ELECTROPHORETIC DETERMINATION OF INTERACTIONS BETWEEN CALMODULIN, Ca^{2+}, Cd^{2+}, AND Ni^{2+}

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Since our recent studies have suggested that both Cd^{2+} and Ni^{2+} can interact with calmodulin (CaM) in the cytoplasm of vascular smooth muscle (VSM) from the shark ventral aorta (Evans et al., this volume), we initiated a study of these putative interactions using the diagonal SDS-PAG electrophoresis technique developed by Otter and Galgoci (Cell Motility and Cytoskeleton 11: 215-216, 1988). This procedure recently has been used to demonstrate interactions between Cd^{2+} and a Ca^{2+}-binding protein in scallop sperm flagellum (Otter, Bull. MDIBL 29: 20-23, 1990).

Ventral aortic VSM from the spiny dogfish, Squalus acanthias, was homogenized in 2 volumes of ice-cold elasmobranch Ringer's (ERS) containing 1 µM PMSF (Phenylmethylsulfonyl Fluoride) using a Tissue Tearor (Biospec Products) on high speed. The homogenate was centrifuged at 16,000 g at 4°C for 30 minutes in microfuge tubes. The supernatant was then diluted 1:1 with 2 X Laemmli buffer (0.25 M Tris-HCl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol (β-MeSH), 0.05% bromophenol blue, pH 6.8), heated at 100°C for 90 sec, then stored at 4°C. Aliquots of 10 µl per well produced good bands on 16% PAGE gels. Shark epaxial skeletal muscle extracts were homogenized in 4 volumes of ERS + phenylmethylsulfonyl fluoride, then centrifuged and evaporated nearly to dryness in a SpeedVac (Savant). The sample was resuspended in approximately four volumes of 1 X Laemmli buffer and stored at 4°C. These extracts were loaded at 5 µl/lane. Low molecular weight standards (Sigma, SDS-7; 14.2-66 kDa) or bovine brain CaM (Calbiochem) were dissolved in 1X Laemmli buffer, heated at 100°C for 2 minutes, stored at 4°C until used at 5 µl/lane or 2 µl/lane, respectively. Samples were run on 0.75 mm, 16% PAGE gels (4% stacking gel) using the formulation provided with the Mini-PROTEIN II cell (Bio-Rad). For the second, diagonal gel, 1.00 mm, 16% gels (no stacking) were used. Lanes from the first gel were cut out, soaked in SDS reducing buffer (1.25 M Tris-HCl, 2% SDS, 10% glycerol, 5% β-MeSH, 0.025% bromophenol blue, pH 6.8) for 15-30 minutes, and sealed on top of the 16% gel with 1% low melting-point agarose. All gels were run at 25 mA for 1.5 hr, and stained with Coomassie blue. For examining the effect of addition of Ca^{2+}, Cd^{2+}, or Ni^{2+}, EDTA (100 µM) was added to the first gel and EDTA (10 µM) plus metal (200 µM) was added to the second gel before polymerization. We found it necessary to add the small amount of EDTA to the second gel because preliminary studies demonstrated that even nano-pure water contained sufficient contamination to move CaM off the diagonal. The metal (200 µM) and 10 µM EDTA were also added to the running buffer for the second gel.

Fig. 1 is an example of the first-dimension gel (100 µM EDTA) with SDS-7 standards (lanes 1,2) and various extracts. It is clear that CaM migrates (lanes 3,4) as a distinct band with a mobility near to the 20.1 kDa soybean trypsin inhibitor standard. Preliminary studies indicated that, as expected with its ca. 17,000 kDa M_r, CaM migrated between that standard and the 14.2 kDa bovine milk α-lactalbumin standard when EDTA was not present. Addition of CaM to the standards solution (lanes 5,6) resulted in a substantial increase in the band at 20.1 kDa. The aortic VSM (lanes 7,8) extract displays distinct bands in this molecular weight range, with a major band at ca. 45 kDa, presumably actin. A relatively indistinct band was visible at ca. 20 kDa, which was significantly enhanced when CaM was added to this extract (lanes 9,10). Shark skeletal muscle displayed many more bands, with a substantial actin band, and a distinct band in

the 20 kDa region. Attempts to enhance the rather indistinct, 20 kDa band in VSM (presumably CaM) by loading the gel with larger extract volumes were unsuccessful, and we were unable to demonstrate a shift of this band off the diagonal in the second dimension, when 200 μM Ca^{2+} or Cd^{2+} were present (see Fig. 2b, Fig. 3b). These results indicate that extracts of shark aortic VSM do not contain sufficient CaM to allow the determination of interactions between CaM and putative metal ligands with this system. Therefore, we used an alternative approach to examine CaM-ligand interactions by running lanes of aortic extract + added CaM (lane 9,10 in Fig. 1) in the second dimension containing 200 μM of either Ca^{2+}, Cd^{2+}, or Ni^{2+} (in the presence of 10 μM EDTA). Figs. 2 & 3 demonstrate that both Ca^{2+} and Cd^{2+} are able to produce a shift of the added CaM off the diagonal; Ni^{2+} was without effect (data not shown). The diagonal on the right of each run in Figs. 2 & 3 is aortic extract without added CaM, in the presence of 200 μM metal.

These data indicate that interactions between CaM added to shark aortic VSM extracts and both Ca^{2+} and Cd^{2+} can be demonstrated using a two-dimensional, SDS-PAG electrophoresis, supporting the hypothesis (Evans et al., this volume) that one site of the vasomotor action of Cd^{2+} toxicity may be direct stimulation of intracellular CaM. However, we could not demonstrate an interaction between Ni^{2+} and CaM electrophoretically, despite our concurrent finding that added CaM enhances the vasoconstrictive action of Ni^{2+} on skinned VSM fibers (Evans, et al., ibid). (Supported by NSF DCB 8916413 to DHE, NIEHS-P30-ESO3828-05 to the Center for Membrane Toxicity Studies, a Pew Undergraduate Faculty Fellowship to AJV, and a Markey Fellowship to T.O.).

Fig. 1. SDS PAG electrophoresis of low M_r standards, calmodulin, shark aortic extracts, and shark epiaxial muscle extracts.

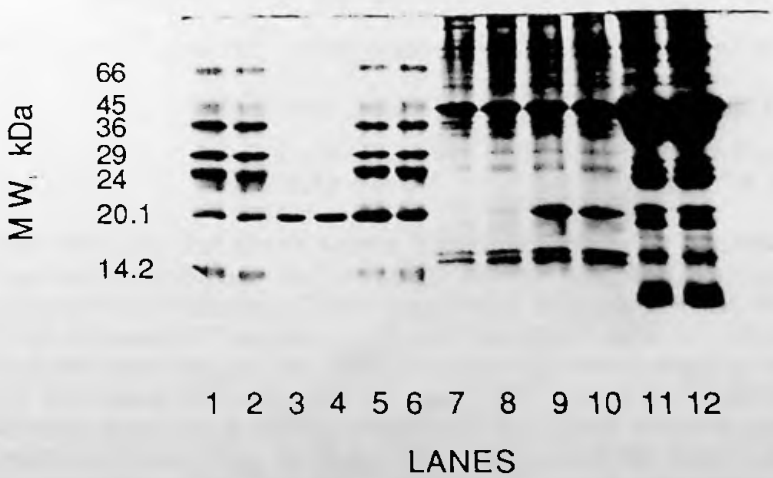

Fig. 2. The effect of 200 μM Ca^{2+} on the mobility of calmodulin added to shark aortic tissue extract. See text for details.

Fig. 3. The effect of 200 μM Cd^{2+} on the mobility of calmodulin added to shark aortic tissue extract. See text for details.