Review

Frequency decoding of calcium oscillations ☆

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ABSTRACT

Background: Calcium (Ca^{2+}) oscillations are ubiquitous signals present in all cells that provide efficient means to transmit intracellular biological information. Either spontaneously or upon receptor ligand binding, the otherwise stable cytosolic Ca^{2+} concentration starts to oscillate. The resulting specific oscillatory pattern is interpreted by intracellular downstream effectors that subsequently activate different cellular processes. This signal transduction can occur through frequency modulation (FM) or amplitude modulation (AM), much similar to a radio signal. The decoding of the oscillatory signal is typically performed by enzymes with multiple Ca^{2+} binding residues that diversely can regulate its total phosphorylation, thereby activating cellular program. To date, NFAT, NF-κB, CaMKII, MAPK and calpain have been reported to have frequency decoding properties.
Scope of review: The basic principles and recent discoveries reporting frequency decoding of FM Ca^{2+} oscillations are reviewed here.
Major conclusions: A limited number of cellular frequency decoding molecules of Ca^{2+} oscillations have yet been reported. Interestingly, their responsiveness to Ca^{2+} oscillatory frequencies shows little overlap, suggesting their specific roles in cells.
General significance: Frequency modulation of Ca^{2+} oscillations provides an efficient means to differentiate biological responses in the cell, both in health and in disease. Thus, it is crucial to identify and characterize all cellular frequency decoding molecules to understand how cells control important cell programs.

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1. Introduction

The key question in the field of calcium (Ca^{2+}) signaling is by what means this simple ion can regulate such a wide spectrum of cellular processes, including fertilization, proliferation, differentiation, muscle contraction, learning and cell death [1,2]. The answer to this question most certainly lies in the huge spatial and temporal diversity of the signal, since a Ca^{2+} response can exhibit infinite patterns [3]. Through an intricate concert of action between several Ca^{2+} transporters in the cell the cytosolic Ca^{2+} concentration can start to oscillate, much like a radio signal. Specific information can thereby be efficiently encoded in the signal and transmitted through the cell without harming the cell itself [4,5]. Already at the beginning of life, when the sperm injects phospholipase C-ζ into the egg, a slow Ca^{2+} oscillatory wave is traveling the egg that triggers fertilization [6]. Transmitting information using oscillating waves can occur by means of amplitude (AM) or frequency modulation (FM) (Fig. 1). However, Ca^{2+} oscillations in vivo are never totally homogenous, but rather have intrinsic variations in oscillation parameters such as frequency and amplitude [7]. This article will review and describe the recent literature on frequency decoding of FM Ca^{2+} oscillations. Thus, spatial aspects of Ca^{2+} signaling, including waves, intercellular communication and microdomains, will not be reviewed here. It should be noted though, that this dichotomy is a simplification since, for example, Ca^{2+} oscillations in mast cells convey transcriptional information only in the neighborhood of CRAC (Ca^{2+} release activated Ca^{2+}) channels [8]. Most studies mentioned in this review employ Ca^{2+} clamping to artificially induce frequency-controlled Ca^{2+} oscillations, either with or without an agonist.

2. Information encoding

Cells are constantly exposed to extracellular stimuli, e.g., mitogens, cytokines, hormones and neurotransmitters, that translate into changes in the cytosolic Ca^{2+} concentration [9]. In addition, environmental cues causing temperature and mechanical stress can result in activation of Ca^{2+} signals [10]. During development, the otherwise stable cytosolic Ca^{2+} level starts to oscillate spontaneously due to largely unknown reasons [11,12]. Depending on the stimuli, that differentially affect Ca^{2+} channels and pumps as well as Ca^{2+} binding proteins, a unique Ca^{2+} signal is induced. The process of building up a unique signal that can be associated with a specific stimulus is called information encoding. In certain circumstances, the stimulus results in persistent oscillatory changes in the cytosolic Ca^{2+} concentration. The biochemistry of such

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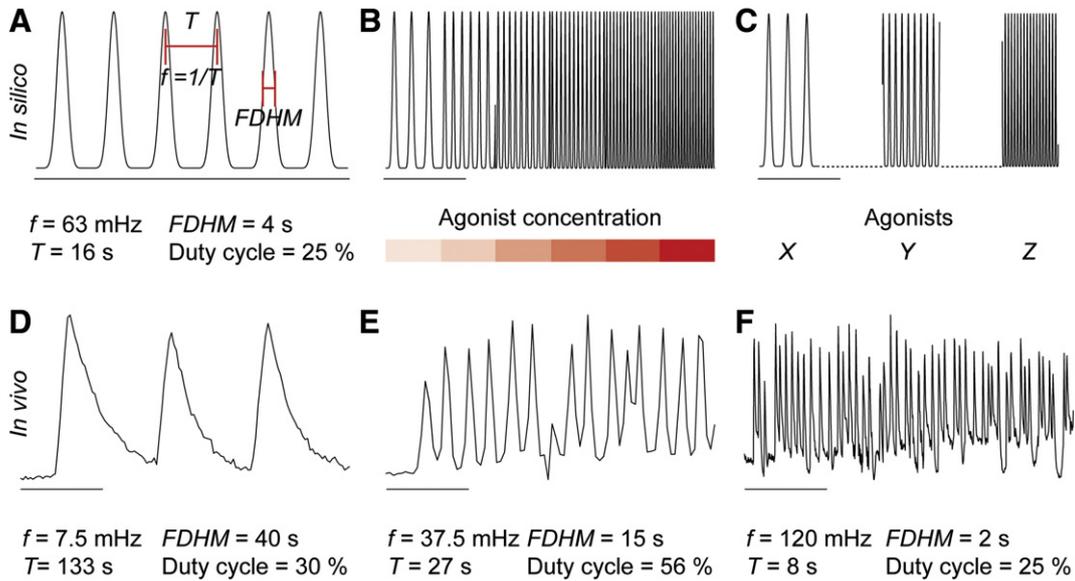E-mail address: per.uhlen@ki.se (P. Uhlén).

Fig. 1. Frequency modulated Ca²⁺ oscillations. (A) A computer generated (*in silico*) oscillating wave with the parameters: period (T), frequency (f), full duration half maximum (FDHM), and duty cycle is depicted. (B) Oscillating wave frequency modulated by agonist concentration. (C) Oscillating wave frequency modulated by the different agonists X, Y, and Z. Three single cell Ca²⁺ recordings of a Fluo-4/AM-loaded neuroblastoma cell (D), HeLa cell (E), and cardiac cell (F) with the parameters T, f, FDHM, and duty cycle stated. Scale bars are 100 s.

Ca²⁺ oscillations has been reviewed elsewhere [13]. The oscillatory frequency can vary from tens of Hz in neurons to tens of mHz in non-excitable cells [14]. Occasionally the frequency is proportional to the amount of stimulus to which the cell is exposed [15–19].

3. Information decoding

Decoding is used by the cell to interpret the information carried by the Ca²⁺ oscillation [20]. This information deciphering occurs when one or several intracellular molecules sense the signal and change their activities accordingly. The process is similar to electromagnetic radiation being received by an antenna on a radio and translated into sound. Mathematical modeling of a generic Ca²⁺ sensitive protein has shown that it is possible to decode Ca²⁺ oscillations on the basis of the frequency itself, the duration of the single transients or the amplitude [21]. Detailed models of real protein-decoders have also been reported [22–24].

At the molecular level, the Ca²⁺ oscillatory frequency regulates the activity of the decoder. The molecular mechanism of decoding is thought to include the on-and-off kinetics of Ca²⁺ binding to kinases and phosphatases, which respectively activate and inactivate target proteins. If the oscillation frequency is much lower than the typical on-off-frequency, no integration will occur and the signal is simply decoded as a sum of single transients. Consequently, maximum frequency sensitivity should be observed for signals with duty cycles between 0 and 0.5. The duty cycle is defined as the ratio of the spike duration to the period (Fig. 1). Oscillating signals are more effective in activating target effectors than constant signals when Ca²⁺ is bound co-operatively and with low affinity (dissociation constants around the peak Ca²⁺ concentration). However, with duty cycle values below 0.5, this requirement is less strict [21]. Several proteins appear to have such characteristics, including phospholipase Cδ [25,26], protein kinase Cβ [27], calmodulin [28], Ca²⁺- and calmodulin-dependent protein kinase II (CaMKII) [29], calcineurin [30], troponin C [31] and the mitochondrial Ca²⁺ uniporter [32]. Salazar et al. define pure frequency encoding as changes in frequency with constant duty cycle, whereas biological frequency encoding is when the duty cycle varies with the frequency as the spike duration remains constant [21]. Both types of frequency encoding as well as amplitude encoding are more efficient than constant signals. In almost all studies mentioned here (except from

[33]), cells exhibit Ca²⁺ oscillations of variable frequency but constant duration, thus modeling biological frequency encoding.

4. NFAT

The transcription factor NFAT (nuclear factor of activated T-cells) has been shown to function as a decoder of Ca²⁺ oscillations [34] and to exhibit working memory properties [35]. In its inactivated state, it is phosphorylated on multiple sites and kept stable in the cytosol [36]. Upon activation by the phosphatase calcineurin, which is modulated by Ca²⁺ binding and calmodulin, NFAT is dephosphorylated and translocated to the nucleus to become transcriptionally active. Upon re-phosphorylation in the nucleus, NFAT can be transported back to the cytosol. The kinetics of the nuclear export varies among different NFAT subtypes [37]. Dolmetsch et al. were first to use frequency-controlled artificially induced Ca²⁺ oscillations on Jurkat cells to show that the NFAT activity is positively correlated with frequencies in the range of 2.5–10 mHz, with a duration of 50 s (duty cycle 0.125–0.5) [34]. The same study showed similar frequency decoding properties for Oct/OAP and NFAT [34]. Interestingly, the NFAT transcriptional activity is both negatively and positively correlated with different Ca²⁺ frequencies in RBL-2H3 cells [38]. The maximum NFAT activity is present at the Ca²⁺ frequency of 16.7 mHz (duty cycle 0.33) with decreasing activity down to 2 mHz and up to 33 mHz, with a duration of 20 s (duty cycle 0.04–0.66). In BHK and Jurkat cells, the frequency of Ca²⁺ oscillations was positively correlated with dephosphorylation and translocation of NFAT4 in the Ca²⁺ frequency range of 1–11 mHz, with a duration of 30 s (duty cycle 0.03–0.3) [35]. The maximum efficiency was gained at 2.8 mHz (duty cycle 0.08) and an oscillatory Ca²⁺ signaling above 5.6 mHz results in stronger nuclear translocation than a sustained Ca²⁺ increase. Higher frequencies of Ca²⁺ oscillations in a genetic model of Noonan syndrome with cardiac myocytes show less NFAT transcriptional activity in the frequency range of 30–190 mHz, with a duration of 4–8 s (duty cycle 0.24–0.76) [39]. However, here no measurements were performed between 30 mHz and 140 mHz, where a maximum might be. By contrast, another study on rat neonatal cardiomyocytes shows that the NFAT activity is positively correlated with Ca²⁺ frequencies in the range of 17–83 mHz (duration 5 s) [40]. In an *in vitro* model of cardiac cell agonist induced hypertrophy, the cell area positively correlated with frequencies in the range of

100–1000 mHz, with a duration of 0.2 s (duty cycle 0.02–0.2) [41]. The cell area was then shown to be regulated by the activity of the NFAT pathway. In rat sympathetic ganglion cells, NFAT activity was measured by live fluorescence upon electrical field stimulation that triggered Ca^{2+} influx [42]. Stimulation with 10 Hz for 5 s, but not 1 Hz, induced NFAT translocation.

The emerging view of a possible role of NFAT as a frequency decoder includes the translocation processes between the cytosol and nucleus [41] in a model called "integrative tracking" [43]. Only a minor fraction of cytosolic NFAT is dephosphorylated by calcineurin upon a brief Ca^{2+} transient. To have a net influx of NFAT to the nucleus, the subsequent transient must arrive well in time; otherwise NFAT will be phosphorylated once again. In correct circumstances, dephosphorylated NFAT will slowly accumulate. This is due to the fact that the rate of dephosphorylation in the cytoplasm is higher than the rate of rephosphorylation in the nucleus [35]. Thus, higher frequencies result in larger accumulation of nuclear NFAT since every single Ca^{2+} transient causes one NFAT unit to translocate to the nucleus. The optimal frequency seems to be approximately 20 mHz and duty cycle around 0.2–0.3 as shown above (Fig. 2). It is unclear why the optimal frequency is around 20 mHz, but one could speculate that frequencies above 20 mHz cause additional kinases to rephosphorylate NFAT, as a negative feedback mechanism. Notably, this decline in NFAT activity above 20 mHz has only been reported in cardiac cells [44], which inherently exhibit high frequency Ca^{2+} oscillations.

5. NF-κB

Several reports demonstrate that the activity of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cell) is dynamically regulated by the frequency of Ca^{2+} oscillations. In its inactive stage, NF-κB is sequestered in the cytoplasm by IκB. Upon calcineurin activation by Ca^{2+} binding, IκB is inactivated and NF-κB translocates to the nucleus to become transcriptionally active [45].

Dolmetsch et al. used Jurkat cells and artificially induced Ca^{2+} oscillations by Ca^{2+} clamping to show that the NF-κB activity is positively correlated with the Ca^{2+} frequency in the domain of 0.56–10 mHz, with a duration of approximately 100 s (duty cycle 0.028–0.5) [34]. Notably, the minimum frequency is 4.5 times lower than for NFAT. This might be due to the ability of NF-κB to stay in the nucleus for longer periods than NFAT [46,47]. Likewise, in human aortic endothelial cells, the frequency of agonist-induced Ca^{2+} oscillations is positively correlated with the NF-κB activity in the range of 1.8–5.3 mHz, with a duration of approximately 160 s (duty cycle 0.29–0.85) [48]. Using another experimental setup with Ca^{2+} clamping in combination with agonist stimulation, vascular endothelial cells were shown to regulate VCAM1 expression with a Ca^{2+} frequency in the range of 1.7–11.7 mHz, with a duration of approximately 29 s (duty cycle 0.049–0.34) [49]. VCAM1 expression was shown to be regulated via the NF-κB pathway and, more importantly, the frequency dependency had different properties if the Ca^{2+} oscillations were only generated by Ca^{2+} clamping or in combination with agonist stimulation.

In human cerebral endothelial cells treated with sarco/endoplasmic reticulum Ca^{2+}-ATPase and inositol 1,4,5-trisphosphate receptor ($InsP_3R$) inhibitors to modulate the Ca^{2+} frequency, the NF-κB activity is positively correlated with frequencies in the range of 0–5.2 mHz (duration 100 s) [50]. Using different doses of histamine to trigger Ca^{2+} oscillations of different frequencies in porcine bronchial epithelial cells, the NF-κB activity is positively correlated with Ca^{2+} frequencies in the range of 7–10 mHz (duration 25 s) [51]. Histamine was also used in human aortic endothelial cells showing that the NF-κB activity is positively correlated with Ca^{2+} frequency in the range of 2–4 mHz (duration 60 s) [52].

Recently, it was demonstrated that the frequency dependence of NF-κB is actually due to the cumulated Ca^{2+} spike duration (equal to
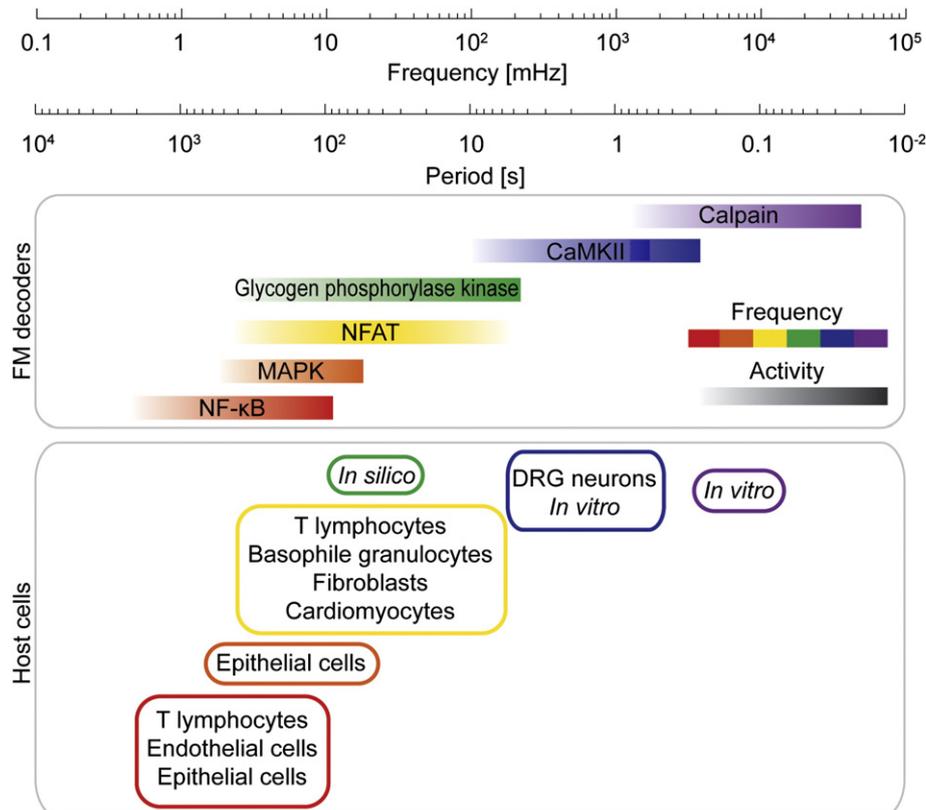

Fig. 2. Frequency decoders and host cells. Illustration showing the frequencies and periods that modulate the different frequency decoders and host cells.

the duty cycle times total signal duration) [33]. When the frequency is kept constant while the spike duration varies (opposite to what was done in almost all other studies mentioned here), the cumulated spike duration also changes as the cell employs pure frequency encoding [21]. In 16HBE cells, the NF-κB transcriptional activity, measured by ELISA of IL-8 mRNA levels, was shown to be positively correlated with the cumulated spike duration for Ca^{2+} frequencies in the range of 3–10 mHz, with duration of 36–52 s (duty cycle 0.36). Sensitivity to the cumulated Ca^{2+} spike duration was lost for frequencies below 3 mHz (2–3 times shorter durations than in Dolmetsch et al. [34]). Most importantly, here the frequency as such was not determining the transcriptional activity of NF-κB. Still, oscillatory Ca^{2+} elevations were reported to be more efficient in activating NF-κB than sustained elevations. Notably NF-κB can also discriminate between different amplitudes and durations of Ca^{2+} transients [46]. The idea that the cumulative Ca^{2+} spike duration conveys information has also been shown during the fertilization process of mammalian eggs, although without any identified molecular decoder [53].

The main difference between NFAT and NF-κB is the higher sensitivity of NF-κB, where frequencies as low as 0.56 mHz suffice. One plausible explanation for this distinction may be the ability of NF-κB to stay longer in the nucleus than NFAT [46,47]. Furthermore, NF-κB seems to be tuned toward higher duty cycles (0.8–0.9) than NFAT. The optimal frequency appears to be around 10 mHz (Fig. 2).

6. CaMKII

Activation of CaMKII is dependent on the frequency of cytosolic Ca^{2+} oscillations [54]. Upon calmodulin and Ca^{2+} binding, the kinase can auto-phosphorylate and become active [55]. In an elegant *in vitro* study (without counteracting phosphatases), auto-phosphorylation of CaMKII was shown to correlate with Ca^{2+} frequency in the interval of 500–4000 mHz, with duration of 200 ms (duty cycle 0.1–0.8). Longer duration results in stronger activation. By exhibiting both trapping of calmodulin upon activation and autonomous Ca^{2+}-independent activity, CaMKII is thought to have a molecular memory [56]. In mouse dorsal root ganglion neurons in culture, CaMKII autonomous activity correlates with the frequency of action potentials in the range of 100–1000 mHz [57]. The action potentials are accompanied by Ca^{2+} spikes, with temporal summation for the high range frequencies. In fertilized mouse eggs, the activity of CaMKII oscillates in synchrony with the slow Ca^{2+} oscillations [58]. Thus for low frequencies, CaMKII may not integrate and decode information (frequency of ~2 mHz with duration of ~60 s).

7. PKC

Activation of protein kinase C (PKC) is dependent on Ca^{2+} and diacylglycerol (DAG) being produced by phospholipase C cleaving plasma membrane phospholipids to DAG and inositol 1,4,5-trisphosphate [59]. More specifically, the degree of PKC-activity is dependent on the frequency of Ca^{2+} oscillations [60]. In a two-state model, both DAG and Ca^{2+} bind to PKC, which causes PKC to translocate to the plasma membrane to become activated. This only occurs for high frequency Ca^{2+} oscillations. Due to a delay in the DAG binding $C1_2$ domain on PKC, multiple Ca^{2+} transients in sequence are integrated as long as the interval is not too long. The tipping point is yet unknown.

8. MAPK

In HeLa cells, Ca^{2+} oscillations optimize activation of Ras and signaling through the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway [61]. The Ca^{2+} sensitivity is mediated *via* two families of Ras guanine nucleotide exchange factors called RasGRFs [62] and RasGRPs [63], as well as *via* the Ca^{2+}-triggered RasGTPase-activating proteins RASAL [64] and CAPRI [65]. Using Ca^{2+} clamping, the phosphorylation of ERK was shown to be positively

regulated by Ca^{2+} frequencies in the range of 1.7–17 mHz with a duration of approximately 50 s (duty cycle 0.085–0.85) [61]. These experimental results were confirmed in a theoretical modeling study showing negative correlation between decreasing Ca^{2+} frequency and activation of MAPK [66].

9. Calpain

The intracellular cysteine protease calpain has been presented as a frequency decoder [67]. In UV-light photolysis experiments, high frequency Ca^{2+} oscillations were generated and calpain activity was measured by MAP2 proteolysis in test tubes without cells. In the frequency range of 1–50 Hz, MAP2 degradation was regulated by frequencies with peak durations of 20 ms (duty cycle 0.1–1). The authors claim that the ability of calpain to sense these fast oscillations may be of potential physiological significance.

10. Mitochondrial dehydrogenases

The Ca^{2+}-sensitive mitochondrial dehydrogenases are enzymes involved in the electron transport chain of mitochondria. In a study on single hepatocytes, cytosolic Ca^{2+} oscillations were efficiently transduced into mitochondrial Ca^{2+} oscillations, which thereby activated these dehydrogenases [68]. By contrast, sustained elevations of cytosolic Ca^{2+} resulted in a transient increase in mitochondrial Ca^{2+} and consequently a transient activation of Ca^{2+}-sensitive mitochondrial dehydrogenases. Discrete mitochondrial redox spikes were observed for cytosolic Ca^{2+} oscillation frequencies below 3.3 mHz and a sustained redox elevation for 8.25–16.5 mHz.

11. Neurotransmitter expression

In a series of studies, Nick Spitzer and co-workers have shown that the identity of neurotransmitters in neurons is dependent on Ca^{2+} oscillations and not only on predefined genetic programs before and after chemical synapse formation [69]. In a homeostatic fashion at the circuit level, more activity results in higher expression of inhibitory neurotransmitters and *vice versa*. In *Xenopus laevis* spinal neurons *in vitro*, the expression of GAD67 (a GABA phenotype) was positively regulated by Ca^{2+} oscillations in the frequency range of 0.28–0.83 mHz [70]. Amplitude, duration and time integral were kept constant, while the frequency varied. These results were later confirmed *in vivo* in *X. laevis* spinal neurons [71]. Glutamate and vesicular transporters were negatively regulated by Ca^{2+} oscillations in the frequency domain of 0–2.8 mHz. Likewise, choline acetyl-transferase expression is suppressed by frequencies in the range of 0–7 mHz. To date, no specific molecular frequency decoder of Ca^{2+} oscillations for neural development has been identified. However, possible candidates are MAPK [72–74], PKA [72–74], PKC [72,73,75], CaMKII [75], CaMKIV [75], CREB [74] and NFκB [74].

12. Orphan decoders

Several proteins are thought to be frequency decoders on theoretical grounds, although their frequency decoding ability has not yet been proven experimentally. These include glycogen phosphorylase, which cleaves glycogen upon hypoglycemia [76]. Glycogen phosphorylase is activated by a kinase that has a calmodulin-like Ca^{2+} sensitive domain [77] and is thought to play a role as a frequency decoder for glycogen phosphorylase activation. The enzyme activity is positively regulated in the following frequency interval of 1.7–170 mHz, whereas the enzyme shows constant activity at higher and lower frequencies.

13. Conclusion

Frequency modulated Ca^{2+} oscillations represent a highly diverse signaling system that can regulate numerous processes in many different cell types. The cell is equipped with frequency decoding molecules that can translate oscillatory Ca^{2+} signals and activate specific cellular programs. As shown in this review article, these frequency decoding molecules exhibit different dependencies on frequency and duration. Intriguingly, when the different FM decoders' active frequency-interval is plotted on a logarithmic scale little overlap is shown (Fig. 2), suggesting their specific roles in the cell. Notably, decoders of high frequency signals (e.g. CaMKII) only involve a single or a few enzymatic steps, whereas decoders of low frequency signals (e.g. NF-κB) involve both enzymatic steps and translocation. Unique expression profiles of these frequency decoding molecules enable different cell types to respond differently to the same treatment. To obtain a complete picture of this intricate decoding system, the total signaling network consisting of kinases, phosphatases and transcription factors needs to be assayed in large-scale screens. With the rapid development of live cell imaging, bio-probes, protein analysis and gene sequencing, we will soon be able to comprehend the basics of frequency decoding of Ca^{2+} oscillations.

Acknowledgements

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