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Review

Ernesto Carafoli: A personal appreciation

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Calcium has been known to be of the greatest importance to a vast range of organisms for over one hundred years. The exceptional organisms are the prokaryotes in the sea, which do not use calcium but must reject it since inside cells sea water calcium concentrations would precipitate many organic molecules. From the beginning of life, then, there has always been a strong gradient of calcium ions across the outer cell membranes. It was this gradient which became of great use to eukaryote cells, since disturbance of the gradient could signal to the internal chemistry of the cell that external conditions, physical or chemical, had changed. The cell then responded. It is this value of calcium as a messenger that interested Carafoli and myself. I shall describe my early interest in calcium first as it was aroused earlier than that of Carafoli, but the major part of this article will refer to Carafoli's work. Unlike myself, Carafoli was an experimentalist working on cell systems while I was initially more a scientist attempting to appreciate the basic principles of calcium gradient importance. Later, about the time Carafoli started his work, we analysed the NMR structure of some calcium-binding proteins. I describe all my own work first in order to present aspects of calcium biochemistry in a coherent manner. Later I indicate where our interests came together.

Given this early knowledge of the calcium gradient and its function, the next steps were to discover the chemistry and biochemistry of how this ion functioned. There are three parts to this question. The simplest is how the calcium and magnesium ions could be separated from one another on the basis of their chemistries. We knew that the magnesium concentration in the sea is higher than that of calcium, but despite this, magnesium did not interfere with calcium biological chemistry. Magnesium has a small gradient across cell membranes and it is weakly bound to organic chemicals different from those binding calcium [1]. The problem I tackled in 1952 was how these differences between the chemistries of the two ions arose.

I showed that the difference was a simple matter of ion size. Crystal structures of inorganic compounds showed that the magnesium was invariably coordinated to no more than 6 ligands in a regular octahedron with ligand distances close to 2.0 Å. By comparison the calcium ion was bound by 7 or 8 ligands in an irregular structure with ligand distances greater than 2.2 Å. A little thought allowed the extension of these facts to the reason that EGTA bound Ca^{2+} to a considerably greater degree than Mg^{2+}, while EDTA was not so discriminating. EGTA could not collapse neatly around Mg^{2+}. Already EGTA had been used to limit Ca^{2+} outside cells by biochemists without any influence on magnesium concentration. The extension of these ideas to both cell membranes and inside cells was obvious, but one needed to show that surfaces, often holes in proteins, could fold around calcium in such a way, to the exclusion of magnesium, as to give the above specific structure for calcium as opposed to magnesium. I chose to use NMR in 1970 to uncover metal-binding sites in proteins. I became aware of the work of Carafoli at that time. For this purpose I needed a small protein [2]. The closest was Troponin C, discovered by Ebashi. Even this was too big for our purposes, but we, Barry Levine and myself [3], learned from Drabikowski that he could split the protein into two halves. We began to study the amino-acids of these half proteins. Already in 1973 Kretsinger, by crystal structure determination, had proposed his model of the calcium-binding sites in the protein, parvalbumin, which involved the special sequence of thirteen amino-acids called the EF-hand. Our NMR work confirmed this structure *in solution* and it was clear that such calcium-binding sites were of 7/8 coordination. Magnesium only bound weakly, some 3 or 4 orders of magnitude less, since the protein fold did not allow collapse to good bond distances for it. Around this time calmodulin became available and we worked on its halves just as with troponin C. It had to wait for a full NMR structure of the whole protein, by Bax in 1995, before we could see the complexity of protein fold changes on calcium binding *in solution*. This analysis was greatly helped by crystal structure determinations. Whereas we were only able to show small relative structure changes within each of the two halves [3], Bax in 1995, showed that the main changes of these proteins were the relative motions of the two halves due to flexibility of the link between them. Such changes in protein structure are basic to cell signalling.

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This account is an introduction of the work of myself and others before I introduce the main theme of this article, based on my warm connection and friendship with Ernesto Carafoli. I begin by saying that while my interest above was in calcium biochemistry, Carafoli was more a physiologist. To see how the relationship arose, I must now show the interrelationship with the above works and Ernesto's work starting from the 1960s. I am not certain, but I believe that Ernesto's interest in calcium biochemistry was awakened after he left Italy and worked with Lehninger at John Hopkins University. Lehninger had become, at that time, one of the leading experimentalists who had shown that mitochondria were able to take up calcium [4] even to the extent that it was precipitated inside these organelles. The possibility arose that when calcium entered a cell to stimulate activity, the subsequent required removal of calcium was due to it being pumped into mitochondria. Ernesto and others, who favoured this view at first, realised that if calcium was to function in the manner described then the uptake into mitochondria had to be fast enough to correspond with calcium function. After much discussion it was clear that mitochondria failed in this respect. It was found that cells contained small vesicles which had the correct uptake rates matching calcium function in activating cells. Much more recently it has been proved that mitochondria shared some of the required properties and Carafoli has been vindicated in his belief of the importance of mitochondria in calcium biochemistry. Carafoli and I often discussed these observations.

I turn now to the particular protein, calcium ATP-ase, which pumped calcium out of cells in a calcium controlled manner. Carafoli and his group became seriously involved in its study [5–7]. It was clear that its pumping functions, were controlled by calcium itself, such that when internal cell calcium reached about 10^{-7} M the pump was switched off. Such switching was found to depend on the binding of calcium to calmodulin, which was itself bound to the ATP-ase. Subsequently, the relationship between these two proteins and their functions has been tackled by Carafoli and his group over many years. The work has been greatly helped by crystal structure determinations. I believe that the interest in calcium in proteins, the structure of which is described above, led to much discussion between us.

Most recently, Carafoli and his group have employed gene technology to discover the value of specific amino-acids in the sequences of calmodulin and the ATP-ase responsible for their cooperative regulation of the pump. This has led to the discovery that mutations in these proteins lead to diseases in man. We now and then exchanged ideas on many of these issues whilst I was active. He and I wrote chapters in a number of books explaining our different understanding and approach to calcium biochemistry.

Carafoli had a quite separate but major influence on the development of calcium biochemistry, both on its functions and its medical significance. He organised a long series, still continuing, of international exchanges between scientists on these issues at a great variety of conference centres in all parts of the world. I assisted in the organisation in a minor way at first, but more recently I have only been a watcher of the details of these meetings. I was always sorry that while Ernesto assisted greatly in these international conferences he refused to play any part in the more local European calcium conferences. One of the striking results of recent studies described at these two sets of meetings had been the uncovering of many different calcium-binding proteins and their actions. It was at one of these meetings that I repeatedly met Ernesto, especially in the presence of Claude Klee, of whom I have equally fond memories. We discussed together the relevance of the selection of calcium, based on binding sites and function. I stopped going to these meetings some years ago, but I am always glad to learn of new calcium-binding proteins and of Ernesto's contributions to the field. Our exchanges were facilitated by visits from Ernesto's co-workers from ETH, mainly Joachim Krebs, to my group, and by the acceptance by Carafoli of Peter James from Oxford.

To conclude: it is very unusual for a scientist to be able to contribute so strongly to an activity in research and in the organisation of meetings. From very early in the 1960s Ernesto had become interested in calcium. My own contributions to the field have been relatively slight as shown in contributions to books. I have also had great pleasure as a listener at his conferences. Many a time this passive activity has been greatly increased in value by personal meetings and discussion. At all times I have to mention with my connection with Carafoli the friendship both of us have enjoyed with Claude Klee. I conclude that Carafoli has made a huge contribution to knowledge of calcium and its functions in his research, his writing and his organisational skills. The following contributions in this book will certainly show this in detail in particular ways.

Conflict of interest

None.

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