Plasma Membrane-Cytoskeleton-Endoplasmic Reticulum Complexes in Neurons and Astrocytes*

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The possibility that certain integral plasma membrane (PM) proteins involved in Ca²⁺ homeostasis form junctional units with adjacent endoplasmic reticulum (ER) in neurons and glia was explored using immunoprecipitation and immunocytochemistry. Rat brain membranes were solubilized with the mild, non-ionic detergent, IGEPAL CA-630. Na⁺/Ca²⁺ exchanger type 1 (NCX1), a key PM Ca²⁺ transporter, was immunoprecipitated from the detergent-soluble fraction. Several abundant PM proteins co-immunoprecipitated with NCX1, including the α2 and α3 isoforms of the Na⁺ pump catalytic (α) subunit, and the α2 subunit of the dihydroproidine receptor. The adaptor protein, ankyrin 2 (Ank 2), and the cytoskeletal proteins, α-fodrin and β-spectrin, also selectively co-immunoprecipitated with NCX1, as did the ER proteins, Ca²⁺ pump type 2 (SERCA 2), and inositol-trisphosphate receptor type 1 (IP₃R-1). In contrast, a number of other abundant PMs, adaptors, and cytoskeletal proteins did not co-immunoprecipitate with NCX1, including the Na⁺ pump α1 isoform, PM Ca²⁺ pump type 1 (PMCA1), β-fodrin, and Ank 3. In reciprocal experiments, immunoprecipitation with antibodies to the Na⁺ pump α2 and α3 isoforms, but not α1, co-immunoprecipitated NCX1; the antibodies to α1 did, however, co-immunoprecipitate PMCA1. Antibodies to Ank 2, α-fodrin, β-spectrin and IP₃R-1 all co-immunoprecipitated NCX1. Immunocytochemistry revealed partial co-localization of β-spectrin with NCX1, Na⁺ pump α3, and IP₃R-1 in neurons and of α-fodrin with NCX1 and SERCA 2 in astrocytes. The data support the idea that in neurons and glia PM microdomains containing NCX1 and Na⁺ pumps with α2 or α3 subunits form Ca²⁺ signaling complexes with underlying ER containing SERCA 2 and IP₃R-1. These PM and ER components appear to be linked through the cytoskeletal spectrin network, to which they are probably tethered by Ank 2.

Cytosolic Ca²⁺ plays a key role as a second messenger in all cells and is responsible for regulating numerous cellular processes simultaneously. Spatial as well as temporal control of the cytosolic Ca²⁺ signals is therefore critical (1, 2). Much of the “signal Ca²⁺ comes from intracellular stores, primarily in the endoplasmic reticulum (ER). Nevertheless, Ca²⁺ signaling depends critically upon the coordination of Ca²⁺ entry across the plasma membrane (PM), Ca²⁺ release from the ER, Ca²⁺ extrusion across the PM, and refilling of the ER stores by re-sequestration and by entry from the extracellular fluid (1, 2). Clearly, the many specific mechanisms responsible for all these Ca²⁺ movements must be coordinated precisely. One possibility is that several functionally inter-dependent mechanisms may be clustered and organized into specialized “Ca²⁺ signaling complexes” (CaSCs). Indeed, recent results suggest that PM microdomains, adjacent “junctional” ER (jER), and the inter-vining tiny volume of cytosol (units we have called “PlasmERosomes”) (2) serve as CaSCs in many types of cells (3–7). Classic examples are the PM-sarcoplasmic reticulum (SR) junctions in skeletal and cardiac muscle (8). Structurally similar PM-SR or PM-ER junctions have been observed by electron microscopy in smooth muscle (9) and neurons (10, 11), even though Ca²⁺ signaling in these cells involves some different Ca²⁺ transporters.

The Na⁺/Ca²⁺ exchanger (NCX) is a Ca²⁺ transporter that helps to control intracellular and jER Ca²⁺ concentrations and cell signaling in many types of cells (12). All three isoforms (NCX1–3), each the product of a different gene, are expressed in the brain (13). NCX1 is the most widely distributed isoform and is prevalent in both neurons and glia (13, 14). The activity of the Na⁺/Ca²⁺ exchanger is driven by the Na⁺ electrochemical gradient across the PM and appears to be functionally linked to Na⁺ pumps with α2 or α3 catalytic subunits in neurons, glia, and other cell types (5, 7, 15, 16). Thus, these particular Na⁺ pumps indirectly modulate Ca²⁺ signaling. Moreover, immunocytochemical evidence indicates that the NCX and Na⁺ pumps with α2 or α3 subunits are confined to PM microdomains that overlie jER (or SR) in neurons, astrocytes, and smooth muscle cells (3, 4). In contrast, Na⁺ pumps with α1 catalytic subunits and the ATP-driven PM Ca²⁺ pump (PMCA) are more uniformly distributed on the surfaces of these cells (3, 4). The Na⁺ pumps with α1 subunits apparently are “housekeepers” that maintain low cytosolic Na⁺ concentrations, whereas those with α2 or α3 subunits, expressed in the same cells, likely regulate Ca²⁺ signaling (7).

Immunocytochemical evidence (17) suggests that store-oper-

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‡The abbreviations used are: ER, endoplasmic reticulum; Ank, ankyrin; CaSCs, Ca²⁺ signaling complexes; DHPR, dihydropyridine receptor; IP, immunoprecipitate; IP₃R, inositol 1,4,5-trisphosphate receptor; jER, junctional ER; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; NCX, Na⁺/Ca²⁺ exchanger; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PM, plasma membrane; PMCA, ATP-driven PM Ca²⁺ pump; SERCA, SR/ER Ca²⁺ pump; SOCs, store-operated channels; SR, sarcoplasmic reticulum; Trk B, a neurotrophin receptor; TRPC, transient receptor potential channel protein; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); NCX1, Na⁺/Ca²⁺ exchanger type 1.
nate was determined with the bicinchoninic acid assay (Bio-Rad), with bo-
immunoprecipitation and immunoblotting. The protein concentration

Sample Buffer (Bio-Rad) (29) and boiled for 5 min. In a few instances,
to rabbit IgG (for pAb) or mouse IgG (for primary mAb),
to the cytoskeletal protein spectrin (25–27). We hypothesized that the cytoskeleton might form a scaffold to link
the relevant PM and adjacent ER transporters. To test this possibility, we used immunoprecipitation (IP) and immunoblot-
ing to isolate and partially characterize the protein complexes
from brain homogenates. Immunocytochemistry was used to verify
the co-localization of some of these proteins in the complexes
in cultured neurons and astrocytes. The results provide
new insight into the structural organization of PM-ER junc-
tions that underlies critical aspects of the spatial regulation
of neuronal and glial Ca2+ signaling. A preliminary report
of some of these findings has been published (28).

EXPERIMENTAL PROCEDURES

Preparation of Tissue Extracts—Brains from Sprague-Dawley rats
(males, 150–200 g) were minced in NaCl/sucrose buffer (~1 mg/ml)
containing 140 mM NaCl, 2 mM EDTA, 10 mM sodium azide, 20 mM Tris
base, pH 7.4, 0.25% sucrose, and protease inhibitor mixture tablets
(Roche Diagnostics). The suspension was homogenized at 4 °C with a
Polytron homogenizer (Brinkmann Instruments) and subjected to cen-
trifugation for 20 min at 27,000 g. The pellet from this
centrifugation step was resuspended in ~15 ml of NaCl/sucrose buffer
containing the non-ionic detergent, IGEPAL CA-630 (1%) (Sigma), and
incubated for 1 h at 4 °C. The lysate was centrifuged for 20 min
at 27,000 × g and 4 °C. The supernatant (detergent extract or “Homoge-
nate”), ~3.3 mg of protein/ml, was stored at −70 °C and used for
immunoprecipitation and immunoblotting. The protein concentration
was determined with the bicinechonic acid assay (Bio-Rad), with bo-
vine serum albumin as a standard.

Immunoprecipitation—Appropriate monoclonal antibodies (mAb; 3 μg)
or polyclonal antibodies (pAb; ~6 μg) were incubated with ~2 × 107
(50 μl) washed magnetic beads (Dynabeads M-450, coated with goat
anti-mouse IgG or M-280 sheep anti-rabbit IgG; Dynal Biotech, Lake
Success, NY) overnight at 4 °C on a rotator (see manufacturer’s direc-
tions). As negative controls, the coated beads were incubated with
either mouse IgG1 (MOPC-21; Sigma) for mAb, or with rabbit γ-glob-
ulin (Jackson Immunoresearch, West Grove, PA) for pAb raised in
rabbits. The beads, with antibody attached, were washed (twice, 200 μl)
with phosphate-buffered saline (PBS). Proteins were immunoprecip-
titated from 1 mg of detergent-extracted total protein by incubation for
4 h at 4 °C with antibody-bound beads. Following incubation, the su-
pernatant and 10 mg MgCl2, 10 mM NaN3, pH 7.4) overnight for 4 h. This
specific cross-reactivity was blocked by incubating the cells for 4 h in 5% normal
rabbit serum (Jackson Immunoresearch) diluted with Antibody
Buffer. The antibodies diluted in Antibody Buffer were applied to the
coverslips overnight at 4 °C. The coverslips were then washed in 3 changes of Antibody
Buffer (0.5 mM NaCl, 10 mM MgCl2, 20 mM NaNO3, 20 mM Tris-Cl, pH 7.4). The neurons
were then washed 3 times with PBS and incubated for 1 h at 22 °C with
different antibodies (Alexa 568-labeled anti-
mouse or anti-rabbit antibodies from Molecular Probes (Eugene, OR)
for NCX1, the α3 subunit of the Na+/pump, or IP-R-1, and fluorescein-
labeled donkey anti-chicken antibody for β-spectrin). The neurons were
then washed again in PBS, and the coverslips were mounted with
Vectashield (Vector Laboratories, Burlingame, CA).

Primary cultured rat astrocytes were fixed for 45 min at 22 °C with
0.45% (w/v) parafformaldehyde in fixation solution (75 mM cycloexyla-
mine (free base), 75 mM NaCl, 10 mM EGTA, 10 mM MgCl2, and 10 mM
PIPES adjusted to pH 6.8 with HCl). The cells on the coverslips were then
permeabilized with fresh fixative containing 0.5% Brij 58 (Sigma)
for 10 min. The coverslips were washed with washing solution (75 mM
Tris-Cl, 10 mM NaNO3, pH 6.7) for 30 min, and then immunostained
for the primary antibody (rhodamine-labeled secondary antibodies
(Alexa 568-labeled anti-rabbit antibody from Molecular Probes (Eugene, OR))
and then incubated for 1 h with appropriate secondary antibody.
Following washing, membranes were incubated with secondary an-
tibodies to rabbit IgG (for pAb) or mouse IgG (for primary mAb).

RESULTS

Immunoprecipitation of the NCX1 from a Detergent-soluble Extract of Rat Brain Membranes—Ca2+ homeostasis in most
cells depends upon two PM Ca2+ transporters, the PMCA (34) and
the NCX (12). The NCX isofrom, which is prevalent in
neurons and glia (see Introduction), appears to be confined
membrane microdomains that form junctional complexes with

- 27,000 g
the adjacent ER (3, 4). Moreover, NCX activity modulates ER-dependent Ca²⁺ signaling (2). To learn if NCX1 forms a complex with elements of the ER, and if these junctional structures contain cytoskeletal structures capable of linking them together, we prepared immunoprecipitates of NCX1 from detergent-solubilized extracts of rat brain membranes. Immunoblots were performed on these IPs to identify other proteins that co-IP with, and may therefore associate with, NCX1 (Figs. 1 and 2).

Immunoprecipitation removed a large fraction (about 73%) of the NCX1 from the detergent extract. Fig. 1A shows that the density of the dominant NCX1 band (140 kDa in this immunoblot)² in the post-IP, detergent-soluble supernatant (IP S) fraction was only about 27% of the band density in the original homogenate (Homog; n = 4 different IPs). For controls, the

² NCX1 protein has a molecular mass of 120 kDa. Following boiling, however, the protein sometimes runs as a 140-kDa band (35).
 TABLE I  

Summary of co-immunoprecipitation data

| Blotted with antibody to | Homogenate immunoprecipitated with antibody to: |
|--------------------------|-------------------------------------------------|
| NCX1                     | Na⁺ pump | Ank 2 | α-Fod | β-Spec | IP3R-1 |
|                          | a1       | a2    | a3    |        |        |
| Na⁺ pump a1              | –        | –     | +     | ++     | ++     | +       |
| Na⁺ pump a2              | –        | –     | X     | +      | –      | –       |
| Na⁺ pump a3              | +        | –     | X     | +      | –      | ++      |
| DHRF a2                  | +        | +     | ±*    | –      | –      | –       |
| PMCA1                    | –        | +     | ±      | +      | –      | –       |
| Trk B                    | –        | –     | –     | –      | –      | –       |
| N-CAM                    | –        | –     | –     | –      | –      | –       |
| TRPC-4                   | ±*       | –     | –     | –      | –      | –       |
| Ank 2                    | +        | ±     | +     | X      | –      | –       |
| Ank 3                    | −        | ±     | –     | –      | –      | –       |
| Hom 1                    | –        | –     | –     | –      | –      | –       |
| Hom 2                    | –        | –     | –     | –      | –      | –       |
| α-Fod                    | +        | −     | +     | −      | X      | −       |
| β-Spec                   | +        | −     | +     | +      | +      | X       |
| IP-R-1                   | +        | −     | +     | −      | X      | −       |
| IP-R-2                   | –        | −     | +     | −      | X      | −       |
| IP-R-3                   | –        | −     | +     | −      | X      | −       |
| SERCA2b                  | –        | −     | +     | −      | X      | −       |

* In one experiment no band was seen; a very weak band was observed in a second experiment (Fig. 4).

Immunoprecipitation of the Na⁺ Pump α Subunit Isoforms—

Additional evidence for the specificity of some of the associations among PM proteins was obtained by generating IPs with antibodies raised against α subunit isoforms 1–3 of the Na⁺ pump (Figs. 3 and 5). Na⁺ pumps are composed of an α and a β subunit. When antibodies raised against the α2 (Fig. 4) or α3 (Fig. 5) isoforms were bound to the Dynabeads and used for immunoprecipitations, both NCX1 and IP3R-1 were found in the IP pellet, as was Ank 2, but the PMCA1 band was weak (α2 IP) or absent (α3 IP). In contrast, IP with an antibody raised against the Na⁺ pump α1 isoform did not co-IP NCX1 or IP3R-1 but did co-IP PMCA1 (Fig. 3; note the stronger 134-kDa band after boiling; Ref. 34); weak Ank 2 and Ank 3 bands were also seen. These data are consistent with evidence that the Na⁺ pumps with α2 or α3 subunits (i.e., those with high affinity for ouabain), but not those with α1 subunits, are functionally coupled to NCX1 and help to modulate Ca²⁺ signaling (5, 7). Moreover, as suggested by immunocytochemistry (4), PMCA1, like Na⁺ pumps with α1 subunits, may be very widely distributed in the PM but excluded from the microdomains that contain NCX1 and Na⁺ pumps with α2 or α3 subunits.

The Na⁺ pump α1 isoform is expressed in virtually all cells (37) and appears to be the “housekeeping” isoform of the catalytic subunit that maintains the low cytosolic Na⁺ concentration (7). In addition, astrocytes express the α2 isoform, whereas mature neurons express α3 (3, 38, 39). Thus, it is not surprising that IP of the detergent-soluble brain membrane fraction with an antibody to the α2 subunit of the Na⁺ pump co-IPs Ank 2 and α-fodrin, both of which are present in astrocytes, but not β-spectrin, which is not present (Fig. 4) (26). In contrast, IP with an antibody to Na⁺ pump α3 (Fig. 5) co-IPs β-spectrin as well as Ank 2 and α-fodrin, all of which are expressed in neurons (26, 27).

Immunoprecipitation of Adaptor, Cytoskeletal, and ER Proteins—As shown above, the detergent extract from rat brain

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3 The PMCA1 antibody detects bands at both ~130 and ~134 kDa that correspond to the PMCA1a and PMCA1b isoforms (34). The lower molecular weight form was most prevalent in the homogenate (e.g., Figs. 1 and 3). When the immunoprecipitated proteins were extracted by boiling the beads, however, the density of the higher molecular weight band increased, and the lower molecular weight band density decreased (Fig. 3); similar results were obtained when the homogenate was boiled (not shown).

4 β-Spectrin in excitable cells can run as multiple bands (26).
membranes contains various adaptor and cytoskeletal elements, some of which appear with PM and ER membrane proteins in the IP fractions generated with antibodies to NCX1. If, as we hypothesize, the adaptor and cytoskeletal proteins link the ER and PM proteins in large multimolecular complexes, then IP of some of these adaptor and cytoskeletal proteins should co-IP PM and ER membrane proteins. IPs were prepared with antibodies to α-fodrin, β-spectrin, and Ank 2 to test this possibility (Figs. 6–8). IP with antibodies to α-fodrin or β-spectrin both co-immunoprecipitated NCX1 and IP3R-1 as well as Ank 2. The IP generated with antibodies to α-fodrin also contained NCX1, the Na\(^+\) pump α2 and α3 isoforms (not shown), β-spectrin, and IP3R-1 (Fig. 6) and that generated with antibodies to β-spectrin also contained NCX1, α-fodrin, DHPR α2, and IP3R-1 (Fig. 7). The IP generated with antibodies to β-spectrin also exhibited a weak band of the α3 subunit of the Na\(^+\) pump but not of the α2 subunit (not shown). The IP generated by antibodies to Ank 2 contained NCX1, α-fodrin, and β-spectrin (Fig. 8). Although we did not test all antibodies by IP, these results suggest that these adaptor and cytoskeletal proteins are present in complexes that contain both PM and ER proteins.

The fact that IP of certain PM or cytoskeletal proteins resulted in co-IP of SERCA2 (or 2b) and IP3R-1 implies that IP of these ER proteins should co-IP some PM and cytoskeletal proteins. This inference was confirmed: NCX1, Na\(^+\) pump α3, Ank 2 (but not Ank 3), α-fodrin, and β-spectrin all co-IP with IP3R-1 (Fig. 9).

**Immunocytochemistry**—Published immunocytochemical data reveal that most of the NCX1 and Na\(^+\) pumps with α2 or α3 subunits are confined to PM microdomains that overlie ER in glia and neurons (3, 4). Only a fraction of the ER markers co-localized with the overlying PM microdomains (4), however, but this is expected because only a small fraction of the ER in these cells closely associates with adjacent PM.

The relationship between the cytoskeleton and the ER or PM microdomains can be expected to be even more complex because the cytoskeleton should be widely distributed under the PM as well as in the cytosol (25, 26, 40, 41). Nevertheless, if there are specific associations between elements of the cytoskeleton and some ER and PM microdomains, as implied by our co-IP data, immunocytochemistry should reveal significant areas of overlap (co-localization) with the cytoskeletal elements. This is exemplified by the similar (although not identical) distribution of β-spectrin and NCX1 at the surface of the cell body in a primary cultured mouse neuron (Fig. 10, A and B; arrows indicate examples of co-labeled structures). The partial co-localization of β-spectrin with α3 Na\(^+\) pumps near the surface of a neuron is illustrated in Fig. 10, C and D. Similarly, the punctate distribution and partial co-localization of β-spectrin with the ER membrane protein, IP3R-1, was detected near the neuron surface (Fig. 10, E and F). In contrast there was no overlap of the β-spectrin stain with that of IP3R-1 in the perinuclear region where much of the ER (and IP3R) is located (Fig. 10, E and F; asterisks). Labeling was specific, as control antibodies failed to label similar structures at a comparable level (Fig. 10, G and H). These results suggest that at least some of the complexes we have examined in co-IP experiments are located at, or immediately adjacent to, the neuronal cell surface and not in intracellular compartments. They are consistent with our hypothesis that the junctional membrane complexes in neurons contain cytoskeletal proteins of the spectrin family as well as integral proteins of the PM and ER.

Astrocytes express the cytoskeletal protein, α-fodrin, but not β-spectrin (26). Therefore, we compared the distribution of the
NCX1, Ank 2, were probed with the blot Abs indicated (abbreviations as in Fig. 2). The post-IP supernatant (were loaded with homogenate (°)

...ter 2, and IP3R-1 all co-IP with α-fodrin. NCX1 and SERCA2 isoforms with that of α-fodrin in primary cultured rat astrocytes. As Fig. 11, A and B, shows, there is striking similarity in the distribution of the NCX1, which exhibits a reticular pattern in these cells, and α-fodrin. SERCA2 is also distributed in a reticular pattern in these cells and partially co-localizes with the α-fodrin (Fig. 11, C and D; E and F is a control that confirms the specificity of labeling). These results, too, are consistent with the presence of junctional membrane complexes containing elements of the spectrin-based cytoskeleton at or near the astrocyte surface.

**DISCUSSION**

**Organization of PM-ER Junctions Involved in Ca²⁺ Signaling**—This study shows that the PM Na⁺/Ca²⁺ exchanger, NCX1, in neurons and glia, is a component of large, multimolecular complexes that include other PM and ER transporters with which NCX1 is functionally associated. The complexes also include cytoskeletal elements as well as adaptor proteins that, presumably, link the PM and ER transport proteins to the cytoskeleton. Taken together, the efficacy of co-IP of these PM proteins, the results of the reciprocal co-IP experiments, and the immunocytochemical evidence of co-localization all demonstrate that both neurons and astrocytes have specialized PM-ER complexes. Also, recent immunocytochemical data indicate that TRPC-1 is confined to PM regions that overlie the ER in astrocytes (17). These results support our hypothesis that the functional interrelationship involving NCX1, Na⁺ pumps with α2 or α3 subunits, SOCs, and the jER (2, 5, 7) is maintained by structural proteins that keep these transporters in close proximity at the PM-ER junctions. The data are consistent with the model diagrammed in Fig. 12. Here the cytoskeletal elements (including α-fodrin and, although we did not examine it, β-fodrin in astrocytes, and both α-fodrin and β-spectrin in neurons) at the PM-ER junctions form a scaffold to which relevant PM and ER transport proteins involved in Ca²⁺ signaling are tethered by Ank 2, and perhaps other adaptor proteins.

The quantitative data for the PM proteins may be relevant. We observed that large fractions of the Na⁺ pump α2 and α3 subunits co-IP efficiently (>25%) with NCX1; TRPC-4 also co-IPs but less efficiently. This agrees with the immunocytochemical evidence (3, 14, 17) that these particular transporters are apparently confined to PM microdomains that overlie jER. Na⁺ pumps with α1 subunits also are very prevalent in neurons and astrocytes. Nevertheless, the IP data suggest that, consistent with earlier immunocytochemical results (3, 4) and

**FIG. 6.** Western blots of IP generated with antibodies raised against α-fodrin and probed with antibodies to PM and cytoskeletal proteins. Detergent extracts were subjected to IP with Dynabeads containing α-fodrin pAb (IP Ab). Control IP beads were prepared with rabbit γ-globulin (rIgG; IP Ab). Proteins were extracted from the beads at 42 °C; thus there was no NCX band at 140 kDa. Gels were loaded with homogenate (1st lane, IP pellet (IP P, 2nd lane), and post-IP supernatant (IP S, 3rd lane; same volume as 1st lane). The gels were probed with the blot Abs indicated (abbreviations as in Fig. 2). The results are representative of data from 3 IPs. The figure shows that NCX1, Ank 2, β-spectrin, and IP3R-1 all co-IP with α-fodrin.

**FIG. 7.** Western blots of IP generated with antibodies raised against β-spectrin and probed with antibodies to PM, ER, and cytoskeletal proteins. Detergent extracts were subjected to IP with Dynabeads containing β-spectrin pAb (IP Ab). Control IP beads were prepared with rabbit γ-globulin (rIgG; IP Ab). Proteins were extracted from the beads at 42 °C; thus there was no NCX band at 140 kDa. Gels were loaded with homogenate (1st lane, IP pellet (IP P, 2nd lane), and post-IP supernatant (IP S, 3rd lane; same volume as 1st lane). The gels were probed with the blot Abs indicated (abbreviations as in Fig. 2). The results are representative of data from 2 IPs. The figure shows that NCX1, Ank 2, α-fodrin, DHPR α2, and IP3R-1 all co-IP with β-spectrin.

**FIG. 8.** Western blots of IP generated with antibodies raised against Ank 2 and probed with antibodies to PM, ER, and cytoskeletal proteins. Detergent extracts were subjected to IP with Dynabeads containing Ank 2 mAb (IP Ab). Control IP beads were prepared with MOPC-21 (IP Ab). Proteins were extracted from the beads at 42 °C; thus there was no NCX band at 140 kDa. Gels were loaded with homogenate (1st lane, IP pellet (IP P, 2nd lane), and post-IP supernatant (IP S, 3rd lane; same volume as 1st lane). The gels were probed with the blot Abs indicated (abbreviations as in Fig. 2). The results are representative of data from 2 IPs. The figure shows that NCX1, α-fodrin, and β-spectrin all co-IP with Ank 2.
functional data (7), the α1 isoform may be excluded from the PM microdomains at these junctions.

Similarly, two PM Ca\(^{2+}\) transporters, PMCA1 and NCX1, are prevalent in the brain membranes. NCX1 is confined to the PM microdomains at PM-jER junctions, however, whereas PMCA1 is more uniformly distributed but does not co-IP with NCX1 and may be excluded from these microdomains (4).

Smaller fractions of the adaptor, cytoskeletal, and ER transport proteins co-IP with NCX1, and cytoskeletal and ER proteins only partially co-localize with NCX1. This is anticipated because the cytoskeleton is distributed widely at the PM, but only a small fraction of the ER is located at PM-ER junctions. The lower yields of some co-immunoprecipitated proteins also may be caused by differences in the stability of different spectrin-based membrane complexes (42, 43), which also may be affected by the binding of particular antibodies. Furthermore, neurons and glia may express different alternatively spliced forms of the cytoskeletal proteins (25, 26, 31), only some of which are designed to participate in junctional complexes. Spectrin at other regions of the PM is likely to be involved in stabilizing the membrane and in immobilizing different classes of proteins into membrane domains with compositions and functions that are distinct from those of the junctional complexes considered here (e.g. at nodes of Ranvier and synaptic densities). The links between spectrin and the integral membrane proteins in these other domains are probably mediated by anchoring proteins other than Ank 2 (e.g. Ank 1, Ank 3 (25), or homer proteins (44)) or by direct binding to spectrin (45).

Our results suggest that these transport proteins associate exclusively with the Ank 2 isoform of ankyrin, in agreement with other reports (25, 27), even though Ank 1 and Ank 3 also are expressed in excitable cells (25, 27). The structural basis of this selectivity remains to be elucidated, but our data and the data Mohler et al. (46) are consistent with the idea that Ank 2 is selectively associated with integral membrane proteins at PM-jER complexes. Furthermore, although our results are consistent with the presence of PM-jER complexes that are linked together into microdomains by spectrin and Ank 2, we still do not know how particular isoforms of PM transporters (e.g. the α2 and α3 isoforms of the Na\(^{+}\) pump but not α1) are targeted to these domains.
that of Procedures

Indeed, sucrose gradient sedimentation studies established that the Na\(^+\)/H\(^+\) pump binds to ankyrin. Results of which only the \(\alpha\) subunit is involved in anchoring the pump to the membrane. The implication is that these PM transport proteins in epithelial form multimolecular complexes that include cytoskeletal elements and an adaptor protein. Whether either transporter in renal epithelia was associated with the ER was not determined nor were the ankyrin or Na\(^+\)/H\(^+\) pump \(\alpha\) subunit isoforms identified, but other studies revealed that the specific isoforms were Ank 3 (49, 50) and the \(\alpha 1\) subunit of the Na\(^+\) pump (51). Other studies demonstrated that proteins of the ER, specifically SERCAs, IP\(_3\)R, and ryanodine receptors, also interact with ankyrins (23, 24, 27, 52, 53), consistent with our findings. Indeed, co-IP of IP\(_3\)R-3 with the \(\alpha 1\) subunit of the Na\(^+\) pump was recently described in renal epithelial cells (54), pretreatment of the cells with cytochalasin D, which depolymerizes the actin cytoskeleton, abolished this co-IP.

The present study expands on these ideas and demonstrates their more general applicability. The IP results verify immunocytochemical and functional evidence that in astrocytes and neurons as in other cell types (1–5, 7, 17), NCX1, Na\(^+\) pumps with \(\alpha 2\) or \(\alpha 3\) subunits and SOCs cluster at PM-ER junctions. These new data suggest that certain PM microdomains are linked, through Ank 2 and a network of spectrin, to the adjacent jER in neurons and astrocytes. If our model (Fig. 12) is correct, both ankyrin and spectrin are critical for both the structure and function of these PM-jER units.

Ankyrins may target a variety of membrane proteins to physiologically appropriate sites (25) and can serve as a bridge between ion transporters in the PM and the cytoskeleton (55). Indeed, a null mutation in one copy of the Ank 2 gene reduces expression of Na\(^+\) pump \(\alpha\) subunits, the NCX, and the IP\(_3\)R in the heart (44). Haploinsufficiency of Ank 2 can cause cardiac arrhythmias and sudden death in man and mouse, probably as a result of partial functional uncoupling of the PM-SR junctions (44). Ank 2 knockout mice, which die within 3 weeks of birth, exhibit a variety of structural defects in the brain including hypoplasia of the corpus callosum and pyramidal tracts and dilation of the cerebral ventricles (56). This is consistent with our hypothesis that the PM-jER (or jSR) complexes play essential roles in Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) signaling (2). Disruption of these units would be expected to affect numerous processes including cell development.

The consequences of Ank 2 deficiency are only one example of the broad functional implications of our findings. Different PM (and ER) transport proteins may be included within the PM microdomains (and the jER or jSR regions) in different cell types or even in different parts of individual cells such as...
neurons. In other words, a single cell may have different junctional complexes with distinct, but specific, functions (2, 6, 26). Thus, the particular cytoskeletal elements to which the PM proteins are linked also may influence function in ways that are not yet resolved. For example, β-spectrin is found in neuronal cell bodies, growth cones, and dendrites but not in axons, which contain β-fodrin (26, 57). Our results suggest that most of the junctional complexes we isolated by IP with antibodies to NCX1 arise from the former compartments, as they contain only β-spectrin. Thus, these PM-β-spectrin-Junctional units likely help regulate postsynaptic, but not presynaptic, Ca²⁺ signaling and Ca²⁺ sensitivity and, hence, neuronal plasticity. Clearly, further exploration of these units should greatly enhance our understanding of these complex phenomena. Indeed, we have tested only a selected group of antibodies in this study. For example, NCX2 and NCX3, as well as the K⁺ channel units distinct from those that contain NCX1. It will be challenging to learn just how heterogeneous the junctional complexes in different tissues are and to elucidate their specific contributions to cellular physiology and plasticity.

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