of rDNA. It is not known whether this fragment is retained within the nontranscribed spacer of the rDNA of Novikoff hepatoma cells or if it serves a function. It may represent fortuitously amplified sequences, "selfish DNA", with no particular function. Future studies on this fragment should provide insight into the mechanism and process of gene amplification.

REFERENCES

Alt, F. W., Kellems, R. E., & Schimke, R. T. (1976) *J. Biol. Chem. 251*, 3063.

Benton, W., & Davis, R. W. (1979) *Science (Washington, D.C.) 196*, 180.

Caizzi, R., & Bostock, C. (1982) *Nucleic Acids Res. 10*, 6587.

Jelinek, W. R., & Schmid, C. W. (1982) *Annu. Rev. Biochem. 51*, 813.

Kominami, R., Urano, Y., Mishima, Y., & Muramatsu, M. (1983) *J. Mol. Biol. 165*, 209.

Mackey, J. K., Brackman, K. H., Green, M. R., & Green, M. (1977) *Biochemistry 16*, 4478.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.

Miller, O. J., Tantravanhi, R., Miller, D. A., Yu, L. C., Szabo, P., & Prensley, W. (1976) *Chromosoma 71*, 183.

Mroczka, D. H., Cassidy, B., Busch, H., & Rothblum, L. I. (1984) *J. Mol. Biol. 174*, 141.

Parker, D. L., Rothblum, L. I., & Busch, H. (1979) *Cancer Res. 39*, 1287.

Parker, D. L., Busch, H., & Rothblum, L. I. (1981) *Biochemistry 20*, 762.

Rothblum, L. I., Parker, D. L., Cassidy, B., Becker, F., Busch, H., & Rodriguez, L. (1981) *Biochem. Biophys. Res. Commun. 101*, 639.

Rothblum, L. I., Parker, D. L., & Cassidy, B. (1982) *Gene 17*, 75.

Schimke, R. T. (1984) *Cell (Cambridge, Mass.) 37*, 705.

Schimke, R. T., Alt, F. W., Kellems, R. E., Kaufman, R. J., & Bertino, J. R. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 649.

Spradling, A. C., & Mahowald, A. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1096.

Stark, G. R., & Wahl, G. M. (1984) *Annu. Rev. Biochem. 53*, 447.

Tiemeier, D. C., Tilghman, S. M., & Leder, P. (1977) *Gene 2*, 173.

Wahl, G. M., Stern, M., & Stark, G. R. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3683.

Wahl, G. M., Padgett, R. A., & Stark, G. R. (1979b) *J. Biol. Chem. 254*, 8679.

Whitney, F. R., & Furano, A. V. (1984) *J. Biol. Chem. 259*, 10481.

External Calcium Inhibits the Efflux of Calcium from Isolated Retinal Rod Outer Segment Disks

H. Gilbert Smith* and Peter M. Capalbo

GTE Laboratories, Inc., Waltham, Massachusetts 02254

Received November 20, 1984

ABSTRACT: Increasing the concentration of calcium in the external buffer flowing past isolated, intact bovine retinal rod outer segment disks immobilized in a flow system reduced the rate of radioactive calcium efflux from within the disks in the dark. We interpret these results as extradiskal calcium acting at an inhibitory binding site to block the calcium efflux. A Scatchard analysis of the external calcium dependence of the efflux yields an apparent dissociation constant of 50 μM, which further suggests that the inhibition is mediated by a specific membrane binding site. The observed inhibition of calcium efflux may represent a functional role for the high-affinity calcium binding site which has been identified by others in previous physical studies of the disk membrane. This external calcium inhibited permeability may explain some of the discrepancies in the reported calcium transport properties of disks. Variations in the external calcium concentration may alter the calcium content of isolated disks, thereby indirectly affecting other transport functions including the measured light-induced release of calcium. No evidence was found for either Na/Ca or Ca/Ca exchange processes across the disk membrane. Lanthanum was even more effective than calcium in inhibiting calcium efflux in the dark. Neither lanthanum nor calcium inhibited the light-induced efflux of calcium from disks, which implies either that light and extradiskal calcium regulate separate permeability processes in the disk membrane or that light greatly reduces the affinity of the inhibitory site for calcium and lanthanum.

The fundamental light-transducing element in vision is rhodopsin, an integral membrane protein which, in retinal rod cells, is found in the intracellular disk organelles within the rod outer segment. In spite of intensive efforts, no process has been proven to be the physiological link between the light energy absorbed by rhodopsin in the disk membrane and the observed change in sodium permeability that hyperpolarizes the plasma membrane to initiate the neural signal which results in vision. Although conclusive proof is not available, substantial evidence has accumulated to support Hagins' hypothesis (Hagins, 1972) which suggests that, upon light exposure, the disks release calcium ions which diffuse to the plasma membrane where they block sodium channels.

This hypothesis is supported by the observed inhibition of the light-sensitive sodium conductance of the rod outer segment plasma membrane by extracellular calcium (Yoshikami & Hagins, 1973). Calcium ionophores increase the sensitivity of the sodium conductance to extracellular calcium, which

0006-2960/85/0424-4233$01.50/0 © 1985 American Chemical Society

suggests that the inhibition is ultimately mediated by an increase in intracellular calcium (Hagins & Yoshikami, 1974). Further support comes from the rapid release of calcium from rod cells in intact retinas upon illumination. This calcium release appears to be the result of Na/Ca exchange acting upon a pool of intracellular calcium which is transiently increased by illumination (Gold & Korenbrot, 1980; Yoshikami et al., 1980). Schröder & Fain (1984) have shown that the total calcium content of the rod outer segment decreases upon illumination.

Efforts to directly measure a light-induced release of calcium from isolated disks have been successful in some laboratories, but there have been discrepancies in both the observed magnitudes and kinetics of the release [see Smith et al. (1977), Szuts (1980), and Kaupp & Schnetkamp (1982) for summaries of the data]. Smith et al. (1977) and Smith & Bauer (1979) observed a light-induced increase in the permeability of the disk membrane at high bleaching levels. Both the rate and magnitude of the resulting calcium release from sonicated disks were smaller than would be expected if such a process initiates vision. George & Hagins (1983) have characterized a nucleotide triphosphate dependent uptake and light-induced release of calcium in suspensions of broken rod outer segments. The measured calcium release which they attribute to transport across the disk membrane had both kinetics and magnitude consistent with calcium acting to initiate visual excitation.

Using flow system measurements, we have found that extradiskal calcium inhibits the efflux of radioactive calcium from intact disks in the dark. We interpret these results in terms of a disk membrane calcium permeability which is inhibited by externally bound calcium. On the basis of differential sensitivities to lanthanum, the extradiskal calcium-sensitive permeability appears to be separate from the light-regulated permeability. This inhibition of calcium efflux by extradiskal calcium may represent a functional role for the high-affinity disk membrane calcium binding site that has been characterized by various physical measurements (Kitano et al., 1983; Schnetkamp, 1979; Hendricks et al., 1977; Hemminki, 1975; Neufeld et al., 1972).

If this permeability mechanism functions in intact rod outer segments, it would allow extradiskal calcium to regulate the calcium content of the disks. In experiments with isolated disks, any procedure which removes extradiskal calcium will increase the calcium-regulated permeability and thereby deplete the intradiskal pool of calcium. The magnitude of the light-induced release of calcium from disks is sensitive to intradiskal calcium concentration (Smith & Bauer, 1979), and differences in the calcium content of the disks used in the various experimental systems may, therefore, explain some of the variations in the results of disk membrane calcium transport studies.

Experimental Procedures

Membrane Preparation. Disks were isolated from bovine rod outer segments by flotation on 5% ficoll in a buffer containing 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 5 mM potassium *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (K-HEPES) at pH 8.0 (Smith & Litman, 1982). The rod outer segments were prepared from frozen dark-adapted retinas (American Stores Packing Co. or Lawson Co., both of Lincoln, NE) by sequential flotation on 45% and 36% sucrose in pH 7.0 0.1 M potassium phosphate buffer. The rod outer segments were stored frozen in the dark under argon. A_{280}/A_{500} ratios for the isolated disks were between 2.3 and 2.6. Succinate dehydrogenase activity measured to monitor mitochondrial contamination was not detectable in the disk preparations using the dichlorophenolindophenol and phenazine methosulfate linked assay of Ackrell et al. (1978). The succinate dehydrogenase activity of the rod outer segment preparations was between 0.08 and 0.10 μmol (mg of rhodopsin)$^{-1}$ min^{-1}. The assay would have detected 5% of this activity in the disks. Also, calcium efflux experiments conducted in the presence of 10 μM ruthenium red or 1 μg/mL oligomycin were indistinguishable from those conducted in the absence of these mitochondrial inhibitors. George & Hagins (1983) point out the necessity of ensuring the absence of contaminating mitochondrial activities for studies of calcium transport in preparations of photoreceptor membranes.

Membrane Permeability Measurements. The intact disks were loaded with calcium by overnight equilibration at 4 °C in a suspension containing 1 mg/mL rhodopsin, 0.1 M KCl, 0.5 mM dithiothreitol, 15 mM $CaCl_2$, and about 30 μCi/mL ^{45}Ca in 0.1 M imidazole chloride buffer (pH 8.0).

The effects of changes in the composition of the external buffer on the efflux of calcium from the calcium-loaded disks were measured in a flow system like that previously used for characterizing the light-induced efflux of calcium (Smith & Bauer, 1979; Smith et al., 1977). A manual valve was added before the peristaltic pump so that the solution entering the flow chamber could be changed as desired without interrupting the flow. The calcium-loaded disks were supported by an about 100-μL bed of controlled-pore glass (2000-Å pore diameters, 200–400 mesh) on an 0.4-μm pore diameter Nucleopore filter in a translucent 13-mm diameter Millipore filter holder. We have also found Celite 545 to be a suitable support, as previously reported by Cavaggioni & Sorbi (1981). The system was thermostated at 30 °C. The flow rate was 0.6 mL/min with samples being collected every minute after a 20-min prewash which removed most of the external radioactivity. Liquid scintillation counting was used to determine the radioactive calcium in each sample. At the end of each experiment, the radioactivity of the glass beads and adhering membranes was measured to determine the remaining trapped calcium. Figure 1 shows a representative set of experiments.

The data from the flow system experiments were analyzed in a manner similar to that used previously for the light-induced release of calcium from sonicated disks. The difference in efflux (Δcpm) between the curve peak (cpm_{max}) and the lowest prepeak fraction (cpm_{min}) was taken as a measure of the change in membrane permeability for a given external buffer change. The magnitude of the resulting calcium release was calculated from the area of the efflux curve above a base line created by fitting the 10 data points taken before the external buffer change to a first-order exponential. The data were treated similarly for experiments such as those summarized in Figure 6 where the increase in efflux was caused by light exposure.

The calcium content of the disks at the time of a stimulus was estimated by the sum of the calcium released into the external buffer by that stimulus and the calcium remaining with the membranes at the end of the experiment. The original amount of calcium accumulated within the disks is difficult to estimate because some of that calcium may leak from the disks during the wash to remove external radioactive calcium. In experiments like those shown in Figure 4, where lanthanum was used to inhibit the leakage of calcium from the disks in the dark, we calculate that the disks had retained about 20 mol of Ca/mol of rhodopsin (Rh).

Calcium Buffers. Calcium buffers were prepared from 0.1 M $CaCl_2$ standard solution (Orion) and citric acid by using the equilibrium constants of Martell & Smith (1974). The

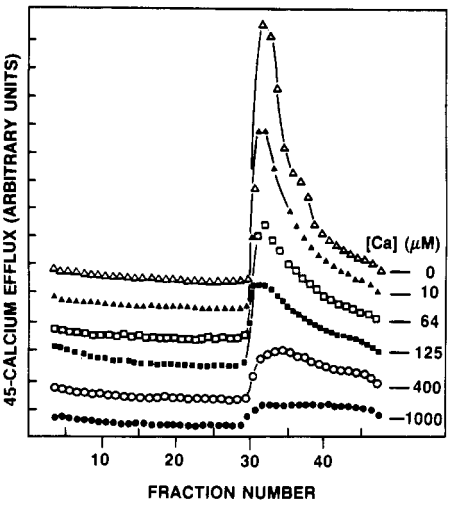

FIGURE 1: External calcium concentration dependence of calcium efflux from disks. The external buffer initially contained 1 mM $CaCl_2$, 0.1 M KCl, and 0.1 M imidazole chloride, pH 8.0. At fraction number 27, the external buffer was switched to one containing 0.1 M KCl, 0.1 M imidazole chloride, 3.8 mM free citrate, and the free calcium concentration shown on the right-hand side of the figure. The collection time for each fraction was 1 min. The peak fraction of the experiment with zero final external calcium contained 1.5 mol of Ca/mol of rhodopsin in the flow cell. This gives an average efflux of 6.9 pmol of Ca cm^{-2} min^{-1} during that peak fraction. The base lines of the experiments are offset for clarity.

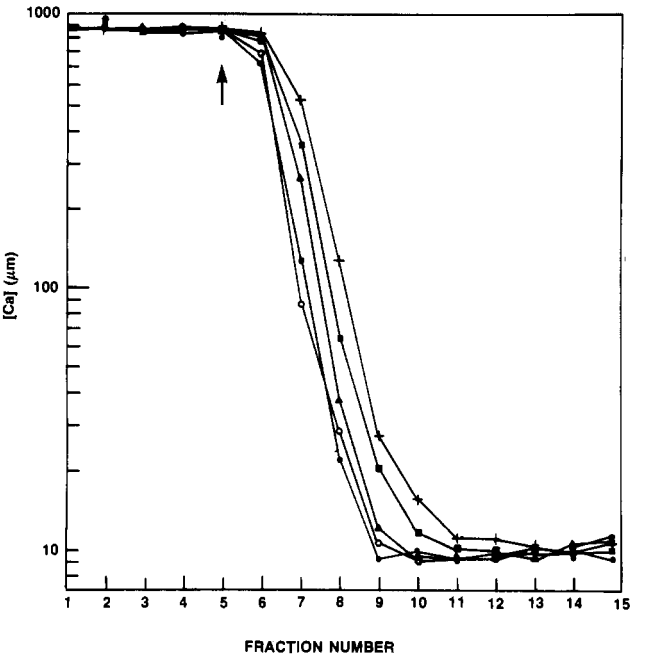

FIGURE 2: Rate at which the calcium concentration of the external buffer can be changed in the flow system. In these experiments, the external buffer initially contained 1 mM $CaCl_2$, 0.1 M KCl and 0.1 M imidazole chloride, pH 8.0. At fraction number 5, the external buffer was switched to one containing 0.1 M KCl, 0.1 M imidazole chloride, 10 μM free calcium, and free citrate at concentrations of 0 (+), 1 (■), 1.9 (▲), 3.8 (●), or 7.6 mM (○). All conditions were like those in the calcium efflux measurements except that the disks contained no radioactive calcium. A calcium-sensitive electrode was used to measure the free calcium concentration in each fraction. At fraction 10 which corresponds to the peak maximum of the calcium efflux experiments, the measured free calcium concentrations were 15.5 μM with no citrate, 11.5 μM with 1 mM citrate, 9.3 μM with 1.9 mM citrate, 9.1 μM with 3.8 mM citrate, and 9.6 μM with 7.6 mM citrate.

resulting free calcium concentrations were confirmed by measurements with a calcium-sensitive electrode (Orion 93-20) which was standardized against appropriate solutions containing no citrate. The total calcium concentrations in the standard solutions were confirmed by using an Applied Research Laboratories Model 35000 inductively coupled argon plasma emission spectrometer.

For experiments like those in Figure 2, the free calcium in each fraction of the effluent from the flow system was measured. To make such measurements in the 0.6-mL volume of each fraction, a sample cell was prepared by drilling a hole just larger than the calcium electrode tip partially into a plexiglass block. This cell was connected through a porous Teflon plug (Coetzee & Gardner, 1982) to a reference compartment containing 0.1 M KCl into which the reference electrode was inserted.

Miscellaneous Procedures. Except during the indicated light exposures, all procedures were conducted in darkness or under dim red light (Kodak Wratten 2 filter or Illumination Technologies Dark Red 20-wall filter for fluorescent fixtures). Rhodopsin concentrations were determined by the change in absorbance at 500 nm upon bleaching samples that were solubilized in 5% *n*-tridecyl decaethylene glycol monoether ($C_{13}E_{10}$ detergent) in 0.1 M imidazole and 0.05 M hydroxylamine at pH 7.0. All buffers contained 0.1 M imidazole chloride and 0.1 M KCl, pH 8.0, unless otherwise specified.

RESULTS

Inhibition of Calcium Efflux by Extradiskal Calcium. The efflux of calcium from disks in the dark was slower when the external buffer contained calcium than when it did not. This was most clearly seen in sets of experiments like those shown in Figure 1 where the external buffer initially contained a standard high concentration of calcium (usually 1 mM) which was then reduced to a lower concentration to initiate the increase in calcium efflux. Conducting the experiments in this manner allowed the calcium concentration of the initial external buffer to be the same for all of the experiments in a given set. Beginning with the high calcium buffer also minimized the loss of intradiskal calcium during the initial part of each experiment. The resulting enhanced retention of intradiskal calcium promoted maximal changes in efflux upon the subsequent reduction in the external calcium concentration. After a 45-min wash with 100 mM imidazole and 100 mM KCl, pH 8, containing no calcium, we found a retention of 3.23 ± 0.80 Ca/Rh (n = 6) which is comparable to that previously reported for sonicated disks (Smith & Bauer, 1979). We found 6 ± 1 Ca/Rh (n = 9) when the external buffer contained 1 mM $CaCl_2$. Lanthanum was even more effective than calcium in inhibiting the calcium efflux. We found retentions of 18 ± 3 Ca/Rh (n = 10) when the external buffer contained 0.1 mM $LaCl_3$.

The calculated amount of calcium retained within the disks in the presence of external calcium was somewhat underestimated in our data analysis because the specific activity of the internal calcium would be diluted by an influx of nonradioactive external calcium. If both the influx and efflux were through the same permeability, then one can represent that permeability by the base-line rate constant estimated by a first-order exponential fit to the 10 fractions taken before the change in external buffer. The estimated rate constant for the base-line efflux was typically about 0.034 min^{-1}. Using that value together with an external calcium concentration of 1 mM and an original internal calcium concentration of 15 mM, we estimate that after the 45-min wash before the buffer change, the internal calcium concentration was 6 mM and the specific activity was 89% of its original value.

Calcium Buffer Effects. Instantaneous changes in the external calcium concentration cannot be achieved in these flow

system experiments. The interface between two solutions containing different concentrations of calcium is broadened by diffusion in the lines running to the filter holder containing the disks and by transient binding of calcium to the glass beads and membranes as the interface passes through the holder. We found that the sharpest change from one calcium concentration to another was achieved when the initial, high calcium, solution contained no citrate buffer. The rate at which the external free calcium could be reduced was enhanced by the presence of free citrate in the second, low calcium, solution, as shown in Figure 2. The final external free calcium concentration was achieved by the time of the maximal calcium efflux in experiments such as those shown in Figure 1, when the external buffer contained 1.9, 3.8, or 7.6 mM free citrate.

There was a small increase in efflux when the external buffer was changed to one containing citrate even when there was no change in the external free calcium concentration (see Figure 1). We attribute this effect, which increased with increasing free citrate, to the changes in ionic strength and osmolarity which accompany the buffer change. For example, a solution with 1 mM free calcium and 7.6 mM free citrate requires 25.0 mM $CaCl_2$ and 31.7 mM potassium citrate whereas a 1 mM free calcium solution with 1.9 mM free citrate contains only 7.01 mM $CaCl_2$ and 7.92 mM potassium citrate. As a compromise between achieving a rapid change in the external calcium concentration and avoiding this base-line artifact, we conducted the experiments reported here with either 1.9 or 3.8 mM free citrate. With these citrate concentrations, the change in base-line efflux at a constant 1 mM calcium concentration was less than 10% of the maximal efflux observed when the calcium concentration was reduced from 1 mM to zero.

Apparent Dissociation Constant. The data from sets of experiments such as that shown in Figure 1 were scaled to the difference in peak efflux rate between the base-line experiment in which the external free calcium concentration was kept constant at 1 mM and the maximal efflux experiment in which the final external free calcium concentration was nominally zero. Control experiments showed that concentrations of external free calcium greater than 1 mM gave no measurable further reduction in efflux rate. The data were thus treated as an inhibition of efflux by increasing external calcium concentrations. Figure 3 shows a Scatchard plot of these data which assumes that the peak efflux at a given external calcium concentration is proportional to the relative number of unoccupied calcium binding sites. The slopes of linear least-squares fits to the data gave apparent dissociation constants of 56 μM for the experiments with 1.9 mM free citrate, 44 μM for those with 3.8 mM free citrate, and 47 μM for the combined data.

We also calculated the apparent dissociation constants from Scatchard plots for each set of experiments like that shown in Figure 1 and averaged the individual values thus obtained. In this determination, we included sets of experiments with fewer than four calcium concentrations which were omitted from Figure 3 so as not to give undue weight to data from experiments with 50 and 100 μM external free calcium. We obtained apparent dissociation constants of 50 $\pm$ 9 μM for 16 sets of experiments where the final free calcium was buffered with 1.9 mM free citrate and 60 $\pm$ 10 μM for another 16 sets of experiments where the final free calcium was buffered with 3.8 mM free citrate. Apparent dissociation constants derived from the intercepts of the Scatchard plots had essentially the same values as those derived from the slopes. Correcting the peak efflux data for estimated variations in the intradiskal calcium concentration also neither improved the fits of the data

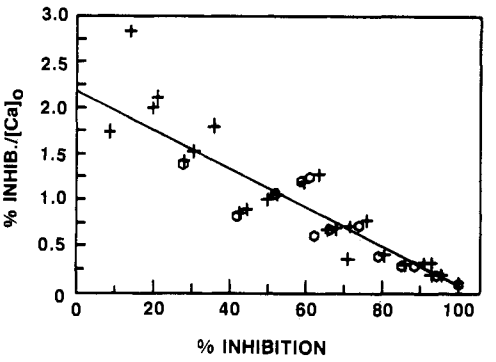

FIGURE 3: Scatchard plot of external calcium-sensitive efflux of calcium from disks. The percent inhibition of maximal efflux divided by the micromolar external calcium concentration is plotted vs. the percent inhibition for sets of experiments like those shown in Figure 1 where the final external calcium was buffered with free citrate concentrations of either 1.9 mM (O, five sets of experiments) or 3.8 mM (+, six sets of experiments). Each set of experiments included efflux determinations with at least four different external free calcium concentrations. An apparent dissociation constant of 47 μM was calculated from the slope of the linear least-squares fit. A value of 46 μM was obtained from the intercept of that plot.

to the Scatchard plots nor significantly altered the dissociation constants derived from those plots. As these data are plotted on a percentage scale normalized to the maximum efflux, one cannot obtain the number of binding sites. The data analysis assumes that in each set of experiments the peak effluxes are due to varying external calcium-dependent permeabilities acting upon equal pools of intradiskal calcium. The analysis does not require the absolute intradiskal calcium concentration to be known.

Competition between Inhibition of Calcium Efflux by External Lanthanum and External Calcium. Lanthanum was even more effective than calcium in inhibiting calcium efflux in the dark. We observed maximal inhibition of the efflux with 100 μM lanthanum chloride in the external buffer. The presence of even 5 μM lanthanum nearly eliminated any change in calcium efflux when the external calcium concentration was reduced from 1 to 0 mM (Figure 4b). The inhibition by lanthanum was slower to reverse than that caused by calcium, and thus, it was not possible to conduct experiments like those in Figure 1 in which the increase in efflux upon a decrease in inhibitor concentration is measured; therefore, a dissociation constant for lanthanum could not be determined.

Relationship between Light-Regulated and External Calcium-Regulated Permeability. Although lanthanum blocked the external calcium-sensitive permeability, it had no such effect on the light-regulated permeability (Figure 4a). The light-induced increase in efflux was larger in the presence of external lanthanum than in the presence of external calcium. This does not necessarily indicate a larger light-induced change in membrane permeability but may instead reflect a larger intradiskal pool of calcium upon which that permeability can act. Because external lanthanum is more effective than external calcium in inhibiting the efflux of calcium in the dark, experiments conducted in the presence of lanthanum will retain more of their original internal calcium during the wash to remove extradiskal radioactive calcium. In the experiments shown in Figure 4a, the internal calcium calculated back to the time of bleaching was 23 Ca/Rh for the experiment with external lanthanum and 14 Ca/Rh for the experiment with external calcium. When experiments conducted under different external buffer conditions are compared, care must be taken to consider variations in the size of the pool of internal

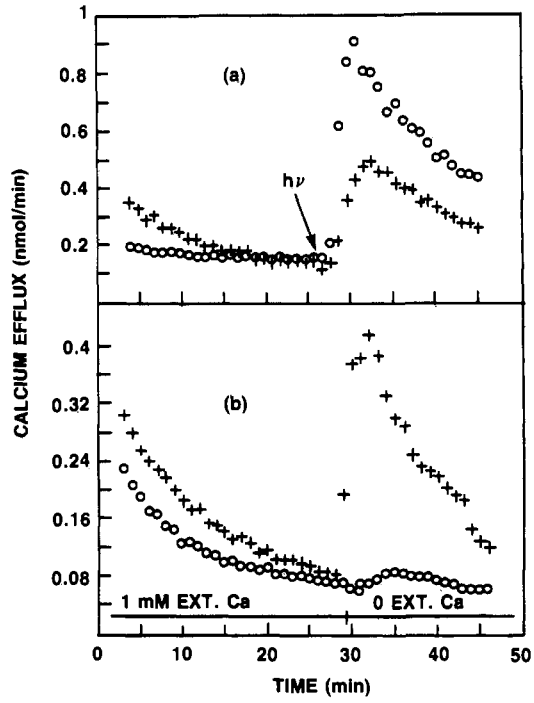

FIGURE 4: Effect of lanthanum on light (a) and external calcium (b) regulated calcium effluxes from disks. In (a), an experiment conducted in the presence of 0.1 mM lanthanum (O) gave about twice the maximum rate of light-induced efflux as did a parallel experiment conducted in the absence of lanthanum (+). In each case, a 30-s light exposure bleached about 50% of the rhodopsin in the sample. In (b), an experiment conducted in the presence of 5 μM lanthanum (O) gave almost no increase in efflux upon the removal of external calcium compared with a parallel experiment conducted in the absence of lanthanum (+).

calcium upon which a permeability change can draw.

From these results, it is unclear whether light and external calcium (or lanthanum) regulate separate permeabilities or whether the effect of light is to remove the ability of external calcium or lanthanum to inhibit a single permeability. This question was addressed by comparing the change in efflux observed when the external calcium concentration was reduced at the same time as light exposure, with the separate efflux changes created either by the reduction in external calcium concentration alone or by light exposure at a constant 1 mM external calcium concentration. If both external calcium and light act upon the same permeability, then the sum of the light-induced permeability increase measured at high (1 mM) external calcium concentration and the separate permeability increase resulting from a given reduction in external calcium concentration should be larger than the permeability increase resulting from simultaneous light exposure and reduction in external calcium. The difference between the sum of the separately measured permeability changes and the simultaneously measured permeability should be greatest when the final external calcium concentration is low. In the limiting case, when the final external calcium concentration is zero, then simultaneous light exposure should give no incremental increase in permeability over the permeability increase created by just the removal of external calcium. On the other hand, if light and external calcium act upon separate permeabilities, then the sum of the separately measured permeability changes should be the same as the simultaneously measured permeability change no matter what final external calcium concentration is used. The ratio of the sum of the separately measured peak efflux changes to the simultaneous peak efflux change from eight sets of experiments like those shown in

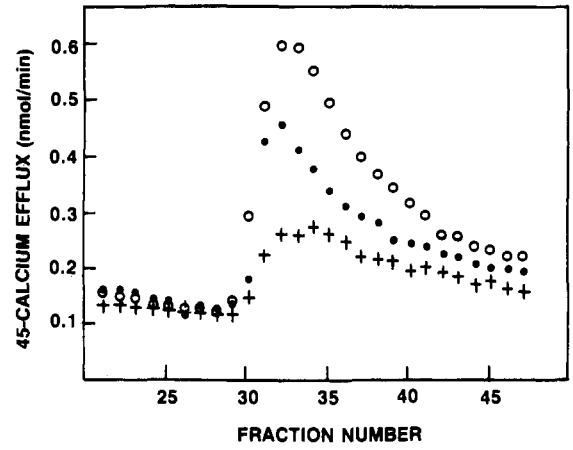

FIGURE 5: Changes in efflux upon both separate and simultaneous light exposure and reduction in external calcium concentration. The external buffer initially contained 1 mM $CaCl_2$. In the lower curve (+), the increase in efflux was caused by a 30-s light exposure which bleached approximately 50% of the rhodopsin in the sample. In the middle curve (●), the increase in efflux was caused by reducing the external free calcium concentration to 50 μM. This final free calcium concentration was buffered with 1.9 mM free citrate. For the upper curve (O), there was simultaneous reduction of the external calcium concentration and illumination. The light exposure was timed to coincide with the arrival of the 50 μM free calcium solution at the disk sample. As in the other experiments, all of the external solutions also contained 0.1 M KCl and 0.1 M imidazole chloride, pH 8.0.

Figure 5, where the final external calcium concentration was 50 μM, ranged from 0.72 to 1.23 with a mean of 0.96 and a standard deviation of 0.15. For experiments with a 100 μM final free calcium concentration, the ratio ranged from 0.64 to 1.26 with a mean of 0.90 (n = 3). For experiments where the final external buffer contained no calcium, the ratio ranged from 0.60 to 1.36 with a mean of 0.94 (n = 3). The large ranges can be partially attributed to the difficulty of creating a change in external calcium concentration at the disk surface which coincides exactly with the light exposure and to inaccuracies in estimating the base line from which the efflux changes are measured. Although we cannot rigorously exclude one mechanism or the other, these results suggest that light and external calcium act upon separate permeabilities.

Influence of Sodium, Potassium, and Magnesium. No additional efflux above the base line was observed in experiments where the external buffer was switched from one containing 1 mM $CaCl_2$ and 0.1 M KCl to one containing 1 mM $CaCl_2$ and 0.1 M NaCl. The increase in efflux observed when the second buffer contained 50 μM free calcium was the same with either 0.1 M KCl or 0.1 M NaCl. Thus, replacing KCl in the external buffer with NaCl altered neither the base-line efflux at high (1 mM) free calcium nor the increase in efflux observed upon a lowering of the external free calcium concentration. These results show that Na/Ca exchange is a significant factor neither in the base-line efflux at high external calcium concentration nor in the increased efflux which appears at lower calcium concentrations.

Although a complete study of the possible inhibition of calcium efflux by magnesium was not made, we did test whether or not external magnesium could replace external calcium as an inhibitor of calcium efflux in the dark. We found that switching the external buffer from one containing 1 mM free calcium to one containing 50 μM free calcium and 950 μM free magnesium buffered with 1.9 mM free citrate produced a 14-fold increase in efflux which was similar to the 12-fold increase observed when the final buffer contained only 50 μM free calcium buffered with 1.9 mM free citrate. Similarly, when the final external buffer contained no calcium and

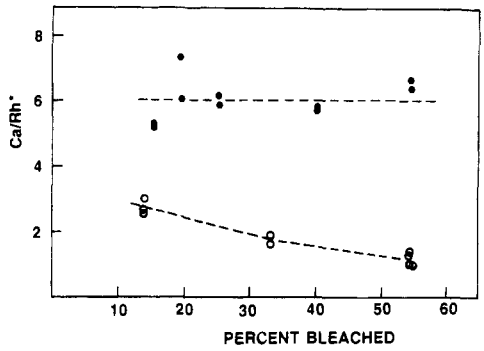

FIGURE 6: Light-induced release of calcium at two concentration levels of internal calcium. The external buffer contained 100 mM KCl and 100 mM imidazole chloride, pH 8.0, with either 1 mM $CaCl_2$ (O) or 0.1 mM $LaCl_3$(●). The trapped calcium calculated back to the time of light exposure was 6 ± 1 mol of calcium/mol of rhodopsin for the experiment with external calcium and 18 ± 3 mol of calcium/mol of rhodopsin for those with external lanthanum. Light exposures of between 2 and 30 s gave the indicated percent bleaching.

1 mM free magnesium also buffered with 1.9 mM free citrate, a 17-fold increase in efflux was observed which is comparable to the 16-fold increase found when the final buffer contained only 1.9 mM citrate with neither calcium nor magnesium. These results demonstrate that if magnesium has an inhibitory effect on calcium efflux, it must bind with a much lower affinity than does calcium.

Effects of Internal Calcium on Light-Regulated Permeability. In experiments with sonicated disks, Smith & Bauer (1979) reported that the magnitude of the light-induced release of calcium depends upon the calcium content of the disk vesicles. In the present experiments, we have found similar results with intact disks. We used external buffers containing lanthanum to greatly increase the retention of calcium by the intact disks in these flow system measurements and as shown in Figure 6 achieved higher magnitudes of light-released calcium than reported previously.

DISCUSSION

These results demonstrate a permeability pathway for calcium through isolated rod outer segment disk membranes in the dark which is inhibited by increasing concentrations of extradiskal calcium. The data presented are consistent with external calcium acting at an inhibitory binding site with an apparent dissociation constant of about 50 μM. This external calcium-regulated permeability appears to be separate from the light-regulated permeability of the disk membrane, but can indirectly influence the light-induced calcium efflux by altering the calcium content of the disks. Both permeabilities act upon the same pool of intradiskal calcium, but the light-induced permeability releases only part of the calcium in that pool whereas the external calcium-regulated permeability can totally deplete that pool. Our experiments cannot differentiate the various possible intradiskal sources of the released calcium whether bound, free, or some combination of the two. Schnetkamp et al. (1981) and Kaupp et al. (1981) have presented evidence that the light-released calcium originates in a bound, intradiskal pool.

The dissociation constant of the inhibitory calcium binding site agrees in magnitude with the high-affinity site reported by others in studies of the binding of calcium to photoreceptor membranes (Neufeld et al., 1972; Hendricks et al., 1977; Schnetkamp, 1979; Kitano et al., 1983). Schnetkamp (1979) presents evidence through the use of ionophores that the binding site is an intradiskal one, whereas the electrophoretic mobility studies of Kitano et al. (1983) indicate an extradiskal site. Our results are most easily explained by an extradiskal site which functions, at least in isolated disk membranes, to inhibit the permeability of the membrane to calcium. The binding can be attributed to a specific protein site in the membrane because the affinity of the site for calcium is much higher than would be expected for binding to even negatively charged lipid head groups (McLaughlin et al., 1981). We have not considered electrostatic effects in our calculation of the dissociation constants and have, therefore, described these as "apparent" constants. The linear Scatchard plots of the data (Figure 3) indicate that such effects are not significant at the ionic strength and calcium concentrations used.

The results which we attribute to an inhibition of unidirectional calcium efflux by extradiskal calcium can be contrasted with those of Schnetkamp et al. (1981) which characterize calcium exchange at an intradiskal binding site. In the exchange process, increases in nonradioactive calcium will exchange with the bound radioactive calcium and thereby reduce the magnitude of the observed light-induced release of calcium from those sites. If there were a calcium exchange mechanism across the disk membrane, then a decrease in extradiskal calcium would decrease calcium efflux rather than increase the efflux as we observe (Figure 1). We find no evidence for either Ca/Ca or Na/Ca exchange across the disk membrane in contrast to the plasma membrane where Na/Ca exchange has been found to control the intracellular calcium concentration (Gold & Korenbrot, 1980; Schnetkamp, 1981) and where a Ca/Ca exchange mechanism has also been reported (Schnetkamp, 1979). Szuts (1980) also observed low rates of Ca/Ca exchange across the disk membrane. In the flow system measurements reported here, increases in extradiskal calcium will inhibit the efflux of radioactive calcium from within the disks in the dark, thereby increasing the size of the intradiskal pool of calcium upon which the light-regulated mechanism can act (Figures 4 and 6).

The trans inhibition of an ionic flux such as that which we have observed is characteristic of the "single file" diffusion mechanism analyzed by Heckmann (1972). Such a trans inhibition can produce a self-limiting process when the movement of ions from one compartment to another increases the concentration of those ions in the second compartment, thereby inhibiting the flux. Such a process exists in neurons where the depolarization-triggered influx of calcium causes an increase in the intracellular calcium concentration which in turn inhibits the calcium influx (Hagiwara & Byerly, 1981). As the disks are intracellular organelles, an efflux of calcium from the disks has the same result as an influx across a plasma membrane. Both processes produce an increase in the intracellular calcium concentration.

The physiological role of the calcium-regulated permeability which we have described is unclear. Extradiskal calcium might serve as a feedback regulator of a light-induced calcium release; however, in the dark, the absence of external calcium would leave the membrane permeable to calcium. The store of calcium upon which the light-induced process acts would, therefore, be depleted. It is, however, possible that some physiological factor other than calcium acts as an efflux inhibitor to regulate the intradiskal calcium concentration.

The external calcium-regulated permeability must be considered in measurements of the calcium content and calcium transport properties of disks. The existence of this permeability may expain some of the discrepancies in the literature and may also resolve some previously poorly understood aspects of the disk calcium transport process. For example, Smith et al. (1977) found that the light-induced calcium release was greatly

reduced by the presence of extradiskal ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). This can probably be attributed to depletion of the intradiskal calcium as a result of the low extradiskal calcium concentration. Similar considerations must be taken into account for experiments which attempt to elucidate the influence of other calcium binding substances such as ATP and GTP on disk membrane properties.

ACKNOWLEDGMENTS

We thank Dr. Peter Cukor for useful suggestions during the early phases of the research and Dr. Charles N. Durfor for his critical review of the manuscript.

Registry No. Ca, 7440-70-2; La, 7439-91-0.

REFERENCES

Ackrell, B. A. C., Kearny, E. B., & Singer, T. P. (1978) *Methods Enzymol. 53*, 466.
Cavaggioni, A., & Sorbi, R. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3964.
Coetzee, J. F., & Gardner, C. W. (1982) *Anal. Chem. 54*, 2625.
George, J. S., & Hagins, W. A. (1983) *Nature (London) 303*, *344*.
Gold, G. H., & Korenbrot, J. I. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5557.
Hagins, W. A. (1972) *Annu. Rev. Biophys. Bioeng. 1*, 131.
Hagins, W. A., & Yoshikami, S. (1974) *Exp. Eye Res. 18*, 299.
Hagiwara, S., & Byerly, L (1981) *Annu. Rev. Neurosci. 4*, 69.
Heckmann, K. (1972) *Biomembranes 3*, 127.
Hemminki, K. (1975) *Vision Res. 15*, 69.
Hendricks, Th., Van Haard, P. M. M., Deamen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 467*, 175.
Kaupp, U. B., & Schnetkamp, P. P. M. (1982) *Cell Calcium 3*, 83.
Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1981) *Biochemistry 20*, 5500.
Kitano, T., Chang, T., Caflisch, G. B., Piatt, D. M., & Yu, H. (1983) *Biochemistry 22*, 4019.
Martell, A. E., & Smith, R. M. (1974) *Critical Stability Constants*, p 161, Plenum Press, New York.
McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., & McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445.
Neufeld, A. H., Miller, W. H., & Bitensky, M. W. (1972) *Biochim. Biophys. Acta 266*, 67.
Schnetkamp, P. P. M. (1979) *Biochim. Biophys. Acta 554*, 441.
Schnetkamp, P. P. M. (1981) *Biochemistry 20*, 2449.
Schnetkamp, P. P. M., Kaupp, U. B., & Junge, W. (1981) *Biochim. Biophys. Acta 642*, 213.
Schröder, W. H., & Fain, G. L. (1981) *Nature (London) 309*, 268.
Smith, H. G., & Bauer, P. J. (1979) *Biochemistry 18*, 5067.
Smith, H. G., & Litman, B. J. (1982) *Methods Enzymol. 81*, 57.
Smith, H. G., Fager, R. S., & Litman, B. J. (1977) *Biochemistry 16*, 1399.
Szuts, E. Z. (1980) *J. Gen. Physiol. 76*, 253.
Yoshikami, S., & Hagins, W. A. (1973) in *Biochemistry and Physiology of Visual Pigments* (Langer, H., Ed.) p 245 ff, Springer-Verlag, New York.
Yoshikami, S., George, J. S., & Hagins, W. A. (1980) *Nature (London) 286*, 395.

Nucleoside and Nucleotide Inactivation of R17 Coat Protein: Evidence for a Transient Covalent RNA–Protein Bond†

Paul J. Romaniuk‡ and Olke C. Uhlenbeck*
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801
Received September 7, 1984; Revised Manuscript Received February 27, 1985

ABSTRACT: R17 coat protein forms a specific complex with a 21-nucleotide RNA hairpin containing the initiation site for the phage replicase gene. The RNA binding activity of the protein is inhibited by prior incubation with 5-bromouridine (BrU). The inactivation occurs with pseudo-first-order kinetics, and the inactive protein is stable to dilution. RNA binding activity of the BrU-inactivated protein is restored upon incubation with dithiothreitol. Inactivation of coat protein by *N*-ethylmaleimide or *p*-(chloromercuri)-benzenesulfonate indicates that a cysteine residue is located near the RNA binding site. Since 5-bromodeoxyuridine does not inactivate coat protein, a specific binding event appears to be required before inactivation can occur. Surprisingly, unmodified cytidine nucleotides also inactivate coat protein, with a specificity similar to the modified analogues. These results are discussed with regard to the formation of a transient covalent RNA–protein bond.

The translational repression of bacteriophage R17 replicase gene expression by the phage coat protein has proven to be an excellent system for the detailed study of a specific RNA–protein interaction (Uhlenbeck et al., 1983). The coat protein binds specifically to a single RNA hairpin in the initiation region of the replicase gene, thereby preventing initiation of translation (Bernardi & Spahr, 1972). A synthetic 21-nucleotide-long RNA fragment corresponding to the binding site was found to have the same affinity as R17 RNA for the binding of coat protein (Krug et al., 1982; Carey et al., 1983a). Subsequent experiments have shown that substitution of one

† This work was supported by Grant GM 19059 from the National Institutes of Health.
‡ Present address: Department of Biochemistry and Microbiology, University of Victoria, Victoria BC V8W 2Y2, Canada.

0006-2960/85/0424-4239$01.50/0 © 1985 American Chemical Society