAADP-sensitive Ca2+ channels. Annu Rev Cell Dev Biol 2000; 16:521-7; PMID:11031246; http://dx.doi.org/10.1146/annurev.cellbio.16.1.521.

13. Kurusu T, Yagala T, Miyao A, Hirochika H, Kuchitsu K. Identification of a putative voltage-gated Ca2+ channel. Nature 1999; 399:159-62; PMID:10335846; http://dx.doi.org/10.1038/20200.

14. Liu Y, Wu Z, Wu Y. 39S Ca2+-dependent modulation of phosphatidylinositol turnover in the plasma membrane of tobacco suspension cultured cells. Biochim Biophys Acta 1993; 1176:427-35; PMID:1604519; http://dx.doi.org/10.1016/0167-4889(93)90140-S.

15. Zhao R, Liu Y, Wu Z, Wu Y. NADP metabolite nicotinic acid adenine dinucleotide phosphate (NAADP) stimulates pulmonary sensory neurons via direct activation of TRPA1. J Appl Physiol 2010; 108:855-67; PMID:20136428; http://dx.doi.org/10.1152/japplphysiol.00904.2009.

16. Michaud E, Lima PT, Borges F, Silva AC, Portes MT, Ruas M, et al. TPC2 is a novel NAADP-sensitive Ca2+ release channel, operating as a dual sensor of luminal pH and Ca2+. J Biol Chem 2010; 286:36039-46.

17. Navazio L, Bewell MA, Siddiqua A, Dickinson GD, Gu Q, Lin RL. Heavy metals zinc, cadmium, and copper stimulate pulmonary sensory neurons via direct activation of TRPA1. J Appl Physiol 2010; 108:855-67; PMID:20136428; http://dx.doi.org/10.1152/japplphysiol.00904.2009.

18. Kamada Y, Muto S. Ca2+ regulation of phosphatidylinerse hydrolysis in neurons of the rat medulla oblongata. Ann Neurol 1981; 10:521-5; PMID:698174.

19. Ratkevicius N, Coia JA, Maione A, Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in Dunaliella salina (Chlorophyta) from heavy metal-enriched environments in northern Chile. Plant Cell Environ 2005; 28:159-44; http://dx.doi.org/10.1111/j.1365-3040.2003.01073.x.

20. van der Graaff AM, Pedersen JD, Pedersen JA, Reuter M, Trebotich J, Vergara E, et al. Copper induces Ca2+-sensitive cell death and mitochondrial apoptosis in Ulva compressa. Plant Signal Behav 2010; 5:197-77; PMID:21139437; http://dx.doi.org/10.4161/psb.5.12.19777.

21. Gu X, Lin RL. Heavy metals zinc, cadmium, and copper stimulate pulmonary sensory neurons via direct activation of TRPA1. J Appl Physiol 2010; 108:855-67; PMID:20136428; http://dx.doi.org/10.1152/japplphysiol.00904.2009.

22. Hu H, Banak J, Moreno MJ, Zha MX, Parsons A, Zn, calcium damage-sensing TRPA1 isoforms. Mar Biotechnol 2005; 7:519-36; PMID:15925784; http://dx.doi.org/10.1007/s10126-005-0157-8.

23. Zhdanov KD, Petz GS, Nandhakumar K, Tanis RW, Bester H. Cadmium supports both inactivation and facilitation of L-type calcium channels. Nat Neurosci 2008; 11:589-96; PMID:18025748; http://dx.doi.org/10.1038/nn.2109.

24. Rakonczay NR, Coia JA, Maione A. Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in Dunaliella salina (Chlorophyta). (from heavy metal-enriched environments in northern Chile). Plant Cell Environ 2005; 28:159-44; http://dx.doi.org/10.1111/j.1365-3040.2003.01073.x.

25. Zazh Z. Biostatistical Analysis. 1998; Prentice Hall, Englewood Cliffs, UK.