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REVIEW

Shuffling the cards in signal transduction: Calcium, arachidonic acid and mechanosensitivity

Luca Munaron

Luca Munaron, Department of Animal and Human Biology, Nanostructured Interfaces and Surfaces Centre of Excellence, Center for Complex Systems in Molecular Biology and Medicine, University of Torino, 10123 Torino, Italy
Author contributions: Munaron L wrote and revised this review.
Correspondence to: Luca Munaron, PhD, Department of Animal and Human Biology, Nanostructured Interfaces and Surfaces Centre of Excellence, Center for Complex Systems in Molecular Biology and Medicine, University of Torino, Via Accademia Albertina 13, 10123 Torino, Italy. luca.munaron@unito.it
Telephone: +39-11-6704667 Fax: +39-11-6704508
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Abstract

Cell signaling is a very complex network of biochemical reactions triggered by a huge number of stimuli coming from the external medium. The function of any single signaling component depends not only on its own structure but also on its connections with other biomolecules. During prokaryotic-eukaryotic transition, the rearrangement of cell organization in terms of diffusional compartmentalization exerts a deep change in cell signaling functional potentiality. In this review I briefly introduce an intriguing ancient relationship between pathways involved in cell responses to chemical agonists (growth factors, nutrients, hormones) as well as to mechanical forces (stretch, osmotic changes). Some biomolecules (ion channels and enzymes) act as "hubs", thanks to their ability to be directly or indirectly chemically/mechanically co-regulated. In particular calcium signaling machinery and arachidonic acid metabolism are very ancient networks, already present before eukaryotic appearance. A number of molecular "hubs", including phospholipase A2 and some calcium channels, appear tightly interconnected in a cross regulation leading to the cellular response to chemical and mechanical stimulations.

Key words: Arachidonic acid; Calcium signaling; Evolution; Mechanosensitivity; Phospholipase A2

Peer reviewers: Christopher M Norris, PhD, Associate Professor of Molecular and Biomedical, Pharmacology and the Sanders-Brown Center on Aging, 131 Sanders-Brown Building, University of Kentucky College of Medicine, Lexington, KY 40536, United States; Shangwei Hou, Senior Research Investigator, D100 Richards, Department of Physiology, University of Pennsylvania, 3700 Hamilton Walk, Philadelphia, PA 19104, United States

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Ca^{2+}: AN ION FOR ALL FUNCTIONS

Calcium is the fifth most abundant element in the earth's crust (3.5%) and is present in the oceans (0.04%). In addition to its extracellular functions as a major material used for the mineralization of bones and shells in *Metazoa*, Ca^{2+} has been recruited as a ubiquitous and universal intracellular messenger since the beginning of life. Its interaction with biomolecules exhibits flexible coordination chemistry, high affinity for carboxylate oxygen (the most frequent motif in amino acids) and rapid binding kinetics[1]. The overall consequence is that cell biochemistry and physiology are Ca^{2+}-dependent. On the other hand, when free cytosolic Ca^{2+} concentration (Ca_c) is too high, it is cytotoxic for both prokaryotes and eukaryotes, triggering aggregation of proteins and nucleic acids, precipitation of phosphates and affects the integrity of lipid membranes[1,2]. This is probably the reason why an

energy-consuming homeostatic system for Ca_c regulation has been established and fixed since the very beginning of biological evolution[1]. Ca_c is usually very low (nmol/L to μmol/L range) compared with the extracellular Ca_c (mmol/L) and the electrochemical gradient (the driving force) is huge, its maintenance demanding substantial energy consumption. Ca_c homeostasis is generally a steady state due to the balance between passive and active mechanisms, respectively, following and against the electrochemical gradient. They are mediated by a number of different membrane proteins, including ion channels, exchangers and pumps expressed by both prokaryotes and eukaryotes[3-7]. The components of the machinery initially acting (at least primarily) on cell survival allowed the evolution of the current Ca_c signaling system through a long history of adaptations and exaptations[1,2,8-12]. The rise of eukaryotic cell organization and the following evolutionary events - the acquisition of polarity, differentiation and multicellularity - correlated with the increasing complexity of biological systems. Consequently, a more efficient signaling apparatus was required to account for coding, control and integration of the biochemical processes in space and time.

Theoretically speaking, we can identify a number of steps in which Ca^{2+} might have played a significant role and, in turn, Ca_c signaling has been drastically modified[1].

The transition from prokaryotic to eukaryotic cell structure led to an increase in intracellular spatial compartmentalization due to the rise in membrane-delimited organelles (nucleus, endoplasmic reticulum-ER, mitochondria, vesicles). ER and mitochondria became Ca^{2+} stores, dynamic intracellular sources for the ion. This new mode of Ca_c regulation added to the preexisting Ca^{2+} entry from the extracellular medium. We know that the so-called Ca^{2+} microdomains, highly localized Ca^{2+} hot spots identified in all cell types, often require the interplay between Ca^{2+} entry (as well as calcium release from ER) and mitochondrial buffering[13,14]. Moreover, new modes for calcium wave generation evolved through the interplay between Ca^{2+} fluxes occurring in the plasma membrane and ER. This is the case for calcium-induced calcium release and store-dependent calcium entry[13,14].

The following evolutionary step, cell polarization, can be viewed as another very intriguing step concerning the modification of tridimensional cell shape, surface/volume ratio as well as of the structural anisotropy. From the diffusional point of view, Ca^{2+} wave propagation within the cell was more dishomogeneous and articulated. Highly polarized epithelial cells from secreting and adsorbing epithelia exhibit a polarized arrangement of Ca^{2+} channel membrane distribution and Ca^{2+}-dependent machinery. A good example is provided by exocrine acinar cells in pancreatic and salivary glands. They evolved sophisticated and complex Ca_c signaling mechanisms that control the secretory events occurring across the apical plasma membrane bordering the gland lumen. In these cells the receptor-evoked Ca_c wave starts at the apical pole, where specific calcium channels are located, and propagates to the basal pole[15,16].

The third step, multicellularity and cell differentiation, definitively strengthened and fixed the great potentiality of Ca_c signaling. Indeed, its omnipresence and versatility was very useful as a framework in cell-cell communication which was required for the functional integrity of multicellular organisms. For this reason, it is not surprising that in *Metazoa* highly diverse extracellular agonists - including hormones, neurotransmitters and growth factors - trigger changes in Ca_c that function as a chemical signal to transduce information for survival, proliferation, motility, differentiation and death in both physiological and pathological conditions. The appearance and diversification of a truly versatile class of "Ca^{2+} effector/sensor" molecules (Ca^{2+}-binding proteins), considered absent in prokaryota until recently, have been claimed as a possible solution to this demand. The most ubiquitous Ca^{2+} sensors are the so-called EF-hand containing proteins, among which the calmodulin family is the best known. EF-hand Ca^{2+} sensors are abundant in yeasts and ubiquitous in multicellular organisms, where they represent 120 families with more than 270 members[1,2,17-19].

The evolutionary association between eukaryotic cell evolution and Ca^{2+} sensors diversification is intriguing, but recent findings do not support this idea. In bacteria, Ca^{2+} is implicated in a wide variety of cellular processes, including the cell cycle and cell division[5-7]. Moreover, a recent analysis of the prokaryotic protein sequences available in the databases revealed the presence of several calmodulin-like proteins. They contain two or more authentic EF-hand motifs, suggesting that calmodulin-like proteins could be involved in Ca_c signaling in bacteria[5].

The same argument is true for eukaryotic transition from single cell to multicellular organization. It is generally believed that the complex Ca_c signaling "toolkit" has arisen from the ancestral multicellular organisms to fit unique physiological roles of specialized cell types. However, the expression of a surprisingly extensive Ca^{2+} signaling "toolkit" in the unicellular choanoflagellate *Monosiga brevicollis* has been reported[12]. Choanoflagellates possess homologues of some animal plasma membrane calcium channels, inositol 1,4,5-trisphosphate receptors, plasma membrane and sarco/endoplasmic reticulum Ca^{2+} ATPases and cation/Ca^{2+} exchanger families. Therefore, a complex Ca_c signaling machinery might have evolved before the emergence of multicellular animals[12]. It may be interesting to investigate the proteome and genome of other unicellular eukaryotes in order to extend and confirm this hypothesis.

These experimental observations prompted us to provide more articulated evolutionary explanations in order to account for a Ca^{2+}-dependent role in the increased complexity related to prokaryota-eukaryota and single-cell/*Metazoa* transitions. The complex structure-function relationship of proteins suggests that new functions do not necessarily arise from structural modifications of

preexisting proteins or "*de-novo*" expression, but also from insertion of the same or a similar protein in a different microenvironment (prokaryotic *vs* eukaryotic cell). The impressive increase in eukaryotic spatial compartmentalization would have driven the reassignment of preexisting proteins into new intracellular sites. Indeed, new functions of Ca^{2+} sensor proteins already expressed and functional in prokaryota may be recruited in the new context of eukaryotic and multicellular framework, leading to a functional enlargement of Ca^{2+} machinery potentiality.

THE TIGHT CONNECTIONS BETWEEN MECHANICAL STIMULI, CALCIUM SIGNALING AND LIPIDS

Mechanical forces are universal and their sensing by living beings, termed mechanosensation, underlies critical physiological processes including osmotic regulation, hearing, touch, balance, proprioception and blood-pressure monitoring[20].

Cell transduction of mechanical stimuli depends on mechanosensitive biomolecules that integrate information into the complex networking biochemical pathways. Such cell components can be considered as functional "hubs". Here we focus our interest on the pivotal role of two well-known and ancient "hubs": Phospholipase A2 (PLA2) with its lipid products and some Ca^{2+}-permeable channels belonging to the superfamily of transient receptor potential (TRP) proteins.

Since the middle of 1990, several authors have reported on the ability of PLA2-related membrane lipids to modulate the activity of Ca^{2+}-permeable channels in different cell types. In particular, arachidonic acid (AA) and some of its metabolites activate a number of TRP channels[21-29] (discussed below). Interestingly, some of these channels are directly or indirectly involved in cell response to mechanical stimuli (mechanotransduction), a very ancient feature of both prokaryotes and eukaryotes. Besides external forces, the composition of the lipid bilayer itself can affect its internal forces and different lipids can change channel gating[20].

Starting from these introductory highlights, the question is: did an intriguing complex partnership between calcium signaling, AA metabolism and mechanotransduction occur since the very beginning of cell evolution? Let us try to dissect this intriguing issue.

PLA2: a mechano- and chemo-regulated intracellular hub for AA release

AA, a 20-carbon omega-6 polyunsaturated fatty acid, is present in the cell membranes of both prokaryotes and eukaryotes[30-34].

In eukaryotic membrane phospholipids, AA is esterified to glycerol. Three phospholipases, A2, C and D, mediate the deacylation reaction that releases the free fatty acid (Figure 1). Free AA interacts with several proteins

Table 1 Properties of phospholipase A2 groups[36,41,43,45,46,54,55,85]

Group	Location	Size (kDa)	Common sources	Calcium requirement
I	Secreted	13-15	Cobra, human	mmol/L
II	Secreted	13-15	Viper, mouse, human	mmol/L
III	Secreted	16-18	Bee, lizard, scorpion, human	mmol/L
IV	Cytosolic	85	Human	< μmol/L
V	Secreted	14	Human	No
VI	Cytosolic	80-85	Human	No
VII	Cytosolic	40	Human, bovine	No
	Secreted	45	Human, bovine	No
VIII	Cytosolic	29	Human	No
IX	Secreted	14	Marine snail	< mmol/L
X	Secreted	14	Human	mmol/L
XI	Secreted	12-13	Green rice	mmol/L
Other	Secreted	14-15	Streptomyces (Bacteria)	mmol/L
	Cytosolic	93	Sporothrix schenckii (Fungi)	< μmol/L
	Secreted	10	Parvovirus	mmol/L

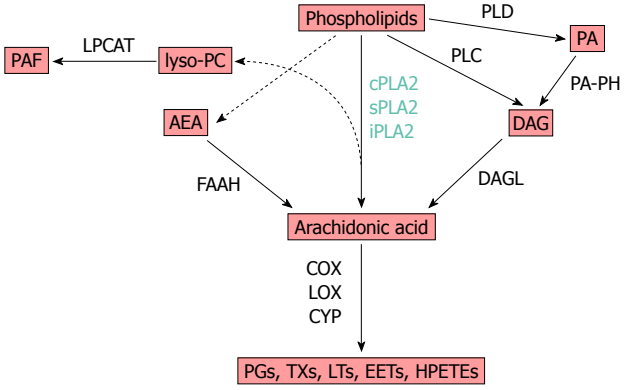

Figure 1 Simplified scheme showing arachidonic acid metabolism. COX: Cyclooxygenase; LOX: Lipoxygenase; CYP: Cytochrome P450 epoxygenase; PGs: Prostaglandins; TXs: Thromboxanes; LTs: Leukotrienes; EETs: Epoxyeicosatrienoic acids; HPETEs: Hydroperoxyeicosatetraenoic acids; DAG: Diacylglycerol; DAGL: DAG lipase; PA: Phosphatidic acid; PLD: Phospholipase D; sPLA2: Secretory phospholipase A2; cPLA2: Cytosolic phospholipase A2; iPLA2: Ca^{2+}-independent phospholipase A2; PLC: Phospholipase C; PA-PH: Phosphatidic acid phosphatase; lyso-PC: Lysophosphatidylcoline; PAF: Platelet activating factor; LPCAT: LPC acetyltransferase; AEA: Arachidonoylethanolamide (anandamide); FAAH: Fatty acid amidohydrolase.

(ion channels, enzymes) in the same cell and freely diffuses giving rise to a paracrine pathway in the neighboring cells. It has a short half-life being the substrate of cyclooxygenases, lipoxygenases and cytochrome P450 monooxygenases, yielding a great number of bioactive lipids and eicosanoids, that act as autocrine/paracrine regulators of a variety of functions in physiological and pathological conditions[35,36]. AA diffusion is also facilitated by its binding to fatty acid binding proteins, a superfamily of ancient proteins expressed in both prokaryotes and eukaryotes[37,38].

While PLA2s release arachidonate in a single-step reaction, phospholipase C and phospholipase D produce the AA-containing lipid products, diacylglycerol (DAG) and phosphatidic acid, respectively (Figure 1)[29,39,40].

PLA2s are a superfamily of diverse intracellular and secreted enzymes, whose activity was first reported in snake venom, more than a century ago (Table 1). They are very ancient proteins also expressed in prokaryotes and viruses[36,41-46]. An intriguing feature of some PLA2 isoforms is their mechanosensitivity, used by both prokaryotes and eukaryotes for the transduction of external mechanical stimuli into the cell[36,41,42,44,47,48]. Several investigations have focused on the structural basis responsible for PLA2 mechanosensitivity[48-51]. A striking feature of lipolytic enzymes, including PLA2, is the so-called interfacial activation[51]. Compared to the hydrolysis of monomeric substrates, their activity is dramatically enhanced when reacting with phospholipid interfaces, such as those present in micelles, monolayers, and bilayers. The activity of PLA2 is modulated by the lipid composition and the phase state of the substrate phospholipids. Accordingly, a number of environmental factors affect the catalytic rate of PLA2. They include negatively charged phospholipids, vicinity to the main phase transition temperature of the substrate, fluid-gel phase coexistence, packing defects, lipid lateral packing density, lipid protrusions, and membrane curvature[50]. A pivotal study reported that in large unilamellar liposomes, osmotic stretching can modulate the sensitivity of the bilayer phospholipids to the action of PLA2s[48]. More recently it has been shown in a number of cell types that cell swelling promotes PLA2 activation (usually cPLA2, see below)[52,53]. Ankyrin repeats, suggested to be involved in the mechanosensitivity of some TRP channels (see below), might play a similar role in PLA2 regulation[44].

PLA2 isoforms are classified into groups which differ in structure, intracellular localization, regulation, calcium dependence and pharmacological inhibition (Table 1)[54,55]. They are also usually distinguished into secretory PLA2, cytosolic PLA2 (cPLA2) and Ca^{2+}-independent PLA2. In particular, the cPLA2s (cPLA2α, cPLA2β, cPLA2γ) are high-molecular weight (60-110 kDa), intracellular PLA2s[36,43,44]. cPLA2α, the best characterized isoform, has a catalytic domain at the C-terminal of the protein and a Ca^{2+}-binding domain in the N-terminal portion. cPLA2α displays high selectivity for glycerophospholipids with choline as the polar head group and the polyunsaturated AA in the sn-2 position[36,43,44]. The catalytic action of cPLA2α is Ca^{2+}-independent, but a submicromolar Ca_c is required for translocation of the enzyme from the cytosol to cell membranes. A key catalytic regulatory step for cPLA2 is due to phosphorylation by members of the mitogen-activated protein kinase (MAPK) family. MAPKs constitute a large family of multifunctional proteins, usually acting as a convergence point for intracellular pathways starting from different membrane receptors. A multitude of paralogous MAPK isoforms has been found in yeast, vertebrates, and other eukaryotes[56,57]. The activity of most MAPKs expressed in animal cells is modulated during osmotic stress and it is likely that MAPKs are evolutionary ancient transducers of osmosensory signals. This hypothesis is further strengthened by the fact that the physiological capacity for osmosensory signal transduction is a highly conserved function not only of animal MAPKs, but also of certain plant and fungal MAPKs[58]. Mechanosensitive PLA2s, already expressed in prokaryotes, might therefore have acquired new regulation by a mechanotransductory MAPK cascade in eukaryotes. This event could account for the increase in complexity in the integration between chemically- and mechanically-stimulated responsivity of eukaryotes, particular of *Metazoa*.

Table 2 No. of transient receptor potential genes in different organisms[60,61,63,67-70,79,88]

Organism	TRP
Arabidopsis (Angiospermae)	0
Chlamydomonas (Chlorophyta)	19
Fungi (unicellular and multicellular)	3
Monosiga brevicollis (Choanoflagellates)	5
Caenorabditis elegans (Nematoda)	17
Drosophila melanogaster (Insecta)	13
Ciona intestinalis (sea squirt, Ascidiacea)	27
Fugu rubripes (Puffer fish)	25
Danio rerio (Zebrafish)	27
Mus musculus (Mammalia)	28
Homo sapiens (Mammalia)	27

Transient receptor potential (TRP) genes are also found in the genomes of a number of protists not shown in table (see text).

AA and calcium signaling: an ancient association

As mentioned before, several calcium-permeable channels in the plasma membrane of eukaryotic cells are regulated by AA and its metabolites[29,39,59].

TRP proteins are cation channels, mostly calcium-permeable, and function as universal polymodal cellular sensors in all eukaryota. Most TRPs can be activated by various chemical or physical stimuli, such as ligand binding, temperature, changes in osmolarity, cell volume, mechanical forces, or voltage[21,23,24,60-66]. The polymodality of TRPs is very ancient as suggested by the biophysical properties of the budding yeast *Saccharomyces cerevisiae* TRPY1. This large conductance cation channel is curiously not located in the plasma membrane but in the vacuolar membrane where it is activated by hyperosmotic stimuli. The subsequent calcium release from the vacuole into the cytoplasm initiates osmotic defense responses[67-70]. Even if the intracellular distribution of TRPY1 appears to be unexpected, it has to be noted that a number of mammalian TRP channels studied thus far, are also found in intracellular membranes. Intracellular TRP channels actively participate in regulating membrane traffic, signal transduction, and vesicular ion homeostasis[71].

Like its animal counterparts, TRPY1 is polymodal, being not only a direct mechanosensor but also a chemosensor activated by indole and other aromatic compounds. Intriguingly, TRPY1 gating is calcium-dependent[62].

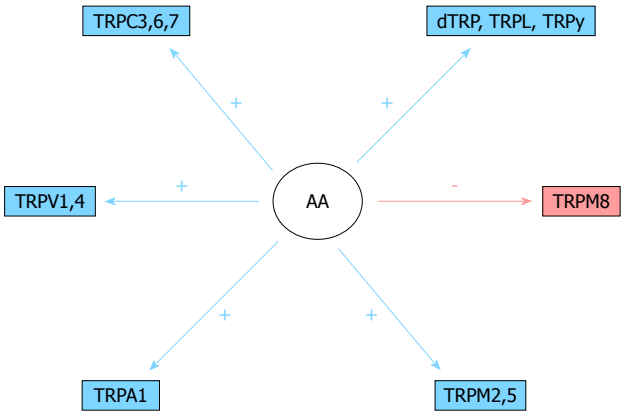

Figure 2 Scheme showing transient receptor potential channels modulated by arachidonic acid and its metabolites. AA: Arachidonic acid.

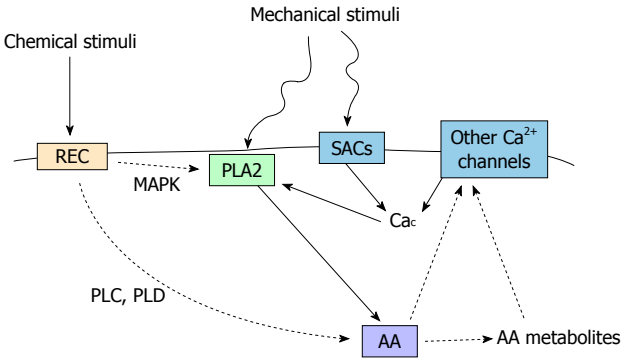

Figure 3 Simplified scheme showing the interplay between mechanical stimuli, arachidonic acid metabolism and calcium signaling. AA: Arachidonic acid; REC: Membrane receptors; SACs: Stretch activated calcium channels; PLD: Phospholipase D; PLC: Phospholipase C; MAPK: Mitogen-activated protein kinase; Ca_c: Ca^{2+} concentration; PLA2: Phospholipase A_2.

First cloned in Drosophila photoreceptors, TRP channels are now classified in 7 subfamilies[60,61,63,72]. Database searches have not (yet) revealed TRP-related genes in eubacteria, archaebacteria and plants (Table 2). TRP-related genes can be found in *Paramecium*, *Tetrahymena*, *Dictyostelium*, *Trypanosoma*, *Leishmania*, and other protists[68-70]. In single-celled fungi (yeasts), one TRP-related gene has been identified (encoding the afore-mentioned TRPY1), which cannot be assigned to any of the known TRP subfamilies in animals[68-70]. The unicellular choanoflagellate *Monosiga brevicollis* displays homologues of 5 mammalian TRP channel families, including TRPC, TRPV, TRPM, TRPML, and TRPA[12]. In animalia, a plethora of TRP-related genes has now been identified (Table 2).

As discussed below, some TRPs are directly activated by lipid intracellular messengers, including DAG and AA (Figure 2). TRPV4, TRPV1, as well as TRPC3 and TRPC6, are substrates for AA and its metabolites[22,26,28,73]. Other calcium channels, evolutionary and structurally unrelated to TRP proteins, are also targets for AA-dependent regulation[74].

These calcium-permeable AA-dependent channels provide a mechanistic link between mechanical stimulation and calcium signaling, through the involvement of PLA2, its product AA (and its metabolites) and regulation of calcium channels[21-23,60].

Interestingly, the functional interactions between PLA2 and TRPs are reciprocal (Figure 3): calcium entry following AA-dependent TRP activation can eventually act as a modulator of the calcium-dependent isoform of PLA2 providing a non-linear network.

Mechanosensitivity of calcium channels

Mechanosensitive (MS) channels are widespread and universal. While some channels are directly mechanically gated, many others acquire mechanosensitivity indirectly through their interaction with true primary mechanosensors including membrane receptors and associated second messengers[75-77].

The bacterial MscL channel was the first stretch-activated channel (SAC) to be cloned and reconstituted into artificial bilayers. It can be directly activated by tension coming from the lipid bilayer itself as well as from the tethered extracellular matrix and/or cytoskeleton[75,78].

Several calcium-permeable eukaryotic channels are sensitive to mechanical stimuli and a number of TRP channels play a key role in animal sensory mechanotransduction. This function is very ancient and shared by *Metazoa*[20,60,68-70,77,79,80].

In eukaryotes, several members of the TRP family are involved in mechanosensation and are recognized as promising candidate MS channels. In the nematode *Caenorhabditis elegans*, Osm-9 is required for nose touch and hypertonic-stress response. In the fly *Drosophila melanogaster*, Nanchung and Inactive (Nan/Iav) of the TRPV subfamily function in hearing, while Nan and water witch (wtrw) of the TRPA subfamily mediate hygrosensation. The vertebrate homologue, TRPV4, plays an evolutionary conserved role in the transduction of osmotic and mechanical stimuli. TRPV4 is expressed in DRG neurons, keratinocytes, and inner-ear hair cells where it can play a critical role in hearing, in addition to the afore-mentioned TRPA1[60,81]. Cell swelling activates TRPV4 by means of the PLA2-dependent formation of AA, and its subsequent metabolization to 5,6-epoxyeicosatrienoic acid (5,6 EET) (Figure 1)[64]. Moreover, in response to vasoactive factors or shear stress, sustained TRPV4-dependent Ca^{2+} entry into endothelial cells contributes to the Ca_c signaling requested for the synthesis and release of vasoactive compounds such as nitric oxide and prostaglandins[82]. Such events globally contribute to the relaxant effects on vascular tone[25,82-84]. This is a paradigmatic example of the interesting complex role of PLA2s in eukaryotic cells. Indeed, in addition to its osmosensitivity, PLA2 enzymatic activity can also be chemically modulated, following cell exposure to growth factors, proinflammatory agents, hormones and neurotransmitters[43,85].

Many other TRPs are activated either by osmotonicity or stress (or both). They include TRPV1, TRPV2, TRPC1, 3,6, TRPP1, TRPM4,7[25,64,81,83,84]. In human kidney, TRPP1

and TRPP2 sense fluid flow and their mutations result in polycystic kidney disease[86]. Recently, it was claimed that TRPC1 and TRPC6 may encode the mammalian SACs, although other reports warrant more detailed evidence and discussion[76,87].

CONCLUSION

Comparative analysis of genomic, proteomic and lipidomic databases allows us to investigate the evolutionary paths of cell transduction machinery. We usually distinguish cell responses to chemical agonists (growth factor, nutrients, hormones) from those triggered by mechanical stimuli due to stretch, pressure and osmotic changes. Nevertheless, we know that several intracellular actors in signal transduction are common and some proteins are directly on indirectly chemically/mechanically co-regulated (Figure 3). In particular Ca_c signaling machinery and AA metabolism are very ancient networks, expressed and functional in both prokaryotes and eukaryotes. Intriguingly, they are regulated and mediate the cellular response to both chemical and mechanical stimulations. Along with the increase in spatiotemporal complexity during prokaryotic-eukaryotic and unicellular-multicellular transitions, new functions have been assumed by these intracellular pathways through a diversification of isoforms, but also (and perhaps more importantly) *via* a rearrangement of the interactions and cross-regulations inside and among the signaling networks. The evolutionary history of calcium signaling and AA metabolism represents a useful conceptual and experimental model to investigate the idea that the structure-function relationship of single biomolecules, as well as of the networks including a number of biomolecules, is critically constrained by the micro/macro environment in which they work.

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