CALCIUM AND CALMODULIN SENSITIVITY OF PERMEABILIZED VASCULAR SMOOTH MUSCLE OF THE VENTRAL AORTA OF THE DOGFISH SHARK (SQUALUS ACANTHIAS)

David H. Evans and Evan Chipouras
Department of Zoology, University of Florida, Gainesville, FL 32611

The endothelium-free, vascular smooth muscle (VSM) rings from the ventral aorta of the dogfish shark is sensitive to a variety of putative, vasoactive substances (Evans and Weingarten, Bull. MDIBL 28: 4-5, 1989; Evans, J. Exp. Biol., in press, 1991) including the heavy metals Cd^{2+} and Ni^{2+} (Evans and Weingarten, Toxicology 61: 275-281, 1990). In an effort to more carefully define this system, which models the mammalian aortic, coronary, and cephalic VSM (e.g., Wolff "Functional Chordate Anatomy", D.C. Heath and Co, 752 pgs., 1991), we have investigated the effect of Triton X-100 treatment, which permeabilizes the sarcolemma and allows the determination of the direct effect of substances on the intracellular, contractile machinery without intervening sarcolemmal or intracellular, Ca^{2+}-store effects (Meisheri et al., In: Grover and Daniel "Calcium and Contractility", Humana Press, pp. 191-224, 1985). Such chemically "skinned" fibers have been used to demonstrate direct interactions between applied Ca^{2+} and various intracellular proteins, such as calmodulin (e.g., Ruegg et al., Basic Res. Cardiol. 78: 462-471, 1983). Indeed, the fact that calmodulin (CaM) applied to skinned fibers significantly increases the sensitivity to Ca^{2+} is the major datum suggesting the importance of the Ca^{2+}-calmodulin complex in the contractile process in smooth muscle (Meisheri et al., op. cit., 1985).

Isolated rings were prepared as described previously (Evans and Weingarten, op. cit., 1990). Ca^{2+} sensitivity of intact rings was determined by exposing rings to a concentration range of 0.05 to 12.8 mM $CaCl_2$ while recording tension, after first eliminating intracellular stores of Ca^{2+} by repeated contraction of rings with norepinephrine or carbachol in the presence of 50 mM KCl and 0.2 mM EGTA in Ca-free Ringer's (McMahon and Paul, Circ. Res. 56: 427-435, 1985). Chemical skinning and subsequent determination of the effect of external Ca^{2+} was performed using a protocol modified from Ruegg and Paul (Circ. Res. 50: 394-399, 1982). Mounted rings were exposed to 50 mM KCl in elasmobranch Ringer's to provide a measurement of response to the opening of voltage-gated Ca^{2+} channels, as an indicator of sarcolemmal integrity. This solution was then aspirated from the tissue chamber and 10 mls of Solution 1 (20 mM imidazole, 5 mM EGTA, 50 mM KCl, 150 mM sucrose) was added for 30 minutes. Solution 1 was then aspirated and Solution 2 (Skinning solution: Solution 1 plus 0.5 mM DTE (dithiothreitol), 2 µg/ml leupeptin and 0.75% Triton X-100) was added for 25-30 minutes. After the skinning period, Solution 2 was aspirated and the rings were rinsed four times in Solution 3 (20 mM imidazole, 4 mM EGTA, 0.5 mM DTE, 2 µg/ml leupeptin, 10 mM $MgCl_2$, 7.5 mM ATP and 1 mM NaN_3). The rings were then exposed to Solution 4 (Contracting solution: Solution 3 plus 50 mM KCl, and 10 mM phosphocreatine and 10 U/ml creatine phosphate as an ATP regenerating system). The lack of response to KCl in Solution 4 demonstrated the completeness of the skinning procedure. Concentration response curves for Ca^{2+} were then generated by adding aliquots of $CaCl_2$ to Solution 4 to produce a concentration range of 0.5 to 3.0 mM. In another series of experiments, we tested the effect of addition of 1 µM calmodulin (CaM; bovine brain, Sigma) on the Ca^{2+}-induced contraction induced in Solution 4. In both series, 0.1 mM carbachol was added at the end of the experiment to also assess the completeness of sarcolemmal permeabilization. In all experiments, the muscarinic agonist was without effect, indicating functional removal of the sarcolemma. Actual free Ca^{2+} concentrations in the Ca-EGTA buffer solutions (Solution 4) were calculated using the computer program of Chang et al. (Comp. Biol. Med. 18: 351-366, 1988). Urea and trimethylamine oxide were not added to any of the

experimental solutions because it has been shown that such additions do not enhance the response of skinned, striated muscle of the dogfish Scyliorhinus canicula (Altringham et al., J. Exp. Biol. 96: 443-445, 1982). Apparent EC_{50}'s were calculated by eye after graphing.

TABLE 1

The effect of chemical skinning and calmodulin on the apparent EC_{50} of Ca^{2+} activation of VSM from the shark ventral aorta

TREATMENT	APPARENT EC_{50}
Intact	500 μM (N = 4-10)
Skinned	10 nM (N = 6)
Skinned + CaM	6 nM (N = 5)

The sensitivity of the intact VSM from the shark aorta to applied Ca^{2+} is in the same range as that described for intact mammalian VSM (ca. 1 mM). Chemical skinning with Triton X-100 produced a substantial increase in sensitivity (50,000-fold) corroborating data on mammalian VSM (Meisheri et al., op. cit., 1985). In fact, the Ca^{2+} sensitivity of chemically skinned fibers of the shark aortic VSM is significantly greater than that described for skinned mammalian VSM (threshold ca. 50 nM, EC_{50} ca. 1 μM; Meisheri et al., op. cit., 1985). This increased sensitivity to Ca^{2+} after chemical skinning is generally assumed to be a direct effect of Ca^{2+} on the intracellular proteins involved in the contractile system (calmodulin, myosin light chain kinase, myosin, actin), without complicating effects of Ca^{2+} channel sensitivity in the sarcolemma or sarcoplasmic reticulum (both of which are permeabilized by the Triton X-100). Thus, our results demonstrate that the chemically skinned shark VSM can be used to study direct effects of modulators of the intracellular proteins that may be involved in the contractile process. The apparent EC_{50} of Ca^{2+} activation in this system is significantly below the 100-200 nM intracellular $[Ca^{2+}]$ described for mammalian vascular smooth muscle using Fura-2 (e.g. Williams et al., Nature 318: 558-561, 1985), suggesting that intracellular $[Ca^{2+}]$ may be lower in shark VSM than mammalian VSM. Interestingly, Table 1 also demonstrates that addition of 1 μM CaM does not appear to increase the Ca^{2+} sensitivity in this system as it does in mammalian skinned smooth muscle. For instance, Ruegg and Paul (op.cit., 1982) found that 4 μM CaM decreased the EC_{50} of Ca^{2+} activation in the skinned hog coronary VSM from ca. 1 μM to 100 nM, and Sparrow et al. (FEBS Lett. 125: 141-145, 1981) found that as little as 0.5 μM CaM decreased the EC_{50} from 5 μM to 0.35 μM in skinned fibers of guinea pig taenia coli. Therefore, our data indicate that intracellular [CaM] is not rate-limiting for Ca^{2+} activation in the skinned shark VSM. This may be due to limited loss of CaM from the skinned fibers because of the short skinning period and low temperature (12°C) in our protocol. This conclusion is supported by the extreme sensitivity of the skinned shark VSM to external $[Ca^{2+}]$. Gardner et al. (Pflugers Arch. 414: 484-491, 1989) have recently shown that exposure of rat caudal artery strips to 0.5% Triton X-100 for one hour at 24°C resulted in loss of 50% of the immunoreactive CaM.

Thus, it appears that the skinned, VSM ring from the spiny dogfish is a viable model to study intracellular events in vascular smooth muscle contraction, such as the roles of various intracellular components and effectors such as Ca^{2+} and heavy metals. (Supported by NSF DCB 8916413 to DHE and NIEHS-P30-ESO3828-05 to the Center for Membrane Toxicity Studies).