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SODIUM GRADIENT DEPENDENT CALCIUM TRANSPORT IN RENILLA LUMISOMES

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SUMMARY. Renilla lumisomes are membrane-bounded bioluminescent vesicles which produce light when the lumisomal membrane is made permeable to Ca^{2+}. During studies of Ca^{2+} transport we found that lumisomes can be made permeable to Ca^{2+} by establishing a Na^+ gradient with the higher Na^+ concentration being on the inside of the lumisomal membrane. No other cation will substitute for Na^+ on the inside but any of several monovalent cations can be used to maintain electroneutrality external to the lumisomes. This Na^+ gradient dependent Ca^{2+} transport appears not to involve active transport and occurs on a millisecond time scale suggesting that it is rapid enough to account for the onset of bioluminescence in Renilla.

Bioluminescence in Renilla is characterized by a Ca^{2+} triggered series of biochemical events leading to the emission of a flash of green light (1,2,3,4). The release of Ca^{2+} is apparently controlled by a nerve net (5,6). The reactions involved have been elucidated, and the three proteins directly involved in Renilla bioluminescence have been purified to homogeneity and characterized (2,7,8). One of these is a Ca^{2+} binding protein, with a Kd for Ca^{2+} of 1.4×10^{-7} M, which serves as a terminal link between nerve excitation and bioluminescence (2). During this process Ca^{2+} apparently acts as a second messenger by binding to this protein which results in the transfer of its bound luciferin to luciferase (2,3).

All of the proteins directly involved in Ca^{2+} triggered Renilla bioluminescence are membrane associated and can be isolated from the organism packaged within membrane-bounded vesicles termed lumisomes (9,10). Previous papers have demonstrated that Ca^{2+} permeability through the lumisomal membrane changes with the ionic environment of the lumisome (1,11). For example, if the ratio of Na^+ inside the lumisome was high, compared to the outside, the membrane became permeable to Ca^{2+} ions (1). In the present paper we show that the

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permeability of the lumisomal membrane to Ca^{2+} is specifically dependent on a Na^{+} gradient which provides the driving force for a rapid influx of Ca^{2+} into the lumisome. We also present evidence for at least two independent types of Ca^{2+} channels in the lumisomal membrane.

MATERIALS AND METHODS. All reagents used were of the best commercial grade available. Stop flow data were obtained using an on-line computer system. Luminescence measurements were determined as previously described (12,13). Renilla reniformis were obtained by collection at Sapelo Island, Georgia and lumisomes were prepared as previously described (1). Lumisomes where were loaded with ions other than sodium were suspended in a buffer of that ion, reisolated by centrifugation, and suspended a second time in the same buffer. All lumisome preparations were centrifuged at 1100 g for 20 min following resuspension. They were prepared in isotonic medium containing 20 mM Tris-HCl, pH 7.5 and 2 mM EGTA.

Lumisomes were assayed by adding 10 to 25 µl of lumisomes to 0.2 ml of isotonic medium (20 mM Tris-HCl , pH 7.5; 2 mM EGTA; XCl at isotonicity) followed by injection of 0.8 ml of test medium (2.0 mM $CaCl_2$; 20 mM Tris-HCl, pH 7.5; XCl at isotonicity). The XCl represents the chloride salt used to maintain isotonicity external to the lumisome, and this salt was left out of the second solution in lysis experiments.

It was possible to reduce experimental variation in the data by using the observation that lumisomes produced the same total photon yield regardless of the experimental condition used. For example, the peak intensity of each experimental determination was normalized against the total number of photons produced in the same experiment. This reduced the variation in the data due to pipetting and other experimental errors. Monitoring of total photons also allowed for an internal check for experimental conditions which might disrupt the bioluminescent system.

The characteristics of lumisome preparations, i.e., their ability, to support Na^{+} gradient dependent Ca^{2+} transport as well as their rates of Ca^{2+} leakages (facilitated diffusion) varied from preparation to preparation. The best preparations came from freshly captured animals. The ratio of the rates of Na^{+} gradient Ca^{2+} transport to Ca^{2+} leakage varied from a high of 40 to nearly one. Most preparations produced a ratio between 13 and 18 and thus preparations with ratios less than 13 were generally discarded. If lumisome preparations were stored in sodium azide at 4°C they were stable for up to three weeks before the above mentioned ratio dropped below 13. Ca^{2+} leakage rate increased with age and changes in this process occurred more rapidly than changes in Na^{+} gradient dependent Ca^{2+} transport.

RESULTS AND DISCUSSION. Evidence for Na^{+} gradient dependent Ca^{2+} transport. The lumisomal membrane is a boundary which Ca^{2+} must cross in order to trigger the luciferin binding protein and thus bioluminescence. This membrane is not impermeable to Ca^{2+} as a low level luminescence is observed when lumisomes are placed in a medium containing Ca^{2+} (Fig. 1A). The lumisome preparation used to produce the data shown in Fig. 1A, was loaded with sodium chloride and placed in a sodium chloride medium. As indicated in Fig. 1A all lumisome

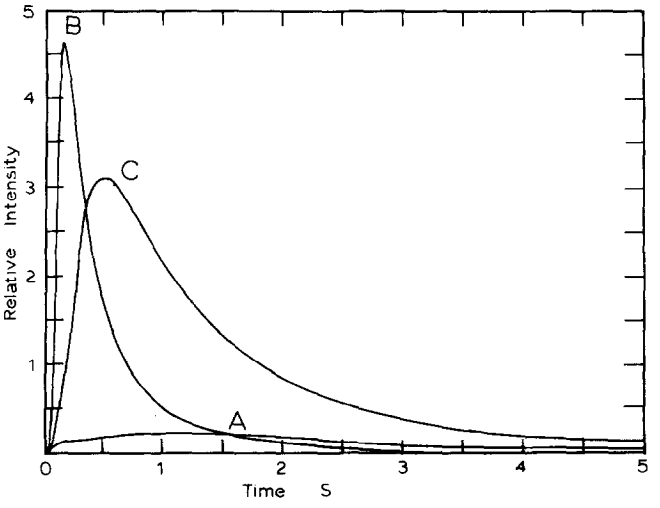
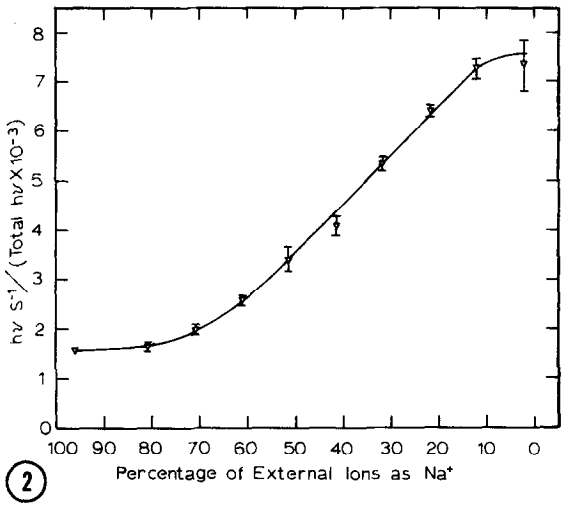


Figure 1. The rate of light emission from Renilla lumisomes. The emission was followed by use of a stopped flow apparatus using a ratio of eight volumes of reaction initiating buffer to 1 volume of Na^+ loaded lumisomes.

Curve A. Leakage. Inject isotonic sodium chloride plus 2 mM $CaCl_2$.
Curve B. Lysis. Inject 2 mM $CaCl_2$.
Curve C. Na gradient dependent transport. Inject isotonic potassium chloride plus 2 mM $CaCl_2$.

preparations show some degree of Ca^{2+} leakage apparently due to a facilitated diffusion process. That is, this Ca^{2+} leakage process, as measured by the luminescence rate, exhibits a saturation effect (see Fig. 3C) and there is no evidence for the requirement of a metabolic energy source. If lumisomes are ruptured by hypotonic stress the initial rate of light emission is at least 20 times greater than that observed during Ca^{2+} leakage (Fig. 1B). Furthermore, when the Na^+ external to the Na^+ loaded lumisomes is replaced by another cation, such a K^+, the membrane now becomes permeable to Ca^{2+}, and a flash of light is seen whose intensity is at least 13 times greater than that observed during Ca^{2+} leakage (Fig. 1C). The total photon yield produced is the same for all three of the above experimental conditions, and therefore the differences in intensity represent differences in the rates of Ca^{2+} access to the luciferin binding protein.



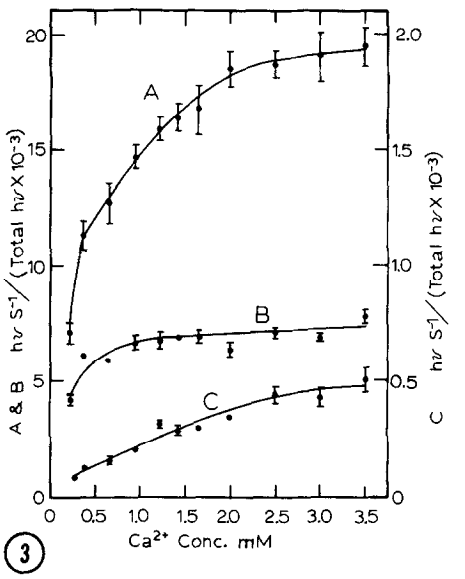


Figure 2. Light emission as a function of the Na^+ gradient. Na^+ loaded lumisomes assayed with decreasing amounts of Na^+ external to the lumisome. Potassium chloride was used to maintain isotonicity.

Figure 3. Effect of Ca^{2+} concentration on the rate of light emission. The Ca^{2+} concentration is the final concentration after mixing with Na^+ loaded lumisomes. The vertical dashed line represents the calcium concentration used in other experiments presented in this paper.

Curve A. Lysis. Inject buffer plus Ca^{2+}.
Curve B. Na^+ gradient dependent Ca^{2+} transport. Inject isotonic KCl plus Ca^{2+}.
Curve C. Leakage. Inject isotonic NaCl plus Ca^{2+}.

Lumisomes can be stored for long periods of time without affecting the rate of Na^+ gradient dependent Ca^{2+} transport (see methods) which suggests that Ca^{2+} transport is dependent on the Na^+ gradient and not on a metabolic energy source. Furthermore, added Mg-ATP has no effect on the Ca^{2+} permeability of the lumisomal membrane and added CN^- or ouabain (10^{-3} M) do not inhibit the Na^+ gradient dependent transport of Ca^{2+}. In fact, if lumisomes are preincubated for 4 min in CN^- (10^{-3} M) the Na^+ gradient dependent transport of Ca^{2+} is stimulated as much as 3-fold without affecting the Ca^{2+} leakage rate. The energy for Ca^{2+} transport into the lumisome must come, then, from the Na^+ gradient, and in fact the rate of Ca^{2+} transport is directly related to the magnitude of

Table I

EFFECT OF MONOVALENT CATIONS ON CALCIUM TRANSPORT

Monovalent Cation External to Lumisome	Monovalent Cation inside the Lumisome					
	Li^+	Na^+	K^+	Rb^+	Cs^+	NH_4^+
Li^+	1.50 ±0.24	29.3 ±2.6	5.41 ±0.43	4.98 ±0.24	11.7 ±0.2	3.67* ±0.12
Na^+	8.26 ±1.11	2.33 ±0.08	8.65 ±0.28	8.22 ±0.47	18.1 ±0.6	4.48* ±0.48
K^+	24.8 ±2.1	65.2 ±2.4	9.95 ±0.95	8.97 ±1.3	23.1 ±0.4	6.43* ±0.27
Rb^+	32.6 ±2.0	70.7 ±2.6	11.8 ±0.6	12.6 ±1.0	26.9 ±0.3	8.16* ±0.60
Cs^+	22.5 ±0.9	79.0 ±2.1	11.8 ±0.6	11.1 ±0.4	21.0 ±0.6	4.07* ±0.55
NH_4	28.1* ±1.7	81.52* ±1.42	6.55* ±0.76	---	---	4.58* ±0.01
$Choline^+$	---	47.6* ±1.6	23.5* ±1.2	---	---	12.3 ±0.3
Lysed Lumisomes	199.5 ±12.8	226.3 ±13.9	232.6 ±17.1	238.2 ±10.3	221.2 ± 7.6	157.99* ± 8.92

Lumisomes were loaded with the monovalent cations shown and assayed as described in methods. *Experiments done with separate lumisome preparation.

the Na^+ gradient (Fig. 2). The linear nature of the relationship indicates that transport is occurring by a mechanism other than Na^+ - Ca^{2+} exchange as described in nervous tissue (14,15,16,17).

Ca^{2+} transport into the lumisome is dependent only on the Na^+ gradient and not on the external ions used to maintain isotonicity (Table I). Potassium, Rubidium, Cesium, Ammonium, and Choline will all support Ca^{2+} transport when Na^+ is inside the lumisome. Li^+ shows some of the properties of Na^+ and will substitute for Na^+ to some degree (Table I). External Li^+ also tends to inhibit

TABLE II

Replacement Ions for Ca^{2+} in Na^{+} Gradient Dependent Transport

Replacement ion at 1.56 mM	PERCENT OF LUMINESCENCE OBTAINED WITH Ca^{2+}	
	In lysed lumisomes	In Na^{+} gradient Dependent transport
Co^{2+}	2.0	6.9
Cd^{2+}	29.4	21.5
Sr^{2+}	32.9	14.2
Mg^{2+}	0	0
La^{3+}	6.8	2.7

Na^{+} loaded lumisomes were mixed with either buffer containing replacement ion (as chloride) or isotonic KCl containing divalent ion.

Na^{+} gradient dependent Ca^{2+} transport as well. When choline is placed inside of the lumisome the total photon yield from the lumisomes dropped indicating that choline might interfere with the bioluminescent system. The choline data was not presented for this reason.

A number of divalent ions and one trivalent ion were tested for their ability to replace Ca^{2+} in the luminescence reaction during the hypotonic lysis of lumisomes and during Na^{+} gradient transport (Table II). Only Cd^{2+} and Sr^{2+} replace Ca^{2+} to any degree although some of the luminescence even with these ions may be due to contamination by Ca^{2+}. These same ions were also tested for their ability to inhibit Ca^{2+} triggered luminescence (Table III). None of the ions tested were strong inhibitors of the luminescence reaction observed with lysed lumisomes but La^{3+} was found to be a reasonable inhibitor of the Na^{+} gradient dependent transport of Ca^{2+} (Table III).

Evidence that Na^{+} gradient dependent Ca^{2+} transport is independent of the Ca^{2+} leakage process. In contrast to the transport process (Table III), La^{3+} does not inhibit the rate of Ca^{2+} leakage across the lumisomal membrane. The two processes are also affected differently by Mg^{2+}. Although Mg^{2+} is a weak inhibitor of luminescence (Table III), Mg^{2+} (300 mM) will completely inhibit

TABLE III

Inhibitors of Na^+ Gradient Dependent Ca^{2+} Transport

Inhibitor ion Added to Ca^{2+} At 1.56 mM	Conc Added ion mM	PERCENT INHIBITION OF LUMINESCENCE OBTAINED WITH Ca^{2+} ALONE In Lysed Lumisomes	In Na^+ Gradient Dependent Transport
Co^{2+}	1.56	3.9	13.8
	3.14	12.2	14.2
Cd^{2+}	1.56	15.9	11.6
	3.14	16.3	24.5
Sr^{2+}	1.56	10.9	0
	3.14	14.2	3.8
Mg^{2+}	1.58	7.0	13.9
	3.16	--	14.9
La^{3+}	1.56	28.0	82.4

Na^+ loaded lumisomes were mixed with either buffer containing inhibitor (as chloride) and 2 mM CaCl, or isotonic KCl containing divalent ions.

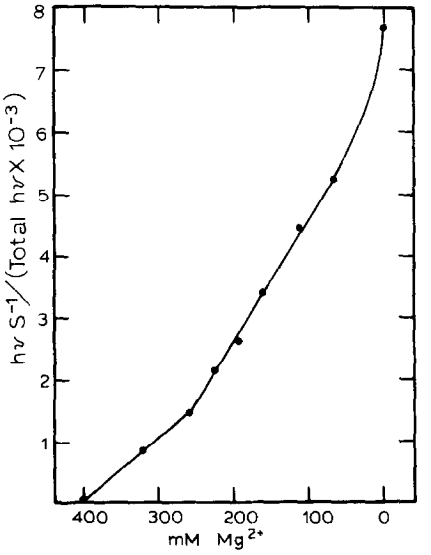


Figure 4. Mg^{2+} inhibition of Na^+ gradient dependent Ca^{2+} transport. Na^+ loaded lumisomes assayed in decreasing concentrations of $MgCl_2$ where KCl was used to maintain isotonicity. Final Ca^{2+} concentration was 1.56 mM.

the Na^+ gradient dependent Ca^{2+} transport process (Fig. 4) whereas the Ca^{2+} leakage rate remains unaffected. The concentration dependency of the two processes for Ca^{2+} are also considerably different (see Fig. 3B and 3C).

Note that the Na^+ gradient dependent transport process saturates at about 0.7 mM Ca^{2+} whereas the leakage process saturates at about 3 mM Ca^{2+}. In addition, pretreatment of lumisomes with mM CN^- results in a 3-fold stimulation of the transport process without affecting the leakage rate. Finally, as lumisome preparations age the Ca^{2+} leakage rate increases much faster than the loss of Na^+ gradient dependent Ca^{2+} transport ability.

The evidence presented above suggest that there exist at least two independent types of Ca^{2+} channels in the lumisomal membrane, one which selects for Na^+ gradient dependent Ca^{2+} transport and one which allows for a relatively low rate of Ca^{2+} leakage. Since a very rapid Ca^{2+} transport is observed in response to a Na^+ specific gradient (Fig. 1C) we suggest that this process may be important in the initiation of a bioluminescence flash in Renilla.

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