Published in final edited form as:
Adv Exp Med Biol. 2013 ; 961: . doi:10.1007/978-1-4614-4756-6_1.

Livin with NCX and Lovin it: A 45 Year Romance*

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Abstract

This conference commemorates, almost to the day, the 45th anniversary of the discovery of the Na^+/Ca^{2+} exchanger (NCX). The discovery was serendipitous, as is so often the case with scientific breakthroughs. Indeed, that is what is so fascinating and romantic about scientific research. I will describe the discovery of NCX, but will begin by explaining how I got there, and will then discuss how the discovery altered my career path.

> "Sit down before fact as a little child, be prepared to give up every conceived notion, follow humbly wherever, whatever abysses nature leads, or you will learn nothing"
>
> Thomas H. Huxley, letter to C. Kingsley, September 20, 1863

For the love of physiology

I was introduced to cell physiology by Howard Schneiderman, a distinguished insect physiologist and developmental biologist, during my undergraduate days at Cornell University. I was interested in neurophysiology and the mind-brain problem, but came under the spell of Daniel Tosteson when I was a medical student at Washington University in St. Louis. Dan convinced me to work on the Na^+,K^+-ATPase ('sodium pump'), which had just been discovered (Skou, 1957). I spent a year-and-a-half in Dan's lab studying the red blood cell cardiotonic steroid-sensitive Na^+ pump. In 1963, after completing medical school and an internship at Boston City Hospital, I was offered a naval commission to work at the US Naval Medical Research Institute in Bethesda, MD (much better than a tour in Vietnam!). Thus, I returned to neurophysiology and, under David Goldman (of the Goldman-Hodgkin Katz equation), I studied the effects of divalent cations and anesthetics on lobster nerve conduction (Blaustein and Goldman, 1966; Blaustein, 1968). I also was fortunate to spend a few weeks at Woods Hole with John Moore and Toshio Narahashi working on tetrodotoxin's action on squid axons (Moore et al., 1967).

I was planning to continue my career in cellular neurophysiology, and arranged for a position in Alan Hodgkin's laboratory in Cambridge, England, with a Special Fellowship from the NIH. My family and I arrived in Cambridge in late August of 1966. After a family trip to Vienna for the International Congress of Biophysics, I left my wife Ellen and our two children (ages 3 and 5) in Cambridge, and headed off to the Laboratory of the Marine Biological Association in Plymouth, England, for the Fall squid season.

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*In memory of Peter F. Baker, David E. Goldman, Alan L. Hodgkin, Howard A. Schneiderman, Daniel C. Tosteson, and Mani Matter.

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“All hands to the pump”

My expectation was to study squid axon electrophysiology, but Peter Baker, Alan’s junior associate, a lecturer at Emmanuel College, Cambridge, took a mini-sabbatical that Fall, and he wanted all Plymouth squid researchers to work on the Na^+ pump. I was paired up with Rick Steinhardt, Richard Keynes’ postdoctoral fellow, and we were tasked with studying the activation of the Na^+ pump by external cations. Richard came to Plymouth at the end of September; he showed us how to dissect squid axons (not knowing of my prior experience) and how to measure $^{22}Na^+$ efflux after injecting the giant axons (0.8-1.2 mm diameter) with a microsyringe that he and Alan designed (Hodgkin and Keynes, 1956). Richard then went off to Homburg (Saar), Germany, to teach in a course on membrane biophysics organized by Hermann Passow and Robert Stampfli (more about this later).

Rick and I began our $^{22}Na^+$ efflux experiments on squid axons, and we rapidly identified a component that depended on external K^+, and was blocked by ouabain, i.e., the Na^+ pump component. When we removed external Na^+ and K^+ (Na_o and K_o, respectively), preparatory to adding back one monovalent cation at a time, we exposed a very large $^{22}Na^+$ efflux that did not depend upon K_o, and was not blocked by ouabain (Figure 1). This efflux persisted when the external NaCl and KCl were replaced by sucrose, LiCl or choline Cl^-. We consulted with Peter Baker, who agreed that internal Na^+ could be exiting with an anion, or exchanging for a cation. It was easiest to remove the other external cations, Ca^{2+} and Mg^{2+}, so we first removed the Ca^{2+} - et, Voila! The large Na^+ efflux was reversibly abolished; i.e., the Na^+ efflux was external Ca^{2+}-dependent (Figure 1). Removal of external Mg^{2+} had negligible effect on the Na^+ efflux, so we had our answer: Na/Ca exchange! This was just the first month of my fellowship!

Peter recalled reading Ralph Niedergerke’s articles on Na^+-Ca^{2+} interactions in frog cardiac muscle, and he suggested that they might be of interest. There was no time to read, however: we were working 14-16 hr days during October because I feared that a gale would interrupt our daily squid supply; the large squid did not survive in the relatively small holding tanks. In fact, the squid usually were injured in the nets and would die before the collection boat docked. Therefore, as soon as squid were caught, the fishermen removed the head and internal organs, and placed the mantle (containing the giant axons) in a thermos of iced sea water. With the Na^+ pumps thus turned off, the axons slowly gained Na^+; the axons didn’t last overnight. As we shall see, this rise in the intracellular Na^+ concentration, $[Na^+]_i$, was fortuitous for the Ca^{2+} influx experiments.

In the meantime, we ordered some $^{45}Ca^{2+}$ to test the Na^+/Ca^{2+} exchange idea directly. The $^{45}Ca^{2+}$ arrived the first week in November, but just before that, we had a gale. Finally, I had a chance to catch my breath. It was a miserable, stormy afternoon, and the laboratory building was deserted; I completed my data analysis for the last experiments, and sauntered down the hall to the library. As soon as I started to read the description (Luttgau and Niedergerke, 1958) of extracellular Na^+-Ca^{2+} ($[Na^+]_o$-$[Ca^{2+}]_o$) antagonism and its influence on frog cardiac contraction (reduced $[Na^+]_o$ induces cardiac contraction), I got very excited. I immediately recognized that NCX must be widely distributed in both tissues and species, including vertebrate heart. Therefore, since NCX apparently functions in the heart, it is the missing link to the puzzle that had stumped me ever since my first studies on the Na^+ pump and, as an intern, my use of digitalis to treat patients with heart failure: How does Na^+ pump inhibition by cardiotonic steroids increase the force of contraction of the heart? Because of both my clinical and research experiences, I frequently thought about this enigma. Here was the answer: raising $[Na^+]_i$ promotes net Ca^{2+} gain by NCX, and thereby enhances cardiac contraction. That ‘Eureka! moment’ was even more thrilling than the discovery of NCX itself. I was, for a brief time, the only one in the world who understood how cardiotonic

steroids enhance cardiac contraction! I was so exhilarated that I went off, alone, to the nearby Green Lantern restaurant, for a fine celebratory dinner with a bottle of claret. Then, slightly inebriated, I returned to the lab to re-read Luttgau-Niedergerke, to be sure I wasn't delusional. It was a great day!

$^{45}Ca^{2+}$ flux studies: verification of Na^+/Ca^{2+} exchange

The following Monday afternoon, Alan Hodgkin came down to Plymouth to see how I was getting on. After we dissected a few axons for the evening's $^{22}Na^+$ efflux experiments, he and I went to dinner with Trevor Shaw, another Plymouth squidder. We talked about the NCX, including my explanation of how Na^+ pump inhibitors exert their cardiotonic effect. Alan asked a few questions but was, otherwise, impassive. I was crestfallen. How could he fail to be enthused by the story? Two days later, however, Alan asked me if I would mind if he remained in Plymouth to perform the $^{45}Ca^{2+}$ flux experiments with me. Would I mind? I was ecstatic! We had won him over.

Alan and I performed the first influx experiments the next Monday. Axons were incubated for 1 hr in artificial sea water (ASW, the external fluid) containing either NaCl or LiCl as the predominant salt, and labeled with $^{45}Ca^{2+}$. The axons were then washed in tracer-free solution, and the axoplasm was extruded and weighed, and ^{45}Ca activity was measured in an old Panax counter with a Nixie tube display. After the first sample from NaCl ASW, with a low count, axoplasm from a LiCl ASW axon was counted: the Nixie tubes lit up with a very high count. Because $[Na^+]_i$ was high in these axons from refrigerated mantles, the differences between the Ca^{2+} influx from Na^+ ASW and Li^+ ASW were large, and easy to detect. Alan's eyes twinkled as he pulled his pipe from his mouth and broke into a broad grin. Reduction of $[Na^+]_o$ increased Ca^{2+} influx (Table 1). Na^+/Ca^{2+} exchange was confirmed! At 2 am, after measuring several replicate samples, we celebrated with a little "medicinal"Scotch before heading off to bed. Another great day!

When I wrote up the results for publication, Alan was reluctant to include the proposed explanation for the cardiotonic action of cardiac glycosides. Therefore, while Alan was on a lecture tour in Eastern Europe, Peter Baker and I contrived to submit the manuscript, including the cardiotonic steroid hypothesis (Baker et al., 1969). [Note: *J Physiol* authorship was alphabetical in those days; there was no jockeying for "first" or "senior" authorship.] Our explanation for the cardiotonic steroid effect was later verified with Ca^{2+} measurements (Wier and Hess, 1984; Altamirano et al., 2006), NCX-knockout mice (Reuter et al., 2002) and NCX blockers (Tanaka et al., 2007).

NCX on the Continent

At the very same time that we were performing these experiments, the Fall of 1966, Harald Reuter, from Mainz, Germany, was attending the Membrane Biophysics Lab Techniques course in Homburg. There, he learned about Na^+/Na^+ exchange, using tracer $^{22}Na^+$, from Peter Caldwell and Richard Keynes (who did not yet know of the results in Plymouth). After completing the course, Harald, a cardiac pharmacologist who was familiar with the Luttgau-Niedergerke articles, immediately set out to look for NCX in cardiac muscle. And, of course, he found it (Reuter and Seitz, 1968). He knew what he was looking for; we were simply lucky, albeit prepared to recognize it when we saw it! As Louis Pasteur put it, "*le hazard ne favorise que les esprits prepares*."

Harald and I first met at the International Physiology Congress in Washington, DC, in August, 1968. I had just returned from England, and was then heading to St. Louis to take a faculty position in the Department of Physiology and Biophysics at Washington University. It was a very amicable meeting that soon led to extremely beneficial consequences. Before

describing those consequences, however, I should mention some other fallout from our Cambridge years.

Cambridge Collateral: Ellen's contributions

While I was off squidding in Plymouth, my 'squidow' (the local name for squidder spouses), Ellen, when she wasn't antiquing or rubbing church brasses, met several Cambridge sabbatical spouses. Upon my return to Cambridge in mid-December, after the 1966 squid season, Ellen invited Marcella and Len Ross for dinner. Len, an anatomist from Philadelphia, told me of his work with Victor Whittaker on the structure of isolated nerve endings, "synaptosomes". I was fascinated, and hypothesized (to myself) that the terminals might reseal and become functionally competent; I planned to test this idea when I returned to the States. In fact, my colleagues and I subsequently showed that synaptosomes do reseal, and that they function like intact terminals. They have functional ion transport mechanisms, including Na^+ pumps and NCX, and they generate ion gradients and membrane potentials (Blaustein and Wiesmann, 1970; Blaustein and Goldring, 1975; Fontana et al., 1995). Synaptosomes can be triggered to release neurotransmitters and to recycle synaptic vesicle membranes (Fried and Blaustein, 1976; Drapeau and Blaustein, 1983), and they sequester Ca^{2+} in the endoplasmic reticulum (Kendrick et al., 1977). We discovered voltage-gated Ca^{2+} channels in synaptosomes that were resistant to dihydropyridines (Nachshen and Blaustein, 1979); this was the first evidence for N, P and Q type Ca^{2+} channels (Catterall, 1998; Cao and Tsien, 2010) that play such an important role in brain physiology. We also used synaptosomes as an assay for the identification of a number of novel Na^+ and K^+ channel inhibitors (Krueger et al., 1980; Blaustein et al., 1991). In all, we published more than 65 articles on synaptosomes, including 6 in *Nature* or *PNAS*. In an extension of the synaptosome studies, we are currently testing the NCX-mediated effects of nanomolar ouabain on Ca^{2+} signaling in cultured neurons and astrocytes. Thus, Ellen's friendship with Marcella Ross paid off in a line of research that has continued to the present day.

Smooth muscle is not such smooth sailing

During our second year in Cambridge, again thanks to Ellen's initial contact while I was in Plymouth, she and I became very friendly with our next-door neighbors, Joy and Mani Matter, from Bern, Switzerland. Mani was the Bern Town Counsel, and was spending a year studying international law in Cambridge; Joy was a teacher who later became Minister of Education for the Canton of Bern. We maintained contact when we returned to our respective home countries, and I visited the Matters in 1970, after presenting my first paper on NCX in synaptosomes at a meeting in Sweden. Ellen, too, was anxious to see the Matters again, so I arranged for a NATO Fellowship-funded mini-sabbatical with Harald Reuter, who had just become chairman of the Department of Pharmacology in Bern (what a fortunate coincidence!). And off we went to Bern, from May to September of 1971. On Harald's suggestion, I agreed to look for NCX in vascular smooth muscle. The experiments were affirmative from the very beginning. We demonstrated antagonism between Na_o and Ca_o (reduced $[Na^+]_o$ promoted Ca^{2+} entry and arterial contraction; (Figure 2A) as well as Na_o-dependent $^{45}Ca^{2+}$ extrusion from guinea pig aortic smooth muscle (Figure 2B).

In a manuscript describing these results, we suggested that NCX helps to regulate vascular tone. We also postulated a close approximation between the plasma membrane (PM) NCX and the sarcoplasmic reticulum; this idea foreshadowed the "buffer-barrier" hypothesis (van Breemen et al., 1986; van Breemen et al., 1995) and the "PLasmERosome" model (Blaustein et al., 1998; Arnon et al., 2000b) of local Ca^{2+} control. The manuscript (Blaustein and Reuter) was rejected by *Nature*, *Science*, and *Experientia*. It was published in a non-refereed journal, *Phil Trans Roy Soc Lond*, because Harald was invited to present the results

at a Royal Society conference on smooth muscle (Reuter et al., 1973); the article has been cited ~300 times.

This novel idea that NCX is functionally important in vascular smooth muscle was, at first, ignored, or even disparaged (Somlyo et al., 1986; Murphy, 1988). Views about this concept slowly began to change more than two decades after its discovery (Murphy, 1993; Somlyo and Somlyo, 1994), once the NCX was cloned (Nicoll et al., 1990) and could be readily identified by immunocytochemistry and immunoblotting (Vemuri et al., 1990; Blaustein et al., 1992; Juhaszova et al., 1994).

Tragically, Mani Matter was killed in an auto accident in 1972. Therefore, we were most fortunate to have spent time in Bern in 1971. Mani had become a Swiss folk-hero because, as an avocation, he founded the Bernese Troubadors, who wrote and sang humorous and satirical songs in Swiss-German dialect that are still popular today.

PLasmERosomes: a structural basis for functional coupling of transport proteins

The NCX, like many other Na^+-coupled transporters, is driven by the Na^+ electrochemical gradient across the PM (Blaustein and Lederer, 1999). Nevertheless, we did not anticipate that the NCX would be co-localized with certain other transporters in PM microdomains adjacent to 'junctional' sarcoplasmic or endoplasmic reticulum (jS/ER) in a variety of cell types including neurons, glia and arterial myocytes (Juhaszova and Blaustein, 1997b). In fact, we were somewhat astonished (but shouldn't have been) that the NCX was confined to the PM microdomains that also contained the Na^+ pumps with a high ouabain affinity catalytic subunit ($\alpha 2$ or $\alpha 3$, depending upon cell type) (Juhaszova and Blaustein, 1997a). Moreover, both immunocytochemical and functional studies indicated that the much more prevalent Na^+ pumps with an $\alpha 1$ catalytic subunit that, in rodents, has low ouabain affinity, were apparently excluded from these microdomains (Lee et al., 2006; Song et al., 2006). Another mechanism that extrudes Ca^{2+} from most types of cells, the PM Ca^{2+}-ATPase (Ca^{2+} pump) may be excluded from these PM microdomains (Lencesova et al., 2004).

Some other Na^+ and Ca^{2+} transporters also co-localize to these microdomains, notably TRPC proteins that are components of receptor- and store-operated channels (ROCs and SOCs, respectively) (Golovina, 2005; Lee et al., 2006; Zulian et al., 2010a). Many ROCs and SOCs are relatively non-selective cation channels, permeable to both Na^+ and Ca^{2+} (Arnon et al., 2000a; Owsianik et al., 2006).

These findings provided a structural basis for the observations that the high ouabain affinity Na^+ pumps, NCX, ROCs and SOCs, and the adjacent jS/ER function cooperatively to regulate Ca^{2+} signaling in a variety of cell types (Arnon et al., 2000a, b; Eder et al., 2005; Poburko et al., 2007). I called this PM microdomain-jS/ER complex, the "PLasmERosome" (Figure 3). PLasmERosomes appear to be widely distributed, fundamental units that play a key role in cell Ca^{2+} signaling. They can modulate Ca^{2+} signaling differently in different types of cells, depending upon: 1. The specific complement of TRPC proteins, and perhaps other channel proteins (e.g., voltage-gated Ca^{2+} channels, TRPM proteins), and 2. Various regulatory molecules that modulate the NCX and other transporters in the PLasmERosomes such as protein kinases, PIP_2 (phosphatidylinositol bis-phosphate) and calmodulin. Not surprisingly, then, these mechanisms may also play prominent roles in pathophysiological processes (Blaustein, 1977), as discussed in the example below.

Linkage to hypertension and other heretical concepts

I learned from Alan Hodgkin that, if you perform careful, controlled experiments, you should be very confident about your own data. So, ignoring the naysayers, I continued to ponder the role of NCX in vascular smooth muscle, and even began to think that it might play a role in hypertension (Blaustein, 1974). This led me to propose a naïve, but useful hypothesis about the roles of an hypothetical endogenous cardiotonic steroid, its Na^+ pump receptor, and NCX, in the pathogenesis of salt-dependent hypertension (Blaustein, 1977). That was before NCX and the Na^+ pump were cloned and their isoforms discovered; and, of course, the endogenous cardiotonic steroid was then just a figment of our imagination.

Despite the previously mentioned skepticism of vascular biologists, however, this hypertension hypothesis aroused great interest in the idea of an endogenous ligand for the Na^+ pump ouabain binding site. In particular, it excited one young man, John Hamlyn, who was a pre-teenager in Plymouth, England, when we discovered the NCX there. John came to my laboratory in 1980, as a post-doctoral fellow, with the idea of trying to purify this hypothetical agent. Ignoring the enormous difficulties, the fierce competition, and the repeated funding rejections from NIH, our team (John and our colleagues at the Upjohn Company) plunged ahead. In 1991, we purified from two tons of human plasma, and identified, by mass spectroscopy, an endogenous compound that, astonishingly, is indistinguishable from plant ouabain, i.e., “endogenous ouabain” or EO (Hamlyn et al., 1991). That paper, too, was rejected by *Nature*: one reviewer had a “*gut feeling*” that our result was an artifact, although another reviewer offered to write a “*News and Views*” article about the discovery. The *Nature* editor, John Maddox, had just taken the heat for publishing articles on “cold fusion” and “infinite dilution”; he apparently didn’t want to take a chance on being burned once more. Fortunately, our friend, Joseph Hoffman, a Na^+ pump expert, agreed to communicate the manuscript to *PNAS*.

Again, many colleagues were skeptical (and some still are), even though the identification of EO was replicated in bovine adrenal and hypothalamic samples by three distinguished groups (Tamura et al., 1994; Schneider et al., 1998; Kawamura et al., 1999). Nevertheless, our Upjohn co-workers, who performed the original mass spectrometry, convinced us of the correctness of the result. Although the EO biosynthetic pathway has not yet been elucidated (funding bodies are not interested in such mundane studies), John Hamlyn and colleagues demonstrated net synthesis of EO by primary cultured bovine adrenocortical cells (Laredo et al., 1994). Furthermore, plasma EO levels are very low in adrenalectomized rodents (Hamlyn et al., 1991) and in humans with adrenocortical insufficiency (Sophocleous et al., 2003).

Ideas about the involvement of EO in the pathogenesis of some forms of hypertension have come a long way since my first, rather simplistic (but perhaps not so far-fetched) thoughts on the subject (Blaustein, 1977). Recent work is summarized to two reviews (Blaustein and Hamlyn, 2010; Blaustein et al., 2011).

NCX: a key player in the pathogenesis of hypertension?

I have already alluded to the central role of NCX in Ca^{2+} signaling that results from its co-localization with certain (ouabain-sensitive) Na^+ pumps and ROCs and SOCs in PM microdomains at PM-S/ER junctions (PLasmERosomes). This fit with the simplistic notion that, if circulating EO levels are elevated in patients with hypertension (Rossi et al., 1995), we would expect the PLasmERosome mechanisms to amplify the Ca^{2+} signals. In fact, this is precisely what we observed in isolated, pressurized rodent small arteries: Ca^{2+} signals and myogenic constriction were augmented by ouabain with an EC_{50} of ~1 nM (Zhang et al.,

2005). Could this account for the increased peripheral vascular resistance and elevation of blood pressure, as I initially proposed (Blaustein, 1977)?

Alas, life is not so simple! (A good thing, because that's what keeps us scientists in business.) As my colleague, Vera Golovina discovered, the situation in hypertension is much more complex. She and her associates were studying arterial smooth muscle acutely isolated from rats in which hypertension was induced by prolonged subcutaneous infusion of ouabain ("ouabain hypertension"). They found that NCX1 (the vascular isoform) and TRPC6 protein (a component of ROCs) were greatly over-expressed in the myocytes, and that this was reflected in augmented Ca^{2+} signaling (Pulina et al., 2010a). The effects were not due to the elevation of blood pressure: both the protein up-regulation and the augmented Ca^{2+} signaling were also induced in primary cultured normal rat arterial myocytes incubated with 100 nM ouabain for 72-96 hr. [In an important twist, digoxin did not up-regulate NCX1 or TRPC6 expression (Zulian et al., 2010b) and, unlike ouabain, it does not induce hypertension in rats (Manunta et al., 2000) – but that's another, as yet incomplete, story.]

Vera and her colleagues also found that NCX1 and TRPC6 were both markedly up-regulated in arterial myocytes from Dahl salt-sensitive rats fed a high salt diet to induce hypertension (Table 2), and in myocytes from Milan hypertensive rats, but not the normotensive strain (Zulian et al., 2010a). In fact, arterial myocyte NCX1 and either TRPC6 or TRPC3 (another component of ROCs) are up-regulated in all models of hypertension that have been tested to date (Table 2). An editorial commentary (Giachini and Tostes, 2010) on the articles from Vera's laboratory stated that, "*Given the… interrelationship among TRPC6, Na^+ influx, NCX, and Ca^{2+}* influx, it seems that this is… the key to better understand the role of Na^+ in hypertension, vascular reactivity, and blood pressure regulation". Vindication! This is the diametric opposite of the view that, "The Na/Ca exchanger does not play an essential role in the regulation of cytoplasmic Ca^{2+} *in smooth muscle*" (Somlyo et al., 1986). The up-regulation of NCX1 and TRPC6 expression may, in fact, be a key mechanism underlying the phenomenon of "whole body autoregulation" (Guyton, 1989) that sustains the increased peripheral vascular resistance and elevated BP in hypertension.

This, of course, is not the end of the story, but just the beginning of the next chapter. The fun in research is that every new discovery leads to new questions. In this case, we must now ask: "What is the mechanism by which ouabain up-regulates NCX1 and TRPC6 expression?" The up-regulation appears to be mediated by a novel activity of Na^+ pumps first elucidated by Zi-Jian Xie, namely ouabain-induced activation of protein kinase signaling cascades (Xie and Askari, 2002; Liu et al., 2007; Liu and Xie, 2010; Liu et al., 2011). [Zi-Jian's new idea, that Na^+ pumps could do something other than pump Na^+ and K^+, was greeted with profound skepticism – and I was originally one of the skeptics – but the data speak for themselves.] It is the anticipation of learning something new at any moment that keeps me young and keeps me going. That is the lesson I most enjoy conveying to students; it is the essence (and the excitement) of scientific research. The fact that such moments are not every-day occurrences makes them so special.

In closing:

I have been most fortunate to have had the opportunity to engage in this exciting adventure. I was well-prepared by my teachers, but I could not have had such success without wonderful students, colleagues and collaborators. My wife, Ellen, in addition to providing extraordinary support, also played a critical part (mentioned above) that neither she nor I could have imagined a priori. And, of course, none of this would have been possible without the many years of financial support from the National Institutes of Health and the American Heart Association.

> "After all, in spite of opinion, prejudice, or error, Time will fix the real value upon this discovery, and determine whether I have imposed upon myself and others, or contributed to the benefit of science and mankind."
>
> William Withering, *"An Account of the Foxglove and Some of its Medical Uses"* Birmingham, England, July 1, 1785.

Acknowledgments

I thank Suzanne Ventura for assistance with the manuscript. Supported by NIH/NHLBI grants HL-045215, HL-078870 and HL-107555.

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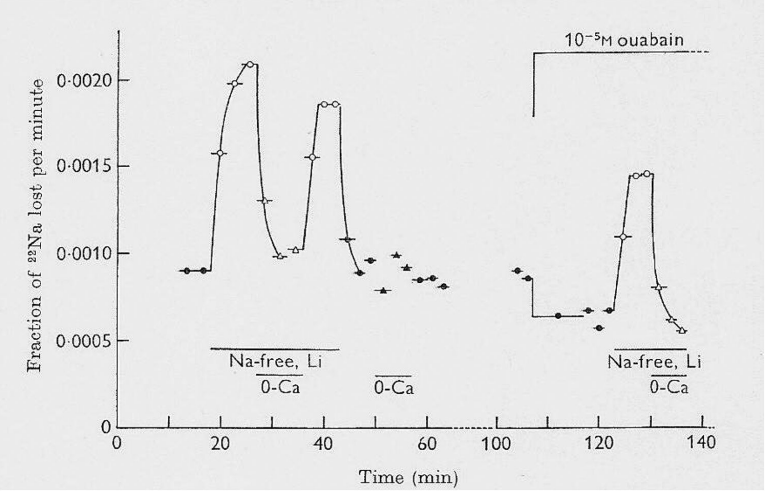

Figure 1.

Reduction of $[Na^+]_o$ activates a large Ca_o-dependent, ouabain-resistant $^{22}Na^+$ efflux in squid axons with high $[Na^+]_i$. Replacement of 460 mM NaCl in the artificial sea water (ASW) by LiCl greatly increased $^{22}Na^+$ efflux, measured as the fraction of $^{22}Na^+$ lost per minute. The increment was abolished by removal of the 11 mM $CaCl_2$ in the ASW ($MgCl_2$ was increased from 55 to 66 mM). This is evidence of "Na_o-Ca_o antagonism", and indirect evidence for Na^+/Ca^{2+} exchange. Ouabain (10^{-5} M) reduced the $^{22}Na^+$ efflux in control Na ASW (i.e., it inhibited the Na^+ pump), but it had no effect on the large Ca_o-dependent $^{22}Na^+$ efflux in Li ASW; right). Reprinted from Baker et al., 1969, with permission.

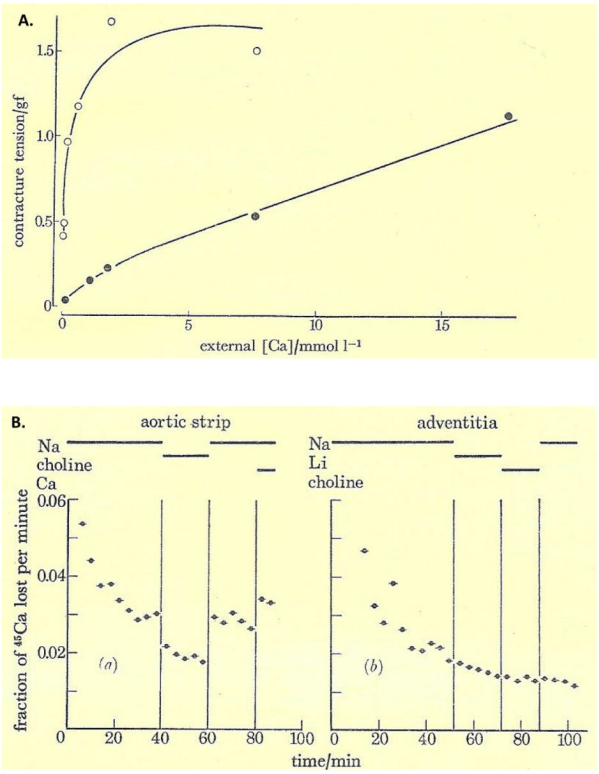

Figure 2.

(A) Effect of the external Ca^{2+} concentration, $[Ca^{2+}]_o$, on contraction of rabbit aorta. Contractile tension (gf) is increased by reduction of $[Na^+]_o$. Either 80% (filled circles) or 100% (open circles) of the NaCl in the standard medium (137 mM) was replaced by equiosmotic sucrose. The greater effectiveness of low $[Ca^{2+}]_o$ in inducing contraction when $[Na^+]_o$ was lowered is evidence of "Na_o-Ca_o antagonism". **(B)** Reduction of $[Na^+]_o$ decreases $^{45}Ca^{2+}$ efflux (fraction of $^{45}Ca^{2+}$ lost per min) in a rabbit aortic strip (left), but not in the isolated adventitia (right). The NaCl in the standard medium (137 mM) was replaced by equimolar LiCl or choline-Cl during the periods indicated by the bars at the top. To avoid influx of Ca^{2+} and dilution of intracellular tracer $^{45}Ca^{2+}$, the efflux (extracellular) solutions were Ca-free and contained 0.5 mM EGTA, except during the period indicated by the "Ca" bar at the end of the experiment on the left. The Na_o-dependent $^{45}Ca^{2+}$ efflux in intact intact aortic strip is evidence of Na^+/Ca^{2+} exchange. Reprinted from Reuter et al., 1973, with permission.

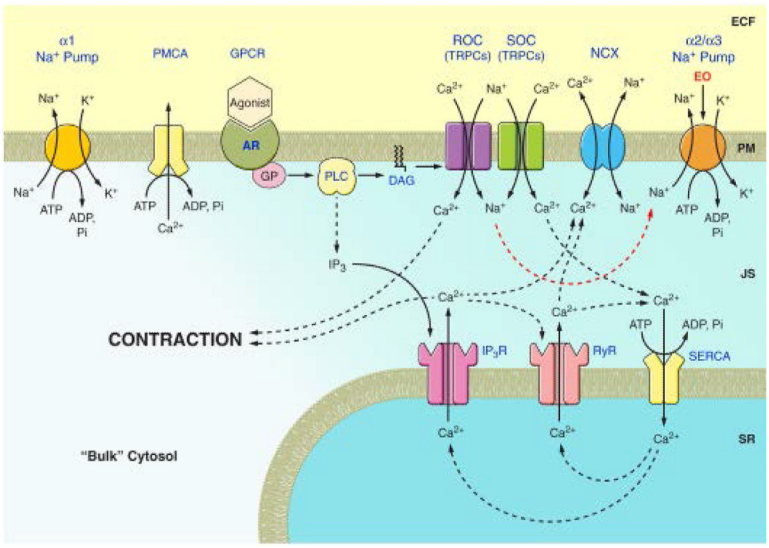

Figure 3.

Diagram of ouabain/EO-regulated α2 Na^+ pump-modulated Ca^{2+} signaling at the plasma membrane-sarco/endoplasmic reticulum junction (PLasmERosome) in arterial smooth muscle. Endogenous ouabain (EO) reduces Na^+ extrusion by the α2 Na^+ pump, thereby increasing local [Na^+] in the junctional space (JS) and reducing Ca^{2+} extrusion by the Na/Ca exchanger (NCX). This enhances Ca^{2+} signaling and, in arterial smooth muscle, contraction. ADP, adenosine diphosphate; AR, agonist receptor; ATP, adenosine triphosphate; DAG, diacylglycerol; ECF, extracellular fluid; GP, G-protein; GPCR, G-protein coupled receptor; IP_3, inositol trisphosphate; IP_3R, IP_3, receptor; Pi, inorganic phosphate; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+} pump; SERCA, sarco-/endoplasmic reticulum Ca^{2+} pump; SR, sarcoplasmic reticulum; SOC store-operated channel, and ROC, receptor-operated channel (both composed of TRPC proteins). Reprinted from Blaustein et al., 2011, with permission.

Table 1

Effects of external cations and of ouabain on $^{45}Ca^{2+}$ influx in squid axons. Reduction of $[Na^+]_o$ increased $^{45}Ca^{2+}$ influx. This is evidence of "Na_o-Ca_o antagonism". The increase in Ca^{2+} influx was much greater in the axons from refrigerated squid mantles (with a high $[Na^+]_i$) than in axons from live squid (with a lower $[Na^+]_i$). This is direct evidence of Na^+/Ca^{2+} exchange. Data from Baker et al., 1969.

External solution	Ca influx (p-mole/cm^2 sec) Mean ± S.E.	Range	*n*
A. Axons from refrigerated mantles			
Collected results*:			
Na sea water, 0 and 10-K, ± ouabain	0.15 ± 0.02	0.04 − 0.57	30
Li sea water, 0 and 10-K, ± ouabain	4.33 ± 0.43	0.90 − 9.50	29
Dextrose sea water, 0 and 10-K, ± ouabain	2.46 ± 0.29	0.80 − 5.30	15
B. Axons from live squid			
10-K, Na sea water	0.23	–	1
10-K, Li sea water	0.66 ± 0.48	0.16 − 1.60	3

n is the number of axons in each group.,

*Li sea water contained 460 mM LiCl in place of NaCl; dextrose sea water contained 720 mM dextrose in place of 460 mM NaCl. In K-free ("0K") sea waters, the 10 mM KCl was replaced by 10 mM NaCl or LiCl, or 20 mM dextrose. Because neither replacement of external K^+ nor addition of 10^{-5} M ouabain affected the Ca^{2+} influx, the 0K and ouabain treatment data were not separated.

Table 2

Expression of Na/Ca exchanger-1 (NCX1) and some TRPC protein components of receptor-operated channels (ROCs) is increased in several hypertensive animal models and in human primary pulmonary hypertension. Modified from Blaustein et al, 2011).

	Hypertension	Artery Smooth Muscle			Reference
		NCX1	ROC		
			TRPC3	TRPC6	
1	Ouabain* (vs vehicle & digoxin)	↑		↑	(Pulina et al., 2010b; Zulian et al., 2010b)
2	DOCA-salt*	ND**		↑	(Bae et al., 2007)
3	Milan hypertension* (vs Milan NT)**	↑		↑	(Zulian et al., 2010a)
4	SHR (vs WKY)**	↑	↑		(Taniguchi et al., 2004; Liu et al., 2009)
5	Dahl salt-sensitive/high (vs low) salt*	↑		↑	VA Golovina (unpublished)
6	$NCX1^{SM.Tg/Tg}$ **	↑		↑	(Iwamoto et al., 2004); J Zhang & MP Blaustein (unpublished)
7	Angiotensin II	↑		ND**	L Chen, M Li & MP Blaustein (unpublished)
8	Human primary pulmonary hypertension	↑	↑	↑	(Yu et al., 2004; Zhang et al., 2007a; Zhang et al., 2007b)

* Hypertension associated with elevated plasma ouabain levels.

** Not determined.

** Abbreviations: NT, normotensive control; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto normotensive control for SHR; $NCX1^{SMTg/Tg}$, smooth muscle-specific NCX1 knockout mouse (Zhang et al., 2010).