PATHWAYS OF Ca^{2+} SIGNALING IN RECTAL GLAND ARTERY (RGA) OF *SQUALUS ACANTHIAS*

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Previous studies performed in the ventral aorta of the dogfish shark, Squalus acanthias, have shown that norepinephrine (NE) and endothelin B (EtB) are constrictors and atriopeptin and prostaglandins are dilators vascular rings (Evans et al. *Am. J. Physiol.* 165:659-664, 1999, 274:R1050-1057, 1998, *J. Exp. Biol.* 157:551-555, 1991). Because salt secretion by the rectal gland (RG) of the shark is crucial to maintaining volume and osmolar homeostasis, the modulators of blood flow to the RG likewise have an important impact on the function of the gland. We determined that NE increases $[Ca^{2+}]i$ and that vasoactive intestinal peptide (VIP) and atriopeptin (CNP) diminish NE-induced $[Ca^{2+}]i$ elevation in shark rectal gland artery (RGA) (Fellner, *MDI Bull.* 40, 2001). To examine the pathways of calcium signaling in RGA, we designed experiments to examine the contribution of calcium entry and of calcium mobilization in vascular smooth muscle (VSM) of RGA.

RGA was isolated and minced in ice cold shark buffer, pH 7.8, containing, in mMol/L, NaCl, 275, KCl, 4, $MgCl_2$, 3, Na_2SO_4, 0.5, KH_2PO_4, 1.0, $NaHCO_3$, 8, urea, 350, D-glucose, 5, TMAO, 72, and Hepes, ~2.5. The complete buffer contained 2.5 mM calcium, whereas no $CaCl_2$ was added to the nominally calcium-free buffer. VSM was loaded with the calcium sensitive fluorescent dye, fura-2AM and $[Ca^{2+}]i$ was measured using a Photon Technology International (PTI) Delta Scan. Nifedipine (10^{-6} M) was used to block L-type voltage-gated channels if present, thapsigargin (10^{-6} M) to inhibit sarcoplasmic reticulum (SR) Ca^{2+} ATP-ase, ryanodine (2 X 10^{-6} M) to stimulate the ryanodine receptor (RyR) and NE (10^{-6} M) to stimulate alpha adrenergic receptors.

In nominally calcium-free buffer, NE stimulated mobilization of Ca^{2+} from the SR, increasing baseline $[Ca^{2+}]i$ from 77 ± 22 nM to 116 ± 50 nM. Subsequent addition of calcium permitted calcium entry and further increased $[Ca^{2+}]i$ to 226 ± 42 nM (n = 7, $p < 0.05$ for both maneuvers). Figure 1 shows a representative tracing of RGA VSM in calcium free buffer. Following addition of NE, mobilization of Ca^{2+} from the SR causes an increase in [Ca2+]i. When external Ca^{2+} is provided, calcium entry causes a step increase in $[Ca^{2+}]i$. Inhibition of refilling of SR calcium by blocking the Ca2+ ATP-ase with thapsigargin raised $[Ca^{2+}]i$ from 81 ± 18 nM to 115 ± 16 nM. Addition of external calcium further increased $[Ca^{2+}]i$ to 213 ± 21 nM (n = 8, $p<0.05$).

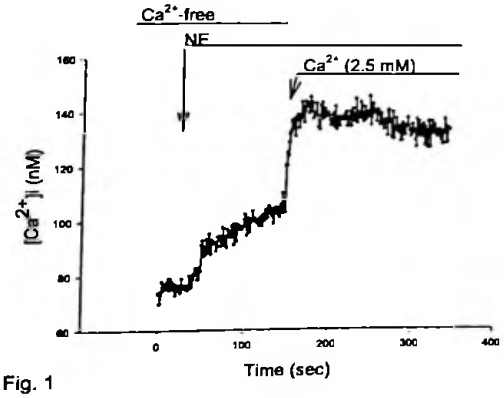


Fig. 1

Similarly, ryanodine caused mobilization of SR calcium, increasing baseline Ca^{2+} from 79 ± 14 nM to 115 ± 22 nM. Addition of external calcium raised [Ca2+]i to 209 ± 19 nM (n = 7, p< 0.05). The 3 different modalities of SR calcium depletion resulted in an approximately 36 nM increase in $[Ca^{2+}]i$. As well, calcium entry following the addition of external calcium was about 100 nM in each.

The typical control study for this experimental design is to add calcium to VSM in nominally calcium free medium in which no agonist has previously stimulated the cells. In mammalian VSM, the plasma membrane is "tight"...that is, there is not a passive leak of calcium into healthy cells. At most, there may be an entry leak of about 10 nM. To my surprise, there was a huge influx of calcium into the RGA VSM in the absence of NE, thapsigargin or ryanodine. Therefore, I performed a dose response to external calcium concentration varying from 0.8 to 5.3 mM (n = 29). Fig. 2 shows a linear increase in $[Ca^{2+}]i$ in that range. The mechanism responsible for the inward calcium leak has not yet been elucidated.

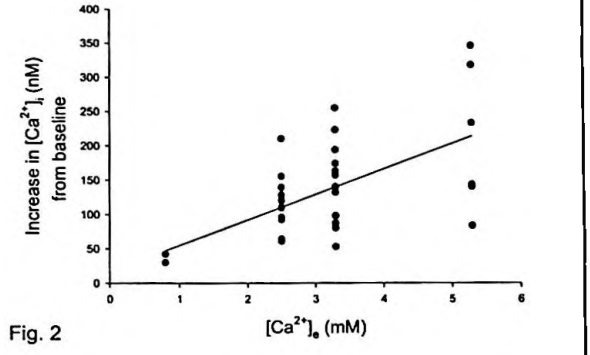
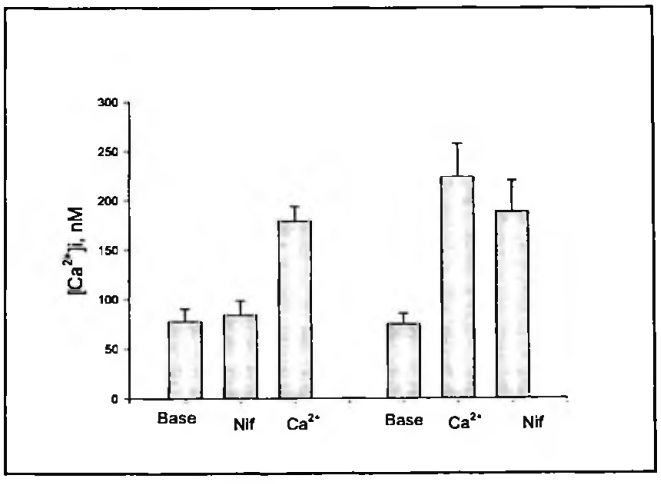


Fig. 2

Nickel is a non-specific inhibitor of several calcium entry pathways including Na/Ca exchange. In order for Na/Ca exchange to be responsible for calcium influx, it must operate in the reverse direction from its usual physiologic mechanism which is to remove calcium from the cytosol. Ni^{2+} (1.2 – 3.6 mM) inhibited calcium entry by about 50% whether it was given before or after the addition of calcium to the RGA VSM (n = 8 both pre and post).



To test the possibility that L-type voltage gated calcium channels were responsible for the observed calcium influx, RGA VSM was treated with nifedipine (10^{-6} M) either before or after the addition of external calcium (n = 9 and 10 respectively). As seen in Fig. 3, nifedipine caused a modest inhibition in the response of VSM to external Ca^{2+}, but these changes were not statistically significant.

Yet to be tested is the possibility that shark RGA VSM has a calcium sensing receptor similar to that found in certain epithelial cells and more recently in the spiral mediolar artery of the cochlea of the gerbil (Wonneberger, K. et al. *J. Membrane Biol.* 175:203-212, 2000). One might hyothesize

that RGA VSM has a uniquely sensitive SR which becomes depleted of calcium after several hours of exposure to calcium free buffer. SR calcium depletion would then set the stage for the opening of store-operated calcium entry channels which would be poised to transport calcium the moment it becomes available in the extracellular space. Each of these hypotheses is testable with specific pharmacologic probes.

These preliminary data show that NE mobilizes Ca^{2+} from the SR and accounts for about 50% of the total response of RGA VSM to NE compared to when it is studied in calcium containing buffer (Fellner, S. *MDI Bull. 40,* 2001). A large calcium influx pathway is present in VSM exposed to calcium free buffer for several hours. The mechanism of this entry is presently unclear. Supported by a New Investigator Award, MDIBL, Salisbury Cove, ME 04672. Laurel Parker was a Hancock County Scholar.