Monoclonal antibodies against the voltage-sensitive Na⁺ channel from mammalian skeletal muscle

(excitable membranes/sarcolemma/immunocytochemistry/Na⁺ channel subunits)

JAN M. CASADEI*, ROBERT D. GORDON*, LOIS A. LAMPSON*†, DONALD L. SCHOTLAND‡,
AND ROBERT L. BARCHI*†§

*Institute of Neurological Sciences and the Departments of †Neurology and ‡Anatomy, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Communicated by Eliot Stellar, June 11, 1984

ABSTRACT A panel of 13 monoclonal antibodies against the voltage-sensitive Na⁺ channel of rat skeletal muscle has been characterized. Each of these antibodies reacted with the purified Na⁺ channel protein in a solid-phase radioimmunoassay. Nine antibodies specifically immunoprecipitated the Na⁺ channel in a form that retained its characteristic high affinity for saxitoxin, and 11 recognized the channel in a crude mixture of solubilized membrane proteins separated on a Sepharose CL-6B column. Six antibodies specifically labeled skeletal muscle in immunofluorescence techniques. In each case, antibody was localized only to the surface membrane of the muscle fibers. Eleven antibodies produced detectable reaction on immunoblot transfers of sarcolemmal membrane proteins; each of these bound to a diffuse 160- to 200-kDa band that comigrated with the large glycoprotein subunit of the purified Na⁺ channel. Further studies were carried out with one of these antibodies, L/D3. In immunoblots of a glycoprotein fraction prepared from muscle that had been homogenized rapidly in a solution containing detergent, EGTA, and protease inhibitors, L/D3 recognized only a single 260-kDa band. Incubation of solubilized muscle proteins at 4°C for 24 hr without EGTA prior to isolation of the glycoprotein fraction resulted in partial conversion of this 260-kDa component to a smaller component between 160 and 200 kDa that comigrated with the principal immunoreactive component of sarcolemma. Based on its immunoreactivity with monoclonal antibodies, the large subunit of the rat skeletal muscle Na⁺ channel appears to be ≈260 kDa in its native state but may be sensitive to proteolysis during the isolation of sarcolemmal membranes.

In most mammalian nerve and muscle cells, a voltage-sensitive Na⁺ channel controls the membrane ionic current that produces the depolarizing phase of an action potential (1). Considerable progress has been made in the biochemical characterization of this unique membrane protein. Voltage-sensitive Na⁺ channels have been isolated from eel electroplax (2), from rat muscle and brain (3, 4), and from rabbit muscle (5). Functional reconstitution of the purified channel has been demonstrated for the protein from rat muscle and brain (6-8) and from rabbit muscle (5). All of these purified Na⁺ channels contain one very large glycoprotein subunit that migrates anomalously in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO₄/PAGE). Although this large component appears to be the only subunit in the eel channel (9), the mammalian Na⁺ channel contains in addition one or more smaller subunits (5, 10, 11).

Monoclonal antibodies provide powerful tools for probing the molecular architecture of complex proteins, for isolating these proteins from mixtures of cellular components, and for locating the protein of interest *in situ*. Availability of such tools would be extremely useful in studies of the mammalian

Na⁺ channel. Monoclonal and polyclonal antibodies have been prepared against the Na⁺ channel from the eel electroplax (12-14); however, these antibodies do not crossreact with the channel from mammalian nerve and muscle (14). A polyclonal antiserum against the rat sarcolemmal Na⁺ channel has been characterized by our group (15). This antiserum will be useful for immunocytochemistry but lacks the fine resolution of monoclonal antibodies for probing channel structure.

We report here the development of a panel of monoclonal antibodies raised against the purified rat sarcolemmal Na⁺ channel. These antibodies have been characterized by radioimmunoassay, immunoprecipitation, immunoblot transfers, and immunocytochemistry, and they should provide the basis for new approaches to the relationship between channel structure and function in mammals.

MATERIALS AND METHODS

Materials were obtained from sources previously identified (3, 5-7, 10, 15) unless otherwise indicated. Saxitoxin was generously provided by E. J. Schantz, University of Wisconsin; the toxin was tritiated and purified as described (3, 16). ¹²⁵I-labeled rabbit antibodies against mouse α, γ, and μ heavy chains and F(ab')₂ fragments were prepared by M. Cancro of the Department of Pathology at the University of Pennsylvania.

Rat skeletal muscle sarcolemma was isolated in the presence of a number of protease inhibitors by differential centrifugation after extraction of the contractile proteins with LiBr (10, 17). The sarcolemma was solubilized in Nonidet P-40 and the Na⁺ channel was purified as described (3, 10).

Preparation of Monoclonal Antibodies. BALB/c mice were immunized by intraperitoneal injections of purified Na⁺ channel protein (5-10 μg of protein per animal) in Freund's complete adjuvant repeated three or four times at 2- to 4-week intervals with the same amount of antigen in Freund's incomplete adjuvant. A final intravenous injection of highly purified channel protein (saxitoxin-binding capacity >2500 pmol/mg of protein) was given 4 days before fusion. Spleen cells from hyperimmune animals were fused with P3/NS-1/1-Ag4 myeloma cells using polyethylene glycol as in the method of Kohler and Milstein (18) as modified by Kennett (19). Positive clones were replated at limiting dilutions to ensure the monoclonal nature of the cell lines. Antibodies were purified from ascites fluid by chromatography on protein A-Sepharose 4B (20).

Solid-Phase Radioimmunoassay. Microtiter plates were precoated for 30 min at 22°C with 0.25 μg poly(L-lysine) per well, and then incubated overnight at 4°C with 10 μl of phosphate-buffered saline (P_i/NaCl; 150 mM NaCl/10 mM sodium phosphate, pH 7.3) containing ≈0.03 μg of Na⁺ channel

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Abbreviations: SA, formalin-fixed *Staphylococcus aureus*; P_i/NaCl, phosphate-buffered saline.

§To whom reprint requests should be addressed.

protein per well. Each well was washed with $P_i/NaCl$, treated for 30 min with 25 μ l of $P_i/NaCl$ containing 10% horse serum, and washed again. Wells were incubated for 2 hr at 22°C with the hybridoma supernatant being tested, then washed extensively with $P_i/NaCl$. The wells were incubated for 2 hr with ^{125}I -labeled rabbit antibody to mouse $F(ab')_2$ (15,000–20,000 cpm per well). The wells were rinsed, dried, and removed from the plate, and radioactivity was measured using a gamma counter.

Immunoprecipitation. Formalin-fixed *Staphylococcus aureus* cells (SA; Calbiochem) were washed (21), precoated with rabbit anti-mouse IgG (22), and suspended (10%, wt/vol) in $P_i/NaCl$. Aliquots (100 μ l) were centrifuged in plastic Eppendorf tubes, and the pellets were resuspended in 200 μ l of hybridoma supernatant and incubated with gentle tumbling at 4°C for 12 hr. Coated SA samples were collected by centrifugation and washed in buffer A (100 mM choline chloride/50 mM potassium phosphate, pH 7.4/0.5 mM $MgCl_2$ /0.5 mM $CaCl_2$) with 0.05% Nonidet P-40. Rat skeletal muscle sarcolemma was solubilized as for a typical channel purification (10). The washed SA pellets were each resuspended in 1 ml of solubilized sarcolemma (diluted to 0.4–0.8 mg of protein per ml with buffer A) and incubated overnight at 4°C. [3H]Saxitoxin was added to a final concentration of 15 nM, with or without the addition of 1 μ M unlabeled tetrodotoxin, for determination of specific saxitoxin binding activity. Toxin associated with channel protein bound to coated SA was separated from free toxin and unbound channel protein by rapid filtration of triplicate 175- μ l aliquots from each sample on Whatman GF/F filters pretreated with buffer A containing 0.05% Nonidet P-40. Filters were assayed by liquid scintillation counting.

Sephacrose CL-6B Chromatography. Sarcolemma was solubilized in 1% Nonidet P-40 (10), and 2–2.5 mg of solubilized protein (saxitoxin-binding capacity = 9–12 pmol/mg of protein) was applied to a 1.0 $\times$ 95 cm column of Sepharose CL-6B equilibrated with buffer A containing 0.1% Nonidet P-40 and asolectin phosphatides (1:5 molar ratio with detergent). Column flow rate was typically 4.3 ml/hr and 0.9-ml fractions were collected. [3H]Saxitoxin binding was measured in each fraction using a minicolumn assay (3, 10). Aliquots (20 μ l) of each fraction were incubated in the wells of microtiter plates coated with poly(L-lysine) and assayed for binding with monoclonal supernatants by solid-phase radioimmunoassay as detailed above.

Immunoblots. Samples were solubilized in 2% NaDodSO₄ and 0.6% dithioerythritol and electrophoretically resolved by the method of Laemmli (23); however, a lower bisacrylamide concentration (12% acrylamide/0.075% bisacrylamide) was used to facilitate subsequent transfer of high molecular weight proteins. The resolved proteins were electrophoretically transferred to nitrocellulose paper in a buffer containing 190 mM glycine/20 mM Tris (pH 8.3), 30% methanol, and 0.1% NaDodSO₄ (24, 25). Antibody binding was assayed as described (24) with ^{125}I -labeled-rabbit anti-mouse $F(ab')_2$ used to locate the bound antibody by autoradiography.

Immunofluorescence Microscopy. Frozen sections 4 μ m thick were obtained from rat anterior tibial muscle and transferred to poly(L-lysine)-coated coverslips. Sections were incubated for 30 min at 22°C in normal goat serum, washed with $P_i/NaCl$, and incubated for 60 min at 22°C with culture supernatants (diluted 1:5 in $P_i/NaCl$) from the clones being tested. P3/X63-Ag8 supernatant was used as a control. Sections were washed well and stained with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) preadsorbed with liver powder.

RESULTS

Spleen cells obtained from mice immunized with the purified rat sarcolemmal Na⁺ channel were fused with myeloma

P3/NS-1/1-Ag4 cells. The purified channel protein used for immunization had an average specific saxitoxin-binding activity of 1250 pmol/mg of protein, about 40% of that expected for a pure 314-kDa channel (26). The protein was used in its mixed micellar form with phospholipid and detergent. For final intravenous boosts, material of higher specific activity ($\approx$ 2500 pmol/mg, $\approx$ 75% pure) was used. To generate the panel of antibodies described below, we screened 1700 clones.

Supernatants were initially screened with a solid-phase radioimmunoassay using purified channel protein immobilized in 96-well microtiter plates. Channel preparations with specific saxitoxin-binding activities of 1000–1500 pmol/mg of protein were used for screening; approximately 0.05 pmol of Na⁺ channel was added to each well of the microtiter plate. As a control for this and all other studies to be described below, supernatant from the plasmacytoma cell line P3/X63-Ag8 was used. P3/X63-Ag8 secretes MOPC21, a $\gamma_1\kappa$ IgG that does not react with any known antigen in muscle. Supernatants were considered to be positive if the binding measured in a well was more than 5 times greater than that observed with P3/X63-Ag8 supernatant. By this criterion, 139 positive supernatants were identified.

Because minor contaminating proteins of high immunogenicity could generate antibodies that would be positive in the solid-phase radioimmunoassay, all positive supernatants were also evaluated with two additional assays. Only those antibodies positive in the radioimmunoassay and one or more of these additional assays were accepted as being directed against the Na⁺ channel.

In the first assay, supernatants were tested for their ability to immunoprecipitate the sodium channel from a mixture of solubilized membrane proteins in a form that retained its specific high-affinity binding for saxitoxin. Antibodies were bound to SA through a rabbit anti-mouse IgG "bridge". The antibody-SA complex was incubated with solubilized sarcolemma and the specific saxitoxin-binding activity bound to the complex was determined with a rapid filtration assay. Nine of 13 monoclonal antibodies that we tested specifically immunoprecipitated the Na⁺ channel; P3/X63-Ag8 supernatant did not (Fig. 1). The positive monoclonals were classified either as strong precipitators (specific saxitoxin binding

Fig. 1. Specific immunoprecipitation of the Na⁺ channel from a crude mixture of solubilized sarcolemmal membrane proteins. Immunoprecipitations were carried out using SA to immobilize the antigen-antibody complex. In this example, SA was precoated with rabbit anti-mouse $F(ab')_2$ and then with various concentrations of either the monoclonal antibody G/B10 or the control IgG MOPC21. The coated SA was incubated with a fixed amount of sarcolemmal membrane protein solubilized in Nonidet P-40. G/B10 (●), but not MOPC21 (○), immobilized channel protein that was capable of specific high-affinity binding of [3H]saxitoxin (STX). mAb, monoclonal antibody (or control IgG).

>5 times the nonspecific background, 6 antibodies) or as weak precipitators (2–5 times the nonspecific background, 3 antibodies).

Since not all antibodies to the Na⁺ channel need be efficient immunoprecipitators, we also evaluated antibodies which were positive in the solid-phase radioimmunoassay for their ability to identify the solubilized channel in its native form in a mixture of solubilized membrane proteins fractionated according to size on a Sepharose CL-6B column (Fig. 2). Since the channel protein used for immunization was purified by ion exchange and lectin affinity chromatography but not on the basis of size, it is unlikely that contaminating proteins would have the same elution coefficient on this column as the channel protein. Individual column fractions were incubated in poly (L-lysine)-coated wells of microtiter plates and antibody binding was determined by the solid-phase radioimmunoassay. Eleven of 13 antibodies that we tested bound to a single peak that coincided with the single peak of specific saxitoxin binding in the elution profile.

Of 139 antibodies identified in the solid-phase radioimmunoassay, 13 were also positive in one or both of the additional assays; these 13 antibodies are the panel we used. Ten have been identified as IgG, 9 of which have heavy chain subclass γ_1 . One is an IgM.

Each of the 13 antibodies has been evaluated as an immunological marker for the Na⁺ channel by indirect immunofluorescence using frozen sections of rat skeletal muscle and a rhodamine-labeled goat anti-mouse second antibody. Six produced strong positive staining. In each case, the antibodies were localized along the surface membrane of muscle fibers (Fig. 3). Cytoplasmic staining was not seen, and the

FIG. 2. Eleven of the 13 monoclonal antibodies recognized the Na⁺ channel in a mixture of solubilized sarcolemmal membrane proteins separated by size on a Sepharose CL-6B column. (A) The sodium channel (●), identified by specific high-affinity [³H]saxitoxin binding, eluted as a single included peak in the leading edge of the total membrane proteins (○) and accounted for <1% of the protein applied to the column. (B) The binding of two representative anti-channel monoclonal antibodies (G/B10, ●; D/C12, ○) and the control IgG MOPC21 (□) to protein in 20-μl aliquots of each column fraction. Binding was assayed by the solid-phase radioimmunoassay using ¹²⁵I-labeled rabbit anti-mouse F(ab')₂.

FIG. 3. Immunofluorescence localization of anti-channel antibody binding in rat skeletal muscle. Frozen sections (4 μm) of rat anterior tibial muscle were incubated with the monoclonal antibody L/D3 or with MOPC21 as a control and bound antibody was localized with rhodamine-conjugated goat anti-mouse IgG. (Upper) L/D3 binding was localized to the surface membrane of skeletal muscle fibers. (Lower) No significant staining was seen with the control antibody, although some fluorescent material was occasionally associated with the extracellular matrix between the muscle fibers.

membranes of connective tissue cells were not labeled. Preliminary electron microscopic studies indicate that the labeling in muscle is at the sarcolemmal and T-tubular membranes but not at the membranes of the sarcoplasmic reticulum.

All of the antibodies that were positive in the radioimmunoassay and one or more of the secondary assays were screened for immunoreactivity with sarcolemmal membrane proteins transferred to nitrocellulose paper after NaDodSO₄/PAGE. Eleven antibodies showed specific reaction in this immunoblot assay. Each labeled a diffuse high molecular weight band that corresponded in the gel system being used to the large glycoprotein of the purified channel, but none labeled components in the 38- to 45-kDa region where the channel's small subunits were found.

The monoclonal L/D3, which showed strong immunoreactivity with the large subunit of the channel on immunoblots, was used to probe for immunoreactive components in less purified preparations of muscle. When sarcolemma, prepared in buffer containing EGTA (0.5 mM) and the protease inhibitors iodoacetamide (1 mM), *o*-phenanthroline (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (1 μg/ml), and pepstatin (0.1 μg/ml), was used to generate transfers, the L/D3 antibody reacted mainly with a diffuse band at 160–200 kDa, which corresponds to the apparent size of the large subunit of the purified channel (Fig. 4, lane 1) (10). Fresh muscle was solubilized directly by homogeni-

FIG. 4. Eleven of 13 monoclonal antibodies produced identifiable immunoreactivity on immunoblots of muscle proteins. Lanes 1 and 2: Sarcoplasmic fraction was isolated in the presence of EGTA (0.5 mM), iodoacetamide (1 mM), *o*-phenanthroline (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (1 μ g/ml), and pepstatin (0.1 μ g/ml) (10). Each of the 11 antibodies stained a diffuse 160- to 200-kDa band corresponding in this gel system to the large glycoprotein of the purified rat sarcolemmal Na^+ channel (lane 1, A), as shown here for the monoclonal L/D3. The control immunoglobulin MOPC21 showed no reactivity with the transferred material (lane 2). Lanes 3 and 4: Fresh muscle was homogenized in a buffer containing 100 mM choline chloride, 50 mM potassium phosphate (pH 7.4), and 1% Nonidet P-40, as well as the protease inhibitors listed above. After centrifugation at $7000 \times g$ for 20 min, the supernatant was applied to a column of wheat germ agglutinin immobilized on Sepharose 4B. The retained glycoprotein fraction, which contained the Na^+ channel protein in ≈ 100 -fold enrichment over the crude homogenate, was eluted with the same buffer containing 50 mM *N*-acetylglucosamine. Samples of the eluate were used for preparation of immunoblots. A single 260-kDa component was immunoreactive with L/D3 (lane 3, B). No staining was seen with the control IgG (lane 4). Lanes 5 and 6: Muscle was homogenized in buffer containing 100 mM choline chloride, 50 mM potassium phosphate (pH 7.4), and the protease inhibitors listed above, but without the addition of detergent or EGTA. After incubation at 4°C for 24 hr, detergent was added to solubilize membranes, and the glycoprotein fraction was obtained as described above. Immunoblots of this fraction showed conversion of the 260-kDa component (lane 5, B) to a smaller component comigrating with the principle immunoreactive component of sarcolemma (lane 5, A). MOPC21 produced no staining in this transfer (lane 6). Lanes 5 and 6 are from a different gel than lanes 1–4.

zation in buffer containing 1% Nonidet P-40 and the same protease inhibitors and a glycoprotein fraction was rapidly prepared by chromatography on immobilized wheat germ agglutinin (see the legend to Fig. 4). This approach avoided the 36 hr required for the preparation of muscle membranes. The resultant glycoprotein fraction contains the Na^+ -channel protein (27). In immunoblots of proteins prepared from this glycoprotein fraction, L/D3 stained a diffuse protein band at 260 kDa. No immunoreactive components of higher mobility were seen (Fig. 4, lane 3). For comparison, fresh muscle was then solubilized in buffer containing detergent and inhibitors, but not EGTA, and held at 4°C for 24 hr before the glycoprotein fraction was isolated. Immunoblots prepared with this material showed partial conversion of the 260-kDa band to the lower molecular weight form seen in isolated sarcolemma (Fig. 4, lane 5). Less prominent immunoreactive species were seen as a diffuse band at 110 kDa and a sharp band at 24 kDa. In further experiments, we found that degradation of the 260-kDa component was reduced by the inclusion of 5 mM EGTA during this incubation.

DISCUSSION

Because of the relatively low percentage of positive supernatants resulting from our fusions, it appears that the purified rat Na^+ channel is not a strong immunogen in the mouse. This is not surprising in view of the apparent similarity throughout the phylogenetic tree of Na^+ channel properties assessed by biophysical methods.

Considering the low immunogenicity of the purified channel and that the preparations used for initial immunization were usually only 40–75% pure (based on saxitoxin binding per mg of protein), the specificity of these antibodies for the channel must be addressed. In this study, only those antibodies that were positive in one or more assays other than the solid-phase radioimmunoassay were considered specific for epitopes on the Na^+ channel. Undoubtedly, other specific antibodies to the channel were discarded using these criteria.

Our results suggest that no single assay can provide a completely reliable indicator of antibody specificity toward the Na^+ channel and that complementary assays are necessary at progressive stages of antibody screening. Overall, immunoprecipitation of specific saxitoxin-binding activity may be the best single test of antibody specificity, but this assay was too time-consuming for initial screening. Most antibodies that were positive in this assay were also able to identify the channel in the Sepharose CL-6B elution profile, although the converse was not true. Positive reaction of an antibody in both of these assays provides strong evidence that the antibody is indeed directed against the channel protein. Antibodies positive in the radioimmunoassay and in one of these assays are also highly likely to be directed against the channel protein. The properties of the 13 antibodies in the different assays (Table 1) indicate five distinct patterns of immunoreactivity. This suggests that the panel contains antibodies to at least five separate determinants on the Na^+ channel.

Antibodies that were positive in the radioimmunoassay

Table 1. Characteristics of anti-sodium channel monoclonal antibodies

Antibody	RIA	Immunoprecipitation	Sepharose 6B	Immunoblot	Immunocytochemistry	Class
M/H1	+	+	+	+	+	IgG1
I/E3	+	+	+	+	+	IgG1
A/B2	+	+	+	+	+	IgG1
F/E4	+	+	+	+	+	IgG1
B/B1	+	+	+	+	–	IgG1
D/C12	+	+	+	+	–	IgG1
G/B10	+	+	+	+	–	IgG1
L/D3	+	–	+	+	+	IgG1
G/G8	+	–	+	+	+	ND
F/G6	+	–	+	+	–	IgG?
G/G9	+	–	+	+	–	IgG1
E/F6	+	+	–	–	–	IgM
B/D6	+	+	ND	–	–	ND

Monoclonal antibodies were assayed as detailed in *Materials and Methods*. + (s), Strong positive; + (w), weak positive; –, negative; ND, not determined.

and in both of the secondary assays bound to a diffuse high molecular weight band that corresponded to the large glycoprotein subunit of the purified Na^+ channel. No antibodies have been identified that labeled the channel's small subunit, either specifically or in addition to the large subunit. Given the relative sizes of the two components, a predominance of antibodies against the large subunit would be probable, but 1 or more antibodies in 11 might be expected to be directed against the small subunits. The absence of such antibodies could reflect very low immunogenicity of this component or could indicate that it is hidden in the mixed micellar form of the channel used for immunization.

Of considerable interest is the fact that these antibodies labeled a diffuse 260-kDa band in transfers prepared directly from muscle homogenized in buffer containing detergent, EGTA, and protease inhibitors but recognized a 160- to 200-kDa band in blots prepared from purified sarcolemma that had been isolated in the presence of the same inhibitors. The location of the 260-kDa band in the former case corresponded to the position of the large glycoprotein subunit of the rabbit skeletal muscle Na^+ channel purified in our laboratory (28) and of the rat brain and eel channels (5, 9, 11), while the 160- to 200-kDa protein from sarcolemma corresponded to the large glycoprotein of the purified rat sarcolemmal Na^+ channel in this gel system (10).

The disappearance of the 260-kDa immunoreactive component concomitant with the appearance of the 160- to 200-kDa component during incubation of detergent-solubilized muscle at 4°C strongly suggests that the smaller arises from the larger by proteolysis. This conversion occurred at pH 7.4 in the presence of a variety of protease inhibitors but was suppressed by EGTA, which would be consistent with proteolysis by a Ca^{2+} -activated neutral protease.

Our data suggest that the large glycoprotein subunit of the rat muscle Na^+ channel is sensitive to proteolytic modification during the preparation of sarcolemmal membranes. The true molecular weight of this Na^+ -channel component of rat skeletal muscle appears to be $\approx 260,000$. The unmodified subunit corresponds in size to the α subunit of the rat brain channel (4), the large subunit of the rabbit skeletal muscle channel (28), and the single subunit of the channel from eel electroplax (9). A large subunit of ≈ 260 kDa appears to be a common feature of all voltage-sensitive Na^+ channels.

We thank Mrs. Nancy Goodman for assistance with the preparation of this manuscript, and Ms. Lois Murphy and Sheri Irons for technical assistance with various aspects of the work. This work was supported in part by National Institutes of Health Grants NS-

18013 and NS-08075 and by a grant from the Muscular Dystrophy Association.

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