Subcellular Distribution of Calcium-binding Proteins and a Calcium-ATPase in Canine Pancreas

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Abstract. Using a ^{45}Ca blot-overlay assay, we monitored the subcellular fractionation pattern of several Ca binding proteins of apparent molecular masses 94, 61, and 59 kD. These proteins also appeared to stain blue with "Stains-All." Additionally, using a monoclonal antiserum raised against canine cardiac sarcoplasmic reticulum Ca-ATPase, we examined the subcellular distribution of a canine pancreatic 110-kD protein recognized by this antiserum. This protein had the same electrophoretic mobility as the cardiac protein against which the antiserum was raised. The three Ca binding proteins and the Ca-ATPase cofractionated into the rough microsomal fraction (RM), previously shown to consist of highly purified RER, in a pattern highly similar to that of the RER marker, ribophorin I. To provide further evidence for an RER localization, native RM were subjected to isopycnic flotation in sucrose gradients. The Ca binding proteins and the Ca-ATPase were found in dense fractions, along with ribophorin I. When RM were stripped of ribosomes with puromycin/high salt, the Ca binding proteins and the Ca-ATPase exhibited a shift to less dense fractions, as did ribophorin I. We conclude that, in pancreas, the Ca binding proteins and Ca-ATPase we detect are localized to the RER (conceivably a subcompartment of the RER) or, possibly, a structure intimately associated with the RER.

EVENTS leading to the production of the second messenger, inositol-1,4,5-triphosphate (IP_3),[1] upon hormonal and neurotransmitter stimulation of plasma membrane receptors have been well described (4). IP_3, in turn, mediates release of calcium from an intracellular store, the site of which has been the subject of debate.

It has recently been suggested that the site of intracellular calcium uptake, storage, and IP_3-mediated release might be distinct from the RER, an organelle termed the "calciosome (10)." The evidence supporting this notion is primarily immunolocalization data using antisera against skeletal muscle calsequestrin and the sarcoplasmic reticulum Ca-ATPase, supplemented by data that IP_3 binding and IP_3 mediated calcium release may fractionate differently from ER markers (10, 19, 22). Nevertheless, a number of other studies suggest that the site of intracellular calcium uptake and release is the RER (2, 9).

We carried out a subcellular fractionation of canine pancreas, then examined the distribution of several putative calcium-binding proteins and a protein recognized by antisera against the cardiac sarcoplasmic reticulum Ca-ATPase. We have found these proteins to cofractionate through the entire fractionation scheme with a well-defined RER marker. Moreover, we demonstrate that, on isopycnic sucrose gradients, the calcium binding proteins and the protein cross-reactive with the Ca-ATPase cofractionate with the RER marker, and behave in a manner predicted for proteins associated with the RER, particularly when the rough microsomes are stripped of ribosomes with puromycin/high salt.

1. *Abbreviations used in this paper*: IP_3, inositol-1,4,5-triphosphate; RM, rough microsome.

Materials and Methods

Materials

^{45}Ca (10–40 Ci/g) and [^{125}I]protein A were from Dupont. Antisera were gifts from the following investigators: Kevin Campbell, University of Iowa (canine cardiac sarcoplasmic reticulum Ca-ATPase monoclonal antibody); David Meyer, Univ. of California, Los Angeles (ribophorin I polyclonal). Purified rabbit muscle calsequestrin was a generous gift of P. Volpe (University of Texas, Medical Branch-Galveston). The amylase assay kit was purchased from Sigma Chemical Co. (St. Louis, MO).

Subcellular Fractionation and Preparation of Rough Microsomes

The canine pancreatic homogenate was fractionated as previously described (16, 17). For studies of the distribution of the Ca-ATPase and various calcium binding proteins, we used approximately "equivalent" fractions derived from the same preparation. Accordingly, an amount of post-mitochondrial supernatant (Fig. 2, lane *3*) analyzed was the amount calculated to yield (in our fractionation scheme) the amount of "post-microsomal" supernatant, "smooth" microsomal interface, cushion and rough microsomal pellet analyzed in lanes *5–8*.

Isopycnic Centrifugation

Freshly prepared RM, or "stripped RM" (with 1 mM puromycin/500 mM

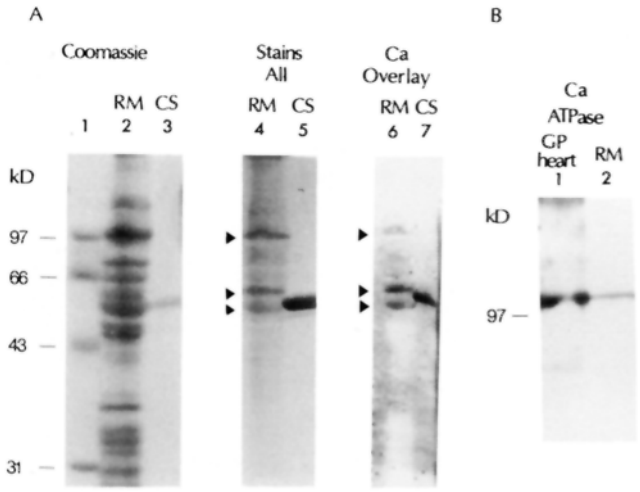

Figure 1. Presence of calcium binding proteins and calcium ATPase in rough microsomes. (*A*) Lane *1*, Coomassie Blue staining of molecular mass markers with masses indicated at left in kilodaltons; lane *2*, Coomassie Blue staining of RM (~100 μg protein); lane *3*, Coomassie Blue staining of skeletal muscle calsequestrin (~1 μg); lane *4*, "Stains All" staining of RM, photographed through orange filter; lane *5*, "Stains All" staining of calsequestrin; lane *6*, ^{45}Ca blot overlay of RM; lane *7*, ^{45}Ca blot overlay of calsequestrin. Calcium binding proteins of apparent molecular masses 94, 63, and 60 kD are indicated by arrowheads. Panel *B*, immunoblot with antisera against canine cardiac sarcoplasmic reticulum calcium ATPase: lane *1*, guinea pig cardiac membranes (crude 100,000-*g* pellet of homogenate); lane *2*, RM.

KOAc, 50 mM TEA HCl, pH 7.5, 5 mM $MgCl_2$) were subjected to isopycnic centrifugation (flotation protocol) through linear 34–65% sucrose gradients as previously described (16).

^{45}Ca Overlay Assay

This was done as described (14), except that the blot was washed with 0.05 mM $MgCl_2$ in water (four changes for a total of 20 min).

"Stains All" Gel Staining

After SDS-PAGE, the gel was stained with 0.0025% "Stains All" as described (7), then photographed through an orange filter.

Results

A canine pancreatic homogenate was fractionated through multiple differential centrifugation steps as previously described (16). Individual fractions have previously been shown to have distinct marker profiles (16, 17). The fractionation pattern of a protein cross-reactive with a monoclonal antiserum against the canine cardiac sarcoplasmic reticulum Ca-ATPase and several calcium binding proteins detected by a ^{45}Ca overlay assay was evaluated in approximately "equivalent" fractions.

About half the RER (defined by the distribution of the RER marker, ribophorin I (13), Fig. 2 *C*) was found to subfractionate into the "nuclear" pellet (lane *2*), consistent with its contiguity with the nuclear envelope. When the postnuclear supernatant (lane *1 C*) was further subfractionated, about half the RER in this fraction distributed into the mitochondrial pellet (lane *4*), as expected (5). Subfractionation of the postmitochondrial supernatant (lane *3*) through a cushion of 1.3 M sucrose yielded four fractions: a postmicrosomal supernatant (lane *5*, representing cytosol), a smooth microsomal interface (lane *6*), the cushion (lane *7*), and a pellet of rough microsomes (RM) (lane *8*). Nearly all the ribophorin I present in the postmitochondrial supernatant (lane *3*) was found in the RM (lane *8*). This pellet was free of secretory granules (as measured by amylase activity, see legend to Fig. 2) and has previously been shown to be uncontaminated by plasma membrane, nuclei and cytosolic markers (16, 17). By EM, this rough microsomal pellet has previously been shown to consist of highly purified RER vesicles (17).

The various pancreatic subfractions were subjected to a ^{45}Ca overlay assay after Western blotting. Calsequestrin, the calcium binding protein of skeletal muscle sarcoplasmic reticulum, served as a control (Fig. 1 *A*, lane *7*). The results of many such assays were as follows. Generally, in RM, three proteins with apparent molecular masses of 94, 61, and 59 kD were detected (Fig. 1 *A*, lane *6*). Three proteins of approximately the same molecular mass retained a blue color (somewhat variably, particularly in the case of the 61-kD protein) when RM proteins separated by SDS-PAGE were stained with Stains-All, a dye which selectively stains calcium binding proteins blue (7) (Fig. 1 *A*, lane *4*), as did calsequestrin (lane *5*). Thus, there appear to be at least three calcium binding proteins in RM, consistent with other reports, which additionally suggest that the 94-kD protein is the luminal RER protein grp 94 (12, 21). The identity of the other proteins is less clear. It is possible that either the 59- or the 63-kD protein is CAB-63 (calregulin) (23). It has been suggested that one of these proteins may be related to calsequestrin (8, 22), though this has been debated (21). None of several antisera against calsequestrin that we were able to obtain cross-reacted with a protein of a similar molecular mass in the RM fraction from canine pancreas, although they recognized purified calsequestrin (not shown). Nevertheless, other antisera against calsequestrin may recognize one of these proteins in canine pancreatic RM.

All three calcium binding proteins detected with the blot-overlay assay fractionated in a manner highly analogous to the RER marker, ribophorin I (compare Fig. 2, *A* and *C*), as well as another RER marker, the 23-kD glycoprotein subunit of signal peptidase (not shown), strongly arguing that they reside in the RER or an RER-associated structure.

A published monoclonal antiserum directed against the 110-kD Ca-ATPase of canine cardiac sarcoplasmic reticulum (11) reacted with a band of the same molecular mass in guinea pig cardiac membranes and in RM (Fig. 1 *B*). Hereafter, this protein will be referred to as the Ca-ATPase, recognizing that we have shown this indirectly. The distribution of this cross-reactive protein in various pancreatic subfractions was highly similar to that of the RER marker, ribophorin I, as well as the three calcium-binding proteins we detected by the ^{45}Ca overlay assay, suggesting that the protein resides in the RER, or an RER-associated structure (compare Fig. 2 *B* with *A* and *C*).

Even though the RM had been previously shown (by EM and marker analysis) to consist of highly purified RER (16, 17), it was still possible that another membrane could fractionate in a fashion similar to the RER. In order to provide additional evidence that the calcium binding proteins and the Ca-ATPase were associated with the RER (and not in a mem-

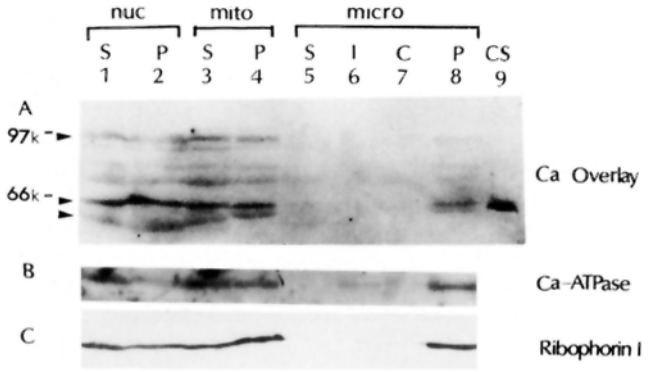

Figure 2. Distribution of calcium binding proteins and calcium ATPase in approximately "equivalent" subcellular fractions. Definition of equivalent fractions and method for overlay assay are given in Materials and Methods. Lane *1*, postnuclear supernatant (145 μg protein); lane *2*, nuclear pellet (230 μg); lane *3*, postmitochondrial supernatant (110 μg); lane *4*, mitochondrial pellet (55 μg); lane *5*, postmicrosomal supernatant (50 μg); lane *6*, interfacial smooth microsomes (35 μg); lane *7*, cushion (10 μg); lane *8*, rough microsomes (45 μg); lane *9*, skeletal muscle calsequestrin (CS). *S*, supernatant, *P*, pellet, *I*, interface, *C*, cushion. (*A*) ^{45}Ca blot overlay assay, with calcium binding proteins indicated by arrowheads; (*B*) immunoblot with antisera against canine cardiac sarcoplasmic reticulum Ca-ATPase; (*C*) immunoblot with antisera against ribophorin I (rough endoplasmic reticulum marker). Relative amylase (secretory granule marker) activities (normalized to RM) for equivalent amounts of the above fractions were as follows: lane *1*, 14.7; lane *2*, 33.6; lane *3*, 14.2; lane *4*, 5.6; lane *5*, 11.4; lane *6*, 5.3; lane *7*, 0.1; lane *8*, 1.0.

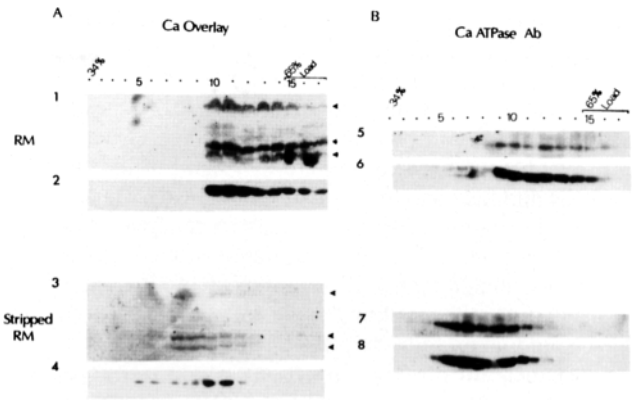

Figure 3. ^{45}Ca blot overlay and immunoblot with Ca-ATPase antisera of fractions upon isopycnic centrifugation of native and stripped RM (flotation protocol). Stripping procedure is referred to in Materials and Methods. Fractions 15–17 are the load zone. The pellet was resuspended in the last fraction. Subpanels *1–4* represent one set of experiments. Subpanels *5–8*, though performed in the same fashion, are a different set of experiments. (*A*) ^{45}Ca blot overlay compared with immunoblot of ribophorin I: subpanel *1*, ^{45}Ca blot overlay of native RM fractions; subpanel *2*, ribophorin I immunoblot of same; subpanel *3*, ^{45}Ca blot overlay of stripped RM fractions; subpanel *4*, ribophorin I immunoblot of same. Calcium binding proteins are indicated by arrowheads. (*B*) Ca-ATPase immunoblot compared with ribophorin I immunoblot: subpanel *5*, Ca-ATPase immunoblot of native RM fractions; subpanel *6*, ribophorin I immunoblot of same; subpanel *7*, Ca-ATPase immunoblot of stripped RM fractions; subpanel *8*, ribophorin I immunoblot of same.

brane that fractionated along with RER), purified RM was subjected to isopycnic centrifugation (Fig. 3). The three calcium-binding proteins (subpanel *1*) equilibrated at high sucrose densities, isodense with the RER marker, ribophorin I (subpanel *2*). Similarly (in a separate experiment), the Ca-ATPase (subpanel *5*) was found in high-density fractions along with the RER marker (subpanel *6*). Moreover, when the RM were treated with puromycin/high salt, a treatment which strips off ribosomes (I), the calcium binding proteins (subpanel *3*), as well as the Ca-ATPase (subpanel *7*), shifted to lighter sucrose densities with ribophorin I (subpanels *4* and *8*, respectively). A density shift such as this would not be expected for a non-RER membrane.

Discussion

Using an "equivalent" cell fractionation scheme, we have examined the subcellular distribution in canine pancreas of three Ca binding proteins detected by a ^{45}Ca overlay assay (Fig. 2 *A*), as well as a single protein recognized by an antiserum against canine cardiac sarcoplasmic reticulum Ca-ATPase that is of the same molecular weight as that detected in heart (Fig. 2 *B*). Proteins of approximately the same mobility on SDS-PAGE as the three calcium-binding proteins we detected in RM by the ^{45}Ca overlay (Fig. 1 *A*, lane *6*) stained blue with Stains All, a dye known to stain certain calcium-binding proteins blue (Fig. 1 *A*, lane *4*). It is not clear whether either of the two proteins of 60 and 63 kD detected by the ^{45}Ca overlay assay and Stains All is related to calsequestrin, which has been previously detected by anticalsequestrin sera in nonmuscle cells (8, 22). The 94-kD calcium binding protein is likely to be the same as that previously described, which appears to be the luminal RER protein grp 94 (12, 21). As can be seen in Fig. 2 *A*, other proteins can also bind ^{45}Ca, albeit variably (notably two proteins, of ~70 and ~80 kD), in the blot overlay assay, which has a high background; however, the three proteins we described consistently bound ^{45}Ca in this assay (compare Fig. 2 *A*, with Fig. 1 *A*, lane *6* and Fig. 3 *A*, subpanels *1* and *3*). Nevertheless, there may be additional calcium-binding proteins in RM that were not clearly detected by this assay. (It should be noted that a role for these proteins in calcium storage has not been unequivocally established.

Our data suggest that the primary site of calcium sequestration in pancreas is the RER or a RER-associated structure. Proteins cofractionating with a well-defined RER marker through several differential centrifugation steps (Fig. 2, compare *A*, *B*, and *C*), which are, in addition, isodense with the RER marker on isopycnic gradients are likely to be in, or associated with, the RER (Fig. 3, compare subpanel *1* with *2*, and subpanel *5* with *6*). The fact that they shift analogously with the RER marker to lighter sucrose densities when rough microsomes are treated with puromycin/high salt provides strong additional evidence for a RER localization (Fig. 3, compare subpanel *3* with *4*, and subpanel *7* with *8*). Thus the physical behavior of the proteins in RM has been correlated with a specific characteristic of RER. It is unlikely that an organelle that is not RER, or RER-associated, would behave in this fashion. Moreover, we have previously

confirmed the high purity of our rough microsome preparation by EM (17) and marker analysis (16, 17).

The calciosome, defined by immunolocalization of proteins cross-reactive with particular muscle calsequestrin and Ca-ATPase antisera to a distinct membrane structure (10, 22), may be a subspecialized compartment or extension of the RER and may thus fractionate analogously. Our findings do not rule out this possibility, at least in pancreas. Although association of the calciosome was demonstrated with several organelles, including the RER, no clear luminal continuity with any organelle was found. Our data, however, argue that the Ca binding proteins detected by the overlay assay and the canine pancreatic protein cross-reactive with antisera against cardiac sarcoplasmic reticulum Ca-ATPase of the same species largely, if not exclusively, colocalize to the RER (or an intimately associated structure). The RER, however, makes up 60% of the total pancreatic acinar cell membrane (6). In other cell types, the ratio of "smooth" to "rough" membranes is often much higher. It is possible that fractionation of these cells may reveal calcium binding proteins and the Ca-ATPase in non-RER compartments as well. It is also possible that there is more than one calcium sequestering compartment in nonmuscle cells (4). Probes other than those we used may detect such a compartment.

In cerebellar Purkinje cells, two immunolocalization studies revealed an IP_3 receptor to be in the endoplasmic reticulum (15, 18). It has been shown that cAMP dependent phosphorylation of the IP_3 receptor inhibits IP_3-mediated calcium release (20). Furthermore, GTP, perhaps by a mechanism involving small GTP-binding proteins, may also modulate intracellular calcium homeostasis (9). Thus the recent localization of cAMP-dependent protein kinase (16) and small GTP binding proteins (17) to the RER may be of functional importance in the context of intracellular calcium homeostasis.

We would like to thank Gunter Blobel for the privilege of performing this work in his laboratory, as well as for helpful discussions.

S. K. Nigam was supported by National Institutes of Health grant DK-01467.

Received for publication 11 December 1989 and in revised form 13 March 1990.

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