Articles

Fusion between Disk Membranes and Plasma Membrane of Bovine Photoreceptor Cells Is Calcium Dependent†

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ABSTRACT: Disk membranes and plasma membrane vesicles were prepared from bovine retinal rod outer segments (ROS). The plasma membrane vesicles were labeled with the fluorescent probe octadecylrhodamine B chloride (R_{18}) to a level at which the R_{18} fluorescence was self-quenched. At pH 7.4 and 37 °C and in the presence of micromolar calcium, an increase in R_{18} fluorescence with time was observed when R_{18}-labeled plasma membrane vesicles were introduced to a suspension of disks. This result was interpreted as fusion between the disk membranes and the plasma membranes, the fluorescence dequenching resulting from dilution of the R_{18} into the unlabeled membranes as a result of lipid mixing during membrane fusion. While the disk membranes exposed exclusively their cytoplasmic surface, plasma membrane vesicles were found with both possible orientations. These vesicles were fractionated into subpopulations with homogeneous orientation. Plasma membrane vesicles that were oriented with the cytoplasmic surface exposed were able to fuse with the disk membranes in a Ca^{2+}-dependent manner. Fusion was not detected between disk membranes and plasma membrane vesicles oriented such that the cytoplasmic surface was on the interior of the vesicles. ROS plasma membrane–disk membrane fusion was stimulated by calcium, inhibited by EGTA, and unaffected by magnesium. Rod photoreceptor cells of vertebrate retinas undergo diurnal shedding of disk membranes containing the photopigment rhodopsin. Membrane fusion is required for the shedding process.

The rod photoreceptors in vertebrate retinas respond to light and initiate the nerve impulse which is transmitted to the brain. The rod cell is divided morphologically into an inner and an outer segment. The outer segment of the rod cell encloses densely packed, closed, flattened membranous disks which are stacked along the length of the outer segment. These disks contain the photopigment rhodopsin. The rod outer segment (ROS)[1] is in a continual state of degradation and renewal, synthesizing prodigious amounts of new membrane daily. Renewal occurs through formation of new disks at the base and shedding of old disks at the apical tip of the ROS. Packets of old, shed disks are phagocytosed by the overlying pigment epithelium. It can be hypothesized that the normal diurnal shedding of old disks requires membrane fusion both to form the packets of disks that are to be shed and to reseal the ROS plasma membrane subsequent to shedding.

While the dynamics of disk degradation and renewal have been reported from morphological data (Besharse et al., 1985; Fliesler et al., 1986; Papermaster et al., 1986), the role of membrane fusion in these processes has not been described nor is the regulation of these processes known. For example, membrane fusion between disks and plasma membrane would provide an interesting mechanism for the initiation of packet formation for disk shedding, but must be controlled so as to not occur indiscriminately along the length of the outer segment.

In this study, we report the observation of fusion between disk membranes and plasma membrane vesicles in vitro. Previously it was reported that normal diurnal disk shedding was stimulated by millimolar calcium, inhibited by EGTA, and unaffected by magnesium (Greenberger & Besharse, 1983). Here we show that fusion between disk membranes and plasma membranes of the ROS required calcium, was inhibited by EGTA, and was unaffected by magnesium. Furthermore, Ca^{2+}-dependent fusion of plasma membrane vesicles and disk membranes required plasma membrane vesicles that were oriented such that the cytoplasmic surface was accessible to the disk membranes (the normal orientation of these two membranes relative to each other in the ROS). These data suggested that calcium-dependent fusion of the disk and the ROS plasma membrane may contribute to the process of disk shedding.

Materials and Methods

Preparation ROS Membranes. Both ROS disk and plasma membranes were prepared as descirbed from the same rod outer segment preparation. Briefly, ROS were isolated from frozen bovine retinas (Lawson; Lincoln, Nebraska) as described by Papermaster and Dreyer (1974). The disk membranes and plasma membranes were isolated from the same sucrose density gradient. The latter was prepared as described by Molday and Molday (1987) and modified by Boesze-Battaglia and Albert (1989). The plasma membrane was eluted from the ricin–agarose (Sigma, St. Louis, MO) with 1 M galactose in 0.1 M sodium borate, pH 8.5. The plasma membrane was pelleted at 28000*g* for 30 min and resuspended in 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 7.5, for labeling with R_{18} or in 0.01 M sodium acetate, 1 mM

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[1] Abbreviations: ROS, rod outer segment; SUV, small unilamellar vesicles; PC, phosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; R_{18}, octadecylrhodamine B chloride.

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$MnCl_2$, and 1 mM $CaCl_2$, pH 5.0 for column chromatography as described below. The buffers used in the isolation of the membrane species were made 1 mM in EDTA, 0.5 mM in DTT, and perfused with argon or nitrogen in order to reduce lipid oxidation. All manipulations of the ROS membranes were done under dim red light.

Isolation of Plasma Membrane Vesicles and Intracellular and Extracellular Orientation. As described previously (Boesze-Battaglia & Albert, 1990), ROS plasma membranes isolated as described above contain vesicles of two orientations. These populations of ROS plasma membrane vesicles were separated using concanavalin-A (Con-A) affinity chromatography as described previously for disk membranes (Shichi, 1983) and modified by Boesze-Battaglia and Albert (1990) for ROS plasma membranes. Briefly, the Con-A column was preincubated with an excess of phosphatidylcholine small unilamellar vesicles prepared as described previously (Boesze-Battaglia & Albert, 1990), to saturate the nonspecific sites. The unbound plasma membranes were eluted from the column with 0.01 M sodium acetate, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 5.0. These ROS plasma membrane vesicles are oriented such that the cytoplasmic surface of the plasma membrane is on the outside of the vesicle (since the Con-A sites were inaccessible) and will be called cytoplasmic side out vesicles in this report. The bound plasma membrane was eluted from the column with 0.1 M α-methylmannoside, 0.01 M sodium acetate, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 5.0. The plasma membrane that eluted with the α-methylmannoside has the extracellular surface of the plasma membrane oriented toward the outside of the vesicle (since the Con-A sites were accessible). This preparation will be labeled extracellular side out vesicles. Greater than 90% of the rhodopsin loaded onto the Con-A column was recovered. The plasma membrane vesicles were pelleted at 28000*g* for 30 min and resuspended in 10 mM Hepes, pH 7.4. The isolated plasma membrane vesicles were labeled with R_{18} as described below.

Labeling of ROS Plasma Membranes. Octadecylrhodamine B chloride (R_{18}) was obtained from Molecular Probes, Inc. (Junction City, OR). Plasma membranes were labeled with the fluorescent lipophilic probe R_{18} at self-quenching levels as described for Sendai virus (Hoekstra et al., 1984). Typically, R_{18} was added to about 3–5 mol % relative to the phospholipid. At this level the fluorescence of the R_{18} is partially self-quenched. Briefly, 10 nmol of R_{18} in 10 mL of ethanol was added for each milligram of rhodopsin in a total volume of 1 mL. The mixture was vortexed and allowed to incubate on ice for 1 h. Labeled plasma membrane was separated from unincorporated R_{18} by passing the mixture through a Sephadex G-75 column and eluting labeled material with 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 7.5.

Preparation of ROS Disk Membranes. ROS disk membranes were isolated from sucrose density gradients as described above. The isolated membranes were washed three times in 10 mM Hepes and 1.0 mM EGTA to remove trace amounts of calcium. Prior to fusion assays the disk membranes were made 1 mmol/mL total phosphate in 10 mM Hepes, based on a phosphate assay.

The amount of free calcium present in the fusion mixture was regulated essentially as described by Robertson and Potter (1984) using a 10 mM Hepes, 1.0 mM EGTA chelating buffer. The amount of free calcium in the fusion assay mixtures was varied from 4.5×10^{-3} mM to 4.5 mM with the addition of $CaCl_2$.

Fusion Assays. The fusion of ROS disk membranes with R_{18}-labeled plasma membrane was measured using an extensively characterized lipid-mixing membrane fusion assay (Hoekstra et al., 1984). All fluorescence measurements were performed on an SLM 8000D spectrofluorometer. The fusion assays were performed essentially as described previously (Hoekstra et al., 1984). To initiate the fusion assay, R_{18}-labeled plasma membranes (initially ~30 μg of protein/mL) were introduced into a suspension of unlabeled ROS disk membranes (~800 μg of protein/mL). The disk membranes were first allowed to equilibrate to the appropriate temperature for 5 min. Then 50 μL of R_{18}-labeled plasma membrane was added to the disk membranes. In order to keep the final volume of the assay mixture constant, 50 μL of 10 mM Hepes, pH 7.4, was added simultaneously with the addition of labeled plasma membrane. In instances where calcium was added to the mixture, the appropriate amount of calcium was added to the disk membranes simultaneously with the addition of labeled plasma membrane. The pH of the solution was approximately 7.4. Fluorescence was monitored with an excitation wavelength of 560 nm and an emission wavelength of 586 nm. The fluorescence intensity obtained without the addition of plasma membrane was taken as a baseline. 100% fluorescence intensity was determined by adding 100 μL of 10% Triton X-100 to the sample. All fluorescence assays were performed under dim light.

Initial rates of fusion were determined from the rate of increase in fluorescence intensity as a function of time as described previously (Hoekstra et al., 1984). This assay has been shown to be sensitive (without artifacts) to the fusion of disk membranes with large unilamellar vesicles of phosphatidylethanolamine as well as disk membrane–disk lipid vesicle fusion (Boesze-Battaglia & Yeagle, 1992). The R_{18} lipid-mixing assay as described here was silent to disk membrane–disk membrane fusion. However, previous results indicated that R_{18}-labeled disk membranes did not spontaneously fuse with disk membranes in this system.

Additional Assays. Phosphate was determined as described by Bartlett (1959) and modified by Litman (1973). Cholesterol was determined as described by Allain et al. (1974). Protein was determined as described by (Lowry et al., 1951). All spectral measurements were performed on an SLM Aminco DW-2 spectrophotometer. Rhodopsin concentration was determined by measuring the difference in absorbance at 500 nm before and after illumination in the presence of 50 mM neutralized hydroxylamine using an extinction coefficient of 40 000. Light-stimulated phosphodiesterase (PDE) activity was assayed by continuous pH monitoring as described in detail previously (Boesze-Battaglia & Albert, 1990). A one-to-one relationship between H^+ produced and cGMP hydrolysis was assumed.

Results

Characterization of ROS Disk–Plasma Membrane Fusion. ROS disk membranes and ROS plasma membranes were obtained from the same ROS preparation. ROS plasma membranes were labeled with R_{18} and mixed with an excess of unlabeled ROS disk membranes. The unlabeled membranes must be in excess in this fusion assay. Since plasma membrane constitutes less than 10% of the ROS membranes (Boesze-Battaglia & Albert, 1989), larger quantities of the ROS disk membranes could be more readily obtained than the relatively scarce ROS plasma membrane.

When labeled plasma membranes were introduced to unlabeled disk membranes in the presence of 4.5×10^{-3} mM free calcium at 37 °C and pH 7.4, an increase in the fluorescence

FIGURE 1: Change in fluorescence of R_{18} upon fusion of labeled plasma membranes with disk membranes. A representative tracing of the change in fluorescence observed with the R_{18} mixing assay is shown. Fusion was initiated with the simultaneous addition of R_{18}-labeled plasma membrane and calcium ($[Ca^{2+}]_{free}$ = 45 mM) to the disk membranes as described under Materials and Methods. This addition is labeled 1a. 100% fluorescence intensity was calculated with the addition of Triton X-100; this addition is labeled 1b.

Table I: Fusion of R_{18}-Labeled Plasma Membrane with Disk Membranes and PC MLVs[a]

	$[Ca^{2+}]_{endo}$	$[Ca^{2+}]_{add}$	$[Mg^{2+}]$	EGTA
R_{18} PM_t–disk	+	+	–	–
R_{18} disk–disk	–	–	ND	–
R_{18} PM_{in}–disk	+	+	–	–
R_{18} PM_{ex}–disk	–	–	–	–
R_{18} PM_t–PC MLV	–	–	–	–

[a] The ability of R_{18}-labeled plasma membrane to fuse with the various target membranes was determined at 37 °C, pH 7.4 as described under Materials and Methods. PM_t refers to total plasma membrane, that is, plasma membrane vesicles as described in Figures 1 and 2. $[Ca^{2+}]_{endo}$ refers to disk membranes that have not been washed extensively in the EGTA chelating buffer, and therefore some endogenous calcium is present. $[Ca^{2+}]_{add}$ refers to the addition of 4.5 mM Ca^{2+} to membranes that have been washed extensively in EGTA buffer to remove trace levels of calcium associated with the membranes. Mg^{2+} was added at 1.0 mM as $MgCl_2$ as described in the text. EGTA was added at 1 and 10 mM concentrations. PM_{ex} refers to plasma membrane vesicles oriented with their extracellular side out, and PM_{in} refers to plasma membrane vesicles oriented with their intracellular side out as described in the legend to Figure 3. PC MLV fusion was carried out as described in the text. ND, not determined.

intensity of R_{18} was observed with time of incubation as shown in Figure 1. This increased intensity was interpreted as a dequenching of the R_{18} fluorescence due to dilution of the probe into the unlabeled membranes upon membrane fusion. When labeled plasma membranes were introduced to disk membranes at 37 °C and pH 7.4, in the absence of free calcium, no change in the fluorescence intensity of the R_{18} was observed (see Table I). Incubation of labeled ROS plasma membrane with egg phosphatidylcholine multilamellar liposomes produced little or no change in fluorescence intensity with time (see Table I) in the presence or absence of calcium.

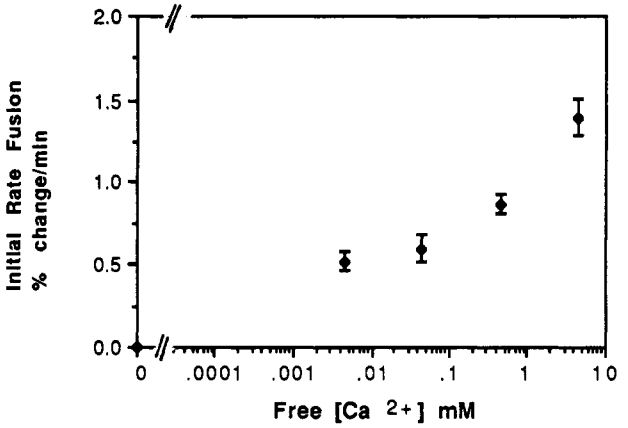

FIGURE 2: Calcium stimulation of ROS plasma membrane–disk membrane fusion. The rates of plasma membrane–disk membrane fusion was determined by the R_{18} lipid-mixing assay. Fusion was initiated with the simultaneous addition of R_{18}-labeled plasma membranes and calcium chloride in a Hepes, EGTA buffer to yield the free calcium concentrations shown. All measurements were taken at 37 °C. The data shown are representative of three independent experiments.

These data indicated that little or no spontaneous exchange of R_{18} occurred from R_{18} plasma membrane as donor, which would have obscured any membrane fusion events. In cases where a minimal increase in fluorescence intensity was observed in the same experiment with measurement of ROS plasma membrane fusion with ROS disk membranes, the apparent initial rate of that exchange was subtracted from the initial rate measured for the plasma membrane–disk membrane interaction. Control experiments also showed no exchange of R_{18} between labeled ROS disk membranes and unlabeled disk membranes. These results indicated that ROS disks did not spontaneously fuse with each other (see Table I).

The tendency of a bilayer to undergo fusion in some systems has been shown to be related to its ability to form nonbilayer structures (Ellens et al., 1989). One nonbilayer structure (the inverted hexagonal II phase) has been observed by electron microscopy in discrete regions of rod outer segments (Corless & Costello, 1981). It has also been shown that the extracted disk lipids undergo a Ca^{2+}-dependent bilayer to hexagonal II phase transition (Albert et al., 1984). Together these data suggest that disk membranes are capable of localized regions of bilayer destabilization and that these regions may be calcium dependent. Furthermore, the previously described fusion results indicate a calcium dependence.

Therefore the calcium dependence of disk–plasma membrane fusion was investigated. The isolated disk membranes were washed extensively in a 10 mM Hepes, 1.0 mM EGTA buffer in order to remove trace levels of calcium that may be associated with the disk membranes. As stated above in the absence of calcium, no detectable fusion was observed between disk membranes and R_{18}-labeled plasma membrane. The calcium concentration in the fusion mixture was then varied from 4.5 μM to 4.5 mM. Calcium was added to the disk suspension simultaneously with the addition of R_{18}-labeled plasma membrane. Figure 2 shows the calcium-dependent fusion of ROS plasma membranes and ROS disk membranes. The fusion of plasma membranes with disk membranes was observed at calcium concentrations as low as 4.5×10^{-3} mM calcium. Strong calcium stimulation was observed between 4.5×10^{-2} mM and 4.5 mM added $CaCl_2$. There was a 3-fold increase in the initial rate of disk membrane–plasma membrane fusion with the addition of millimolar calcium at pH 7.4, 37 °C. These results are representative of three inde-

Table II: Comparison of Cytoplasmic Side Out and Extracellular Side Out Plasma Membrane Vesicles

vesicles	rhodopsin total protein	chole-sterol/PL[a]	PL/protein	PDE activity[b]
cyto-out[c]	0.49	0.36	77	0.26
extra-out[d]	0.41	0.35	72	0.0

[a] PL_3 phospholipid by phosphate analysis. [b] PDE, phosphodiesterase activity, micromoles of H^+ per second per mole of rhodopsin at full flash. [c] Cytoplasmic side out plasma membrane vesicles. [d] Extracellular side out plasma membrane vesicles.

pendent preparations, each of which was done in triplicate. This represents the first example in the visual system of a calcium-dependent disk membrane–plasma membrane fusion in vitro, using a quantifiable assay of membrane fusion.

The specificity of calcium-stimulated fusion was examined by testing the ability of Mg^{2+} to substitute for calcium. The effect of $MgCl_2$ on disk membrane–plasma membrane fusion is shown in Table I. Magnesium was added to the disk suspension simultaneously with the addition of R_{18}-labeled plasma membrane. Mg^{2+} at 10 μM, 1 mM, and 10 mM had no effect on the initial rates of fusion observed. Thus magnesium will not substitute for calcium in this stimulation of membrane fusion at pH 7.4, 37 °C.

Specificity of Plasma Membrane Orientation in Relation to Disk–Plasma Membrane Fusion. A primary event in disk membrane shedding is the formation of packets of disks at the apical tip of the ROS. It is postulated here that the formation of these disk packets may involve a disk membrane–plasma membrane fusion event. On the basis of the orientation of the disk membranes and the plasma membrane in the ROS, fusion between disks and plasma membrane would involve the cytoplasmic surface of the plasma membranes fusing with the cytoplasmic surface of the disk membranes. The plasma membrane preparation contained vesicles with both possible orientations. In order to characterize the relationship between plasma membrane sidedness and the disk–plasma membrane fusion observed above, ROS plasma membranes oriented "cytoplasmic side out" or "extracellular side out" were isolated as described under Materials and Methods. These vesicles were separated according to their ability to bind to a Con-A column (extracellular side out) or not to bind (cytoplasmic side out). Further characterization of these vesicles appears in Table II. The sidedness suggested by the Con-A chromatography was confirmed by the phosphodiesterase activity. No ability to activate phosphodiesterase was exhibited by the extracellular side out vesicles. Such vesicles should have their rhodopsin oriented such that the G-protein binding site was occluded (facing the inside of the vesicles) and thus would not be expected to activate phosphodiesterase. Further characterization (Table II) showed that the rhodopsin content, the cholesterol content, and the phospholipid/protein ratio of the two vesicle populations was otherwise the same [and distinctly different from disk membranes (Boesze-Battaglia & Albert, 1989)]. SDS–polyacrylamide gel electrophoresis of the two plasma membrane preparations revealed no significant differences between them [although as reported previously (Molday & Molday, 1987; Boesze-Battaglia & Albert, 1989) significant differences were observed between plasma membrane and disk membrane]. Therefore the preparations in cytoplasmic side out and extracellular side out plasma membrane appear to differ from each other only in their orientation.

When plasma membrane vesicles (cytoplasmic side out) were introduced to calcium-depleted ROS disk membranes in the absence of added free Ca^{2+}, no change in fluorescence intensity was observed (see Table I). This is consistent with

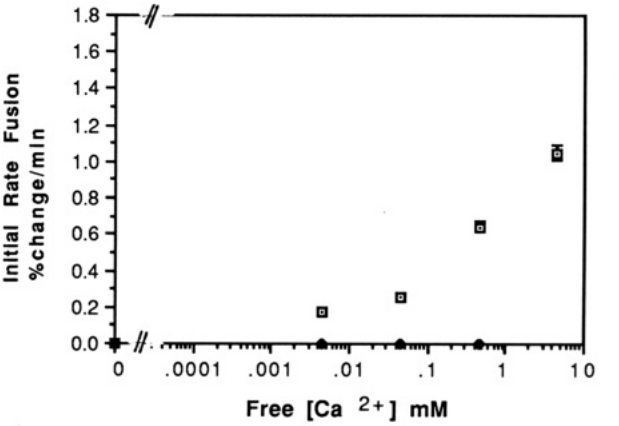

FIGURE 3: Fusion of cytoplasmic side out and extracellular side out plasma membrane vesicles with disk membranes. The rates of intracellular side out and extracellular side out plasma membrane vesicle–disk membrane fusion were determined by the R_{18} lipid-mixing assay. Fusion was initiated with the simultaneous addition of R_{18}-labeled plasma membranes and calcium chloride in a Hepes, EGTA buffer to yield the free calcium concentrations shown. All measurements were taken at 37 °C. The data shown are representative of three independent experiments.

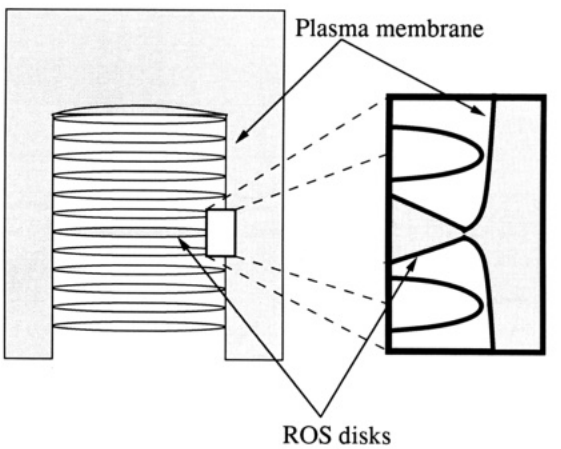

FIGURE 4: Schematic representation of the postulated fusion event between disk membranes and plasma membrane of the retinal ROS. The shading represents the continuity between the extracellular space and an intradiskal space permitted by such membrane fusion.

the results described above where no change in fluorescence intensity was seen upon mixing total R_{18}-labeled plasma membrane with disk membranes.

However, when the free calcium concentration in this mixture was established at 4.5 μM, an increase in the fluorescence intensity was observed. The calcium-dependent fusion of ROS disk and (cytoplasmic side out) plasma membrane fusion is shown in Figure 3. The calcium dependence of these fusion events closely mirrors the calcium dependence of disk–total plasma membrane fusion seen in Figure 2. Mg^{2+} could not substitute for calcium in these experiments (see Table I). Thus disk–plasma membrane fusion is a calcium-dependent event. This calcium-dependent membrane fusion occurs between plasma membrane vesicles that have their cytoplasmic surface exposed and disk membranes. This is the orientation of the membrane surfaces that is found in the ROS under physiological conditions.

When extracellular side out plasma membrane vesicles were introduced to calcium-depleted ROS disk membranes, no change in fluorescence intensity was observed except at the highest calcium concentration studied (see Figure 3). Thus plasma membrane oriented with the extracellular surface on the outside of the vesicle showed no fusion with disk mem-

branes except at very high calcium concentrations.

Discussion

Ca^{2+} levels in the rod cell have been reported to range from 1.4 nM to 0.5 mM depending on cellular activity (Korenbrot & Miller, 1989; Lambrecht & Koch, 1991). It was recently proposed that Ca^{2+} is involved in the sensitivity of the photoreceptor. On a molecular level little is known about Ca^{2+}-regulated processes in the rod cell. Presently, Ca^{2+} levels have been shown to indirectly regulate guanylate cyclase through recovrin (Dizhoor et al., 1991).

The work described here has revealed a novel Ca^{2+}-regulated process in the ROS. We have shown that the fusion between ROS plasma membranes and disk membranes is a Ca^{2+}-dependent event. This fusion can be supported by micromolar calcium concentrations.

Shedding of disk membranes likely involves a membrane fusion event. In order for a particular fusion event to be physiologically relevant, the sidedness of the membranes undergoing fusion must be addressed. Membrane fusion between disks and plasma membrane must involve, for topological reasons, fusion between the cytoplasmic surface of the disks and the cytoplasmic face of the plasma membrane. We have shown here that the calcium-dependent fusion event described is dependent on the sidedness of the plasma membrane vesicles isolated. As described above, fusion between plasma membrane vesicles and disk membranes was observed exclusively with (cytoplasmic surface out) plasma membrane vesicles. R_{18}-labeled plasma membrane vesicles oriented with their extracellular surfaces exposed are unable to fuse with disk membranes under any of the conditioned tested. Thus fusion between ROS disks and ROS plasma membrane is specific for contact between the membrane surfaces that normally face each other in the intact ROS.

Lucifer yellow labeling experiments suggested potential fusion events between disk and plasma membranes of the ROS. These labeling experiments revealed intrusion of dye specifically in the region of packet detachment in the ROS during shedding. This staining by lucifer yellow was localized and did not spread throughout the ROS. The staining occurred in single or multiple bands across the ROS. It was suggested that the restraint of the staining to relatively narrow bands was due to the involvement of ROS disks (Matsumoto & Besharse, 1985). In previous experiments, ROS vesiculation and tubulation (which required membrane fusion events) have been observed within the ROS in the region of old disk packet formation (Besharse & Dunis, 1982; Currie et al., 1978).

The specificity of the Lucifer yellow dye penetration could be simply explained as a fusion of a disk or closely spaced disks with the plasma membrane, allowing a continuity between specific intradiskal space and the external medium. A disk–plasma membrane fusion event would permit dye penetration into a band in the ROS, while not allowing access to the totality of the cytoplasmic space of the ROS. The observation that only relatively small molecules could enter this region indicates that the fusion zone is restricted. Such restriction could be readily achieved with disk–ROS plasma membrane fusion as represented schematically in Figure 4.

The fact that large molecules cannot enter this dye-stained region of the ROS again speaks to a disk–plasma membrane fusion. While an infolding of the plasma membrane could result in the complete separation of the packet of disks that are to be shed, the size restriction noted in this staining experiments would be lost, because an infolding of the plasma membrane would be expected to allow access to large molecules.

It is interesting to speculate that an early event in the shedding process involves a fusion between one or several old disks in the distal region of the ROS and the plasma membrane of the ROS. A signal of the initiation of this process could be the loss of integrity of disk morphology in that region. Previous observations of disk vesiculation in the region where the packet of old disks is ultimately separated from the ROS by definition suggest membrane fusion events (Matsumoto & Fabrie, 1990). Such vesiculation could be the aftermath of the initial potentiation of fusion which might initially involve plasma membrane and some disks in a localized region. However, this remains speculation since neither disk membrane–disk membrane fusion nor plasma membrane–disk membrane fusion have been examined fully in vivo.

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Mapping the Lipid-Exposed Regions in the *Torpedo californica* Nicotinic Acetylcholine Receptor†

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ABSTRACT: To identify regions of the *Torpedo nicotinic* acetylcholine receptor (AchR) interacting with membrane lipid, we have used 1-azidopyrene (1-AP) as a fluorescent, photoactivatable hydrophobic probe. For AchR-rich membranes equilibrated with 1-AP, irradiation at 365 nm resulted in covalent incorporation in all four AchR subunits with each of the subunits incorporating approximately equal amounts of label. To identify the regions of the AchR subunits that incorporated 1-AP, subunits were digested with *Staphylococcus aureus* V8 protease and trypsin, and the resulting fragments were separated by SDS-PAGE followed by reverse-phase high-performance liquid chromatography. N-terminal sequence analysis identified the hydrophobic segments M1, M3, and M4 within each subunit as containing the sites of labeling. The labeling pattern of 1-AP in the α-subunit was compared with that of another hydrophobic photoactivatable probe, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). The nonspecific component of [^{125}I]TID labeling [White, B., Howard, S., Cohen, S. G., & Cohen, J. B. (1991) *J. Biol. Chem. 266*, 21595-21607] was restricted to the same regions as those labeled by 1-AP. The [^{125}I]TID residues labeled in the hydrophobic segment M4 were identified as Cys-412, Met-415, Cys-418, Thr-422, and Val-425. The periodicity and distribution of labeled residues establish that the M4 region is α-helical in nature and indicate that M4 presents a broad face to membrane lipid.

The nicotinic acetylcholine receptor (AchR)[1] from *Torpedo* electric organ is a ligand-gated cation channel composed of four homologous, transmembrane subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Raftery et al., 1980). One of the best characterized membrane-bound allosteric proteins, it contains both the acetylcholine binding sites and the ion channel [for reviews, see Popot and Changeux (1984), Hucho (1986), Stroud et al. (1990), and Galzi et al. (1991)]. Electron microscopic image analysis of the AchR indicates that the subunits are arranged pseudosymmetrically around a central axis with all five subunits contributing structurally to form the lumen of the ion channel (Mitra et al., 1989; Toyoshima & Unwin, 1990).

The complete primary structures of the subunits have been established by cDNA cloning and sequencing (Noda et al., 1982, 1983a,b; Claudio et al., 1983). Each subunit contains four hydrophobic segments 20-30 amino acids in length, referred to as M1-M4, that are proposed to be membrane-spanning α-helices. Within each subunit, the helices might be organized as a four-helix bundle, with one segment from each subunit associating at the central axis to form the ion channel. Inspection of the subunit sequences revealed that M4 was the most hydrophobic and likely to be oriented at the periphery in greatest contact with lipid, while arguments could be made favoring the positioning of either M1, M2, or M3 at the central axis [reviewed in Popot and Changeux (1984)].

Affinity labeling studies with noncompetitive antagonists (Giraudat et al., 1986, 1987, 1989; Hucho et al., 1986; Oberthur et al., 1986; Revah et al., 1990; Pedersen et al., 1992) as well as combined mutagenesis and electrophysiological experiments (Imoto et al., 1986, 1988; Leonard et al., 1988; Charnet et al., 1990; Villarroel et al., 1991; Revah et al., 1991) provide evidence that M2 segments from each subunit are α-helical and associate around the central pore. In addition, the M1 segment may also be close to the ion permeation pathway since amino acids within that segment of the α-subunit are also labeled by a photoaffinity noncompetitive antagonist (Di Paola et al., 1990).

A variety of photoactivatable hydrophobic probes have been used to identify regions of the AchR interacting with lipid. Each AchR subunit is labeled by photoactivated phospholipids (Giraudat et al., 1985; Blanton et al., 1990, 1991) and cholesteryl diazoacetate (Middlemas & Raftery, 1987), as well as by small hydrophobic molecules, including [^{125}I]iodonaphthalazide (Tarrab-Hazdai et al., 1980, 1982), pyrenesulfonylazide (PsyA; Clarke et al., 1987), [^{3}H]adamantane-

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[1] Abbreviations: AchR, nicotinic acetylcholine receptor; 1-AP, 1-azidopyrene; [^{125}I]TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine; PsyA, pyrenesulfonylazide; 43K protein, the basic, membrane-bound 43-kDa protein of *Torpedo* postsynaptic membranes; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; Me_2SO, dimethyl sulfoxide; V8 protease, *Staphylococcus aureus* V8 protease; TPS, *Torpedo physiological* saline (250 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0).

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