Article Addendum

**Ca²⁺ signaling in smooth muscle**

TRPC6, NCX and LNats in nanodomains

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Following the recent observation of localized cytosolic subplasmalemmal [Na⁺] elevations (LNats) in rat aortic smooth muscle cells, we discuss here the current evidence for the structural and molecular roles of cytosolic nanodomains at close junctions of the plasma membrane (PM) and sarcoplasmic reticulum (SR) in the generation of LNats. These junctions, the loss of which might contribute to vascular aging and disease, provide a platform for ion metabolism signalplexes and the interaction of localized Na⁺ and Ca²⁺ gradients. We moreover suggest the existence in the junctions of a Na⁺ diffusional barrier as a necessary condition for the generation of LNats. LNats are likely a fundamental feature of near membrane ion signaling in many cell types, and their discovery offers new possibilities for elucidating the mechanism, function and pathogenesis of Na⁺ and Ca²⁺ signaling nanodomains.

Introduction

We recently reported the first observation of transient, peripherally localized [Na⁺] elevations (LNats) in agonist-stimulated smooth muscle (SM) cells, using the Na⁺ dye CoroNa green.1 In this addendum, we attempt to integrate this new knowledge into the current understanding of how junctional membrane complexes provide a structural and mechanistic basis for localized ionic signaling in SM, with an emphasis on relating LNats to the reversal of the plasma membrane (PM) Na⁺/Ca²⁺-exchanger (NCX).

The NCX is a unique integrator of localized changes in [Ca²⁺]i and [Na⁺]i immediately beneath the PM and an important pathway for Ca²⁺ entry that refills sarcoplasmic reticulum (SR) Ca²⁺ stores in stimulated SM. The direction of NCX activity is determined by membrane potential and transmembrane Na⁺ and Ca²⁺ gradients.² At rest, forward NCX extrudes Ca²⁺ from the cells, while during agonist-induced stimulation NCX reversal mediates the SR refilling that is required to maintain an oscillatory Ca²⁺ signal.³ However, under physiological conditions reversal of the NCX (to Ca²⁺ entry mode) requires [Na⁺]i elevations of a magnitude that is not commonly observed in the bulk cytosol of stimulated SM. Our group's discovery of LNats of much higher local [Na⁺] than the bulk [Na⁺], resolves the above paradox and highlights the need to better understand the structural and dynamic components that shape the LNats as well as the localized Ca²⁺ gradients. These LNats were causally associated with Na⁺ entry through TRPC6, which we hypothesize to be peripherally clustered in patches of the PM that overlie the SR and form the external boundary of 20-nm-wide cytoplasmic nanodomains bound internally by sheets of peripheral SR (PM-SR junctions).

Cytoplasmic Nanodomains and Functional Linkage of Ion Transporters in Smooth Muscle

The concept of localized signaling by virtue of signalplexes is currently gaining wide acceptance. The term is applied to the structural alignment of a varied number of receptors, transducers, enzymes and translocators by means of membrane associated scaffolding proteins.⁴ It is important to realize that the signaling molecules are not only confined to limited membrane domains, but that they are bathed in an equally specialized cytosolic nanodomain, characterized by local ionic concentration gradients. Here we discuss the specific example of the SM cell PM-SR junction, which contains a critical nanodomain for oscillatory Ca²⁺ signaling. Close associations between peripheral SR and the PM were clearly demonstrated in visceral SM by Gabella³ and in vascular SM (VSM) by Devine and Somlyo.⁶ The first evidence that diffusion in the nanodomains between PM and SR and the bulk cytoplasm was restricted came from work by van Breemen et al. in the late seventies showing that Ca²⁺ entry from the extracellular space could be preferentially routed to the SR,⁷,⁸ without prior equilibration with the bulk cytoplasm. This same 1978 review⁸ also introduced the class of receptor-activated channels (RAC,
subsequently referred to as ROC\(^9\)), which includes the TRPC6 shown to generate LNats in VSM cells.\(^1,10\) Since as much as 100 micromoles of Ca\(^{2+}\) could be transported into and out of a liter of cells by external Na\(^+\) depletion and subsequent repletion, we proposed the involvement of NCX in the preferential transport between the extracellular space and SR lumen in both directions.\(^8,11\) By 1986 we had developed the superficial Ca\(^{2+}\) buffer barrier theory that described cycling of Ca\(^{2+}\) between the SR lumen and extracellular space involving ionic diffusion across a restricted space between the PM and SR.\(^12\) In other words, the NCX at PM-SR junctions mediates both SR Ca\(^{2+}\) refilling during activation and SERCA assisted Ca\(^{2+}\) extrusion during rest.\(^13\) PMCA-mediated Ca\(^{2+}\) extrusion occurs in parallel, but from the bulk of the PM lying outside the PM-SR junctions.\(^14\)

Arnon and Blaustein showed that NCX blockade inhibited adrenergic activation of VSM and concluded that NCX reversal contributes to agonist-induced vasoconstriction.\(^15\) This fitted well with the earlier observation by Daniel\(^16\) that Na\(^+\) depletion abolished agonist-induced activation of SM. However, based on the generally accepted two-phase activation of smooth muscle (SR release followed by Ca\(^{2+}\) influx), NCX reversal could well directly supply Ca\(^{2+}\) to the myofilaments. It was Iino’s discovery\(^17\) that norepinephrine-induced VSM activation is based on repetitive Ca\(^{2+}\) waves that led us to show that NCX reversal was linked to SERCA-mediated SR Ca\(^{2+}\) refilling across the 20 nm wide PM-SR junctional space.\(^5,18\) Although most of the above was deduced from functional experiments, the latter conclusion was greatly strengthened by structural observations related to the localization of ion channels and transporters at junctions between cellular membranes. Moore and Fay first demonstrated extensive co-localization of NCX with calsequestrin, which is preferentially concentrated in the peripheral SR.\(^19\) They also showed co-localization between NCX and NKA, which was later shown by Blaustein to be the NKA\(\alpha_4\) isomer tethered to ankyrins in the junctional PM.\(^20,21\) This is important since the NKA\(\alpha_4\) has a low affinity for Na\(^+\) and therefore allows for a greater transient increase of [Na\(^+\)]\(_i\) within the junctional space, i.e., generation of the LNat that is crucial for NCX reversal.\(^22\)

**Figure 1.** Left: ultrastructural micrograph of a rabbit inferior vena cava smooth muscle cell region containing a PM-SR nanodomain. Right: the superimposed drawings illustrate the hypothesized causal link between Na\(^+\) entry, LNat generation and NCX reversal (and SR Ca\(^{2+}\) refilling, for completeness). A random walk diffusive trajectory of a representative Na\(^+\) ion is also shown (white trace), as well as one for a Ca\(^{2+}\) ion (blue trace). During vasoconstriction, NCX reversal is linked to SERCA-assisted SR Ca\(^{2+}\) refilling. (Maximum magnification 60,000x).

**Nanodomains Limit Diffusion and Guide Ion Fluxes**

How do nanodomains determine the flow of Na\(^+\) or Ca\(^{2+}\) from a channel or exchanger (the source) to its target transducer or pump (the sink), while preventing loss of ions to the bulk cytoplasm? The answer requires detailed knowledge of the ultra-structure of the PM-SR junction, which was obtained through 3D reconstruction of serial EM sections.\(^18\) Monte Carlo random walk simulations, using the observed shape and dimensions of the diffusion limiting space and the diffusion coefficient of Ca\(^{2+}\), provided estimates of the probabilities of Ca\(^{2+}\) either hitting its target site on SERCA within the apposing SR membrane or escaping into the bulk cytoplasm from the edge of the PM-SR junction. In this manner, we estimated that more than 90% of the Ca\(^{2+}\) entering the SM cells via reverse NCX would be taken up into the SR via SERCA, using the average geometry and prevalence of the PM-SR junctional nanodomains and previously published transport characteristics of NCX and SERCA. We are currently applying a similar modeling approach to study the diffusion of Na\(^+\) entering the junction through TRPC6 and generating the LNats responsible for reversing NCX and secondarily reloading the SR. With a diffusion coefficient of free Na\(^+\) in cytosol about three times greater than its Ca\(^{2+}\) counterpart, a quick calculation shows that Na\(^+\) diffusion alone (from even several TRPC6 channels) could not account for the observed [Na\(^+\)]\(_i\). Barring the existence of cytosolic Na\(^+\) buffers, we can hardly avoid the hypothesis that Na\(^+\) accumulation into LNats requires physical obstruction to diffusion. As originally shown by Devine and Somlyo,\(^6\) bridging structures are present in PM-SR nanodomains and are illustrated at high magnification in an interpretive electron micrograph in Figure 1. We propose that rather than serving as a transduction mechanism between outer membrane and SR as in skeletal muscle, the principal function of the connecting structures seen in figure 1 is to define the architecture of the nanodomain and slow the diffusion of Na\(^+\) and thus permit generation of LNats and NCX reversal.\(^1\)
Physiological Importance and Clinical Relevance of LNats

There is no doubt that the interaction of Na⁺ permeant channels with NCX is important for the function of all types of muscle, nerves and even vascular endothelium. In our previous communications, we showed that PM-SR junctions (likely functioning to generate LNats) are required for the localized reversal of the NCX that provides the Ca²⁺ entry to refill the SR during physiological asynchronous Ca²⁺ oscillations. There are strong indications that the complex function of the PM-SR junctions is diminished with disease. Sturek reported that superficial SR, decreased NCX activity and suppression of the superficial buffer barrier in a swine model of diabetes, and we have preliminary data indicating that the PM-SR junctions deteriorate with age and vascular disease.

Although LNats have thus far only been visualized in vascular smooth muscle, we speculate that they might be present in other cell types. In the neonatal heart the linkage of TRPC and NCX is very similar to that in SM, and in endothelial cells Ca²⁺ supplied by rev-NCX selectively/locally activates eNOS. Finally, the NCX appears to play a role in synaptic facilitation as Zucker has shown that the transient increase in [Na⁺] in the nerve terminals resulting from a tetanic pulse of action potentials can reverse NCX to induce presynaptic potentiation.

It seems therefore that spatially and temporally defined local Na⁺ elevations, like LNats, and their important connection to Na⁺ transporters in the PM as well as to NCX need to be regarded as one of the fundamental mechanisms underlying nanodomain facilitated Ca²⁺ signaling.

Conclusion

The discovery of LNats has opened new possibilities for elucidating the mechanism, function and pathogenesis of the interactions between Na⁺ and Ca²⁺ within cellular nanodomains, which is a crucial part of site and function specific ionic signaling. The observation that local [Na⁺] gradients can occur also suggests that a confined space on the scale of nanometers, and likely additional mechanisms to limit ionic diffusion, are a necessary stage on which Ca²⁺ signaling takes place.

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