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# SYNERGISM OF TRIGGERED LUMINESCENCE BY SIMULTANEOUS TREATMENT OF pH TRANSITION AND POTASSIUM ADDITION IN CHLOROPLASTS

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## Summary

KCl-induced luminescence in relation to slow delayed light emission (>3 s) and pH shift-triggered luminescence was studied in preilluminated chloroplasts. An activation pathway for KCl-induced luminescence similar to that for acid-base-triggered luminescence but different from that for delayed light emission is suggested.

When the chloroplasts were subjected to a small amount of pH transition together with a simultaneous addition of KCl, a synergistic enhancement of triggered luminescence was observed. The synergism was not observed when the pH transition was increased. The results are interpreted according to the protonation model for stimulated luminescence.

## Introduction

A number of physical and chemical treatments stimulate delayed light emission from preilluminated chloroplasts: pH transitions, salt addition, electric field applications, temperature jump and organic solvent injection [1—8]. In a previous paper [9], acid-base-triggered luminescence in relation to slow delayed light emission (>3 s) was studied. The possibility of the presence of an activation pathway for acid-base-triggered luminescence which differs from that of slow delayed light emission was postulated.

In this paper, the relation between slow delayed light emission and KCl-triggered luminescence was examined first. Then simultaneous treatments of chloroplasts with pH transition and KCl addition were made to elucidate the interaction between the two kinds of triggered luminescence.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

## Materials and Methods

Chloroplasts were prepared from market spinach leaves by methods described previously [9,10] and were suspended in a preparation medium diluted to one half (0.2 M sucrose, 5 mM NaCl and 10 mM Tricine · NaOH, pH 7.9). The chloroplasts were stored at 0° C before use. The chlorophyll concentration was determined following the method of Arnon [11]. Luminescence was measured using the same apparatus as described previously [9].

Chloroplasts containing 0.08—0.1 mg of chlorophyll in 0.1 ml were added to a glass vial containing 0.1 ml of 0.2 M Tris · HCl with or without valinomycin (2 $\mu$M) and then mixed by shaking (pH 8.4). The vial was inserted immediately into a photometer and the chloroplasts were preilluminated for 20 s with 669 nm light of 3.3 $W \cdot m^{-2}$ obtained from an incandescent light source with an interference filter [9]. The photometer shutter was opened 3 s after preillumination. At 6 s after preillumination, 0.2 ml of a KCl solution was injected into the vial with an automatic pipette (mixing time 0.3 s) in order to observe the triggered luminescence.

For pH shift experiments, chloroplasts were added to a vial containing 0.1 ml of 10 mM succiniate and 10 mM Tris with valinomycin (2 $\mu$M) at pH 4.8 or 6.9. At 6 s after preillumination, 0.2 ml of a base solution containing 0.2 M Tris · HCl was injected into the vial to bring the chloroplasts to pH 8.4. For simultaneous treatment of pH shift and KCl addition, KCl was added to the base solution. The dilute concentration of valinomycin used (final concentration 0.5 $\mu$M) affected neither the pH shift-triggered luminescence nor the delayed light emission.

For quantitative estimation of the triggered luminescence, integrated light emission counts from 6 to 12 s after preillumination were recorded by selecting the photometer readings.

All experiments were carried out at room temperature of about 20° C.

## Results

*KCl-triggered luminescence*

Fig. 1 shows typical time courses of delayed light emission and superimposed KCl-induced luminescence with and without valinomycin. KCl-triggered luminescence was much increased when valinomycin (final concentration 0.5 $\mu$M) was added prior to preillumination. Only small triggered luminescence was observed when NaCl, instead of KCl, was injected, independently of the presence or absence of valinomycin (data not shown). These reuslts indicate that the luminescence must be triggered by a transient evolution of diffusion potential due to KCl and the thylakoid membrane as shown by Barber and Kraan [4].

Similar to the acid-base-induced luminescence mentioned in a previous paper [9], the decay of delayed light emission after the transient burst of triggered luminescence was found to return to the original curve (Fig. 1), although a slight decrease of the delayed light emission, probably due to the dilution of the chloroplast suspension, was sometimes observed. It may be concluded that the precursor for KCl-induced luminescence is not identical with that of delayed light emission.

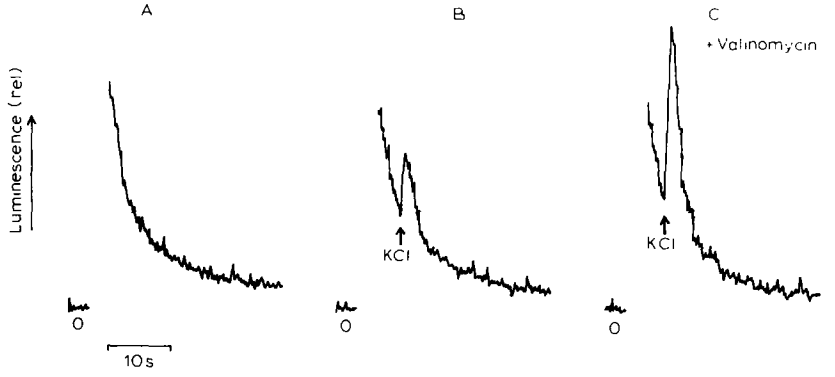


Fig. 1. Time courses of (A) delayed light emission, (B) KCl-induced luminescence and (C) KCl-induced luminescence with valinomycin (final concentration 0.5 μM, added prior to the preillumination). Preillumination ended at time 0. The arrows indicate the addition of KCl to the chloroplast suspension. The suspending medium was 0.1 M Tris · HCl, pH 8.4. Chlorophyll concentration 0.4 mg/ml in A and 0.2 mg/ml after the addition of KCl (final concentration 0.5 M) in B and C.

Several characteristics of KCl-triggered luminescence, which are quite similar to those of the acid-base-triggered one (cf. Figs. 2, 3 and 4, ref. 9), were observed: (a) A parallel decay of delayed light emission and KCl-triggered luminescence was observed when KCl solutions were added at various darkness periods. (b) Considerable increase of the triggered as well as the delayed light emission was observed (without changing the type of overall time courses) when DCMU (final concentration $1.4 \cdot 10^{-5}$ M), an electron transport inhibitor on the reducing side of Photosystem II, was added prior to preillumination. (c) The uncoupler Atebrin (final concentration $1.2 \cdot 10^{-4}$ M) only inhibited KCl-induced luminescence leaving the delayed light emission unaffected. (d) The uncoupler CCCP inhibited both triggered luminescence and delayed light emission; the extent of inhibition depended on its concentration (0.1—20 μM).

One clear difference between acid-base- and KCl-triggered luminescence was that an uncoupler $NH_4Cl$ added (up to the final concentration of 5 mM) prior to preillumination did not inhibit the latter in contrast to the former (cf. Fig. 4A, ref. 9). $NH_4Cl$ itself induced no triggered luminescence when injected after preillumination. Therefore, it is probable that the mechanism of KCl-induced luminescence differs somewhat from that induced by the acid-base transition.

*Synergism of triggered luminescence by simultaneous treatment of pH transition and KCl addition*

Fig. 2A shows the combined effect of pH transition and KCl addition on the triggered luminescence of chloroplasts. Curve a in Fig. 2A, 0.2 ml of chloroplast suspension at pH 6.9 was preilluminated for 20 s, then 0.2 ml of base solution containing 0.2 M Tris was injected at 6 s after preillumination to bring the chloroplasts to pH 8.4. A small triggered luminescence appeared due to the small difference of pH between the two stages. Curve b in Fig. 2A shows the triggered luminescence when 0.1 M KCl was injected into the chloroplasts at pH 8.4. When a base solution containing both 0.2 M Tris and 0.1 M KCl was

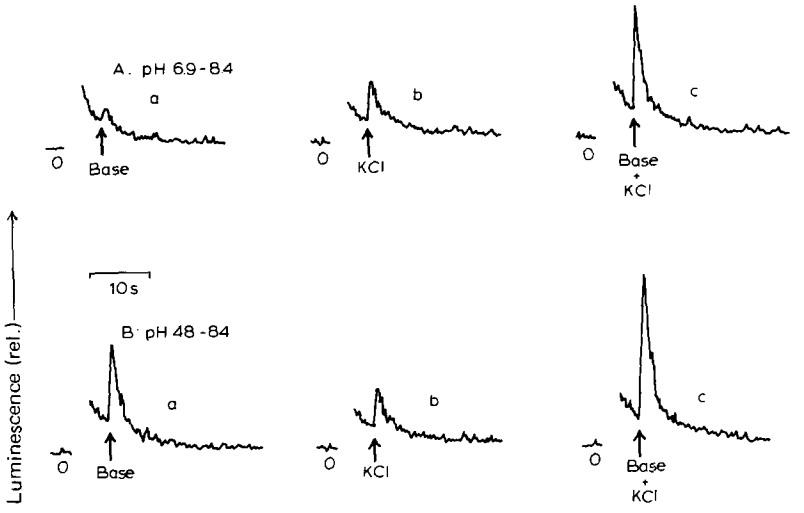


Fig. 2. (A) Time courses of (a) pH shift-triggered luminescence (pH 6.9—8.4), (b) KCl-triggered luminescence (at pH 8.4) and (c) luminescence triggered by simultaneous treatment of pH shift and KCl addition. (B)Time courses of (a) pH shift-triggered luminescence (pH 4.8—8.4), (b) KCl-triggered luminescence (at pH 8.4, the same curve as in Fig. 2A, b), and (c) luminescence triggered by simultaneous treatment of pH shift and KCl addition. Final concentration of KCl was 50 mM. Valinomycin (final concentration 0.5 μM) was added before preillumination. The arrows indicate the addition of base solution or KCl or both. For explanation, see Materials and Methods and Fig. 1.

injected into the chloroplasts at pH 6.9 to bring them to pH 8.4, a considerable enhancement of triggered luminescence was observed as shown curve c in Fig. 2A. The luminescence (initial peak) was found to be 2.3 times larger than the sum of each of the two triggered luminescences.

This is not the case when the pH transition is increased, e.g. pH transition from 4.8 to 8.4, as shown in Fig. 2B. The luminescence (initial peak) triggered by simultaneous treatment of acid-base transition and KCl addition (curve c in Fig. 2B) did not much exceed the sum of each of the triggered luminescences (1.3 times).

To confirm these observations quantitatively, triggered luminescence amounts were compared directly by reading the photometer countings. The triggered luminescence count starting at 6 s (at the time of injection) to 12 s after the preillumination was read first. Almost all triggered luminescence was terminated by 12 s under the experimental conditions used. Then the amount of delayed light emission (without the triggered luminescence, see Fig. 1) was read over the same time range (6—12 s). Subtracting the latter reading from the former gives the amount of each triggered luminescence. The relationship between the total output of luminescence and the initial peak has found to be linear [5].

Results obtained from experiments in which two different ranges of pH transition were combined with the addition of three different concentrations of KCl are summarized in Table I. Experimental procedures were similar to those in Fig. 2. Synergistic enhancement of triggered luminescence compared with the sum of each of the two triggered luminescences is evident in Table IA.

Degree of the synergism, i.e. the ratios of luminescence caused by simultaneous treatment of pH transition and KCl addition to the sum of each of the two separate triggered luminescences, were 2.2, 2.8 and 2.3 for 5, 50 and

TABLE I

SYNERGISM OF THE TRIGGERED LUMINESCENCE CAUSED BY THE SIMULTANEOUS TREATMENT OF pH SHIFT AND KCl ADDITION IN CHLOROPLASTS

Sizes of the pH shift were from 6.9 to 8.4 (A) and from 4.8 to 8.4 (B), and the final concentrations of KCl added were 5, 50 and 500 mM. Experimental conditions as for Fig. 2.

| Experiment | KCl concentration (mM) | pH shift-induced luminescence * | KCl-induced luminescence * (at pH 8.4) | Luminescence induced by pH shift and KCl addition * | Enhancement |
|---|---|---|---|---|---|
| A (pH 6.9—8.4) | 0 | 14 | | | |
| | 5 | | 5 | 41 | 41/14 + 5 = 2.2 |
| | 50 | | 28 | 119 | 119/14 + 28 = 2.8 |
| | 500 | | 64 | 176 | 176/14 + 64 = 2.3 |
| B (pH 4.8—8.4) | 0 | 122 | | | |
| | 5 | | 5 | 142 | 142/122 + 5 = 1.1 |
| | 50 | | 28 | 183 | 183/122 + 28 = 1.2 |
| | 500 | | 64 | 223 | 223/122 + 64 = 1.2 |

* Relative values (photometer countings).

500 mM KCl, respectively, in Table IA and 1.1, 1.2 and 1.2, respectively, in Table IB. The ranges of the ratios obtained from several other experiments in pH transition from 6.9 to 8.4 were 2.2—1.4 (3 experiments), 2.0—1.5 (7 experiments) and 2.3—1.4 (7 experiments) for 5, 50 and 500 mM KCl, respectively, and those in pH shift from 4.8 to 8.4 were 0.96—0.81 (5 experiments), 1.2—0.88 (9 experiments) and 1.2—0.56 (7 experiments), respectively.

Although KCl-triggered luminescence was measured at pH 8.4 throughout the experiments in Table I and those described above, the luminescence

TABLE II

pH DEPENDENCY OF KCL-TRIGGERED LUMINESCENCE

Photometer countings (relative values) as for Table I. Chloroplasts containing 0.08 mg of chlorophyll in 0.1 ml were added to the vial containing 0.1 ml of 0.1 M Tris · HCl (pH 8.4) or 10 mM succinate and 10 mM Tris (pH 6.5 and 4.8). Valinomycin was added (final concentration 0.5 μM) in all experiments. At 6 s after preillumination, 0.2 ml of 0.1 or 1 M KCl was injected (final concentration 50 and 500 mM, respectively) and the luminescence was counted from 6 to 12 s. For details, see Materials and Methods and also Results.

| pH | KCl | |
|---|---|---|
| | 50 mM | 500 mM |
| Experiment 1 | | |
| 8.4 | 64 | 96 |
| 6.5 | 50 | 77 |
| 4.8 | 22 | 71 |
| Experiment 2 | | |
| 8.4 | 72 | 98 |
| 6.5 | 57 | 62 |
| 4.8 | 13 | 57 |

decreased at pH values less than 8 (Table II). Therefore, the average values of the synergism would be larger than those described above when KCl-triggered luminescence was measured at pH values in lower pH stages. In experiments where various pH transition ranges were combined with KCl addition, maximum synergism was obtained when the pH shifts were from approx. 7 to 8.4 rather than from 4—6 to 8.4.

In the presence of uncoupler $NH_4Cl$ (5 mM), the triggered luminescences caused by the simultaneous addition of base and KCl solution were almost the same as those by the injection of KCl alone.

## Discussion

As shown in Fig. 1, KCl-triggered luminescence was much increased when valinomycin was present in the chloroplast suspension. Therefore, luminescence is supposed to be caused by a diffusion potential produced by a selective increase of permeability of $K^+$ through the thylakoid membranes (inside positive) [4,12,13]. This assumption explains why the uncoupler $NH_4Cl$ does not inhibit KCl-triggered luminescence.

The effects of the electron transport inhibitor DCMU, and uncouplers Atebrin and CCCP were similar for both KCl- and acid-base transition-triggered luminescence [9]. However, the fact that $NH_4Cl$ did not inhibit KCl-triggered luminescence apparently indicates the presence of a different pathway of activation between the two kinds of luminescence.

On the other hand, some interactions were observed between KCl-stimulated and pH transition-triggered luminescence under certain experimental conditions. As clearly shown in Table IA (cf. Fig. 2A), triggered luminescence caused by the simultaneous addition of base and KCl solution exceeds the sum of each of the two triggered luminescences obtained separately when the $\Delta$pH is small (from pH 6.9 to 8.4). This synergism does not appear when the pH shift is extended from 4.8 to 8.4, accompanying the increased contribution of the acid-base-triggered luminescence.

The synergism observed can be explained if one supposes that the two kinds of triggered luminescence originate from common substrates and that the stimulating pathways of the two align in a series.

If the two kinds of triggered luminescence have separate substrates and pathways for activation, then no interaction would be observed. If the two luminescences have common substrates and separate pathways connecting independently to the substrates, then enhancement could not be explained, either. On the contrary, when the two luminescences have common substrates and separate pathways connecting to the substrates in a series, one can expect a synergistic enhancement of the luminescence under certain conditions. If the two pathways were activated simultaneously and cooperatively, a synergism could be observed as long as available substrates remain. This is presumed to be the case in the experiments shown in Fig. 2A and Table IA. When stimulation of both triggered luminescences were suboptimal, considerable enhancement could be observed by both treatments simultaneously.

The above considerations can be well explained by the model proposed by

Kraan et al. [12] (see also ref. 14). According to this hypothesis, the reduced primary electron acceptor $Q^-$ and oxidized donor $ZH^+$ of System II are located on the outside and inside of the thylakoid membrane and react in a pH-dependent equilibrium as

$$Q^- + H^+ \rightleftharpoons QH \text{ (outside)} \tag{1}$$

and

$$ZH^+ \rightleftharpoons Z + H^+ \text{(inside)} \tag{2}$$

Luminescence occurs only upon back reaction of $Q^-$ and $ZH^+$ as substrates (see Fig. 6 in ref. 12). The acid-base transition would favor a shift of the equilibrium of reactions 1 and 2 towards the formation of $ZH^+$ and $Q^-$, thus in turn stimulate the luminescence. Addition of salts such as KCl produces a diffusion potential which is positive on the inside with respect to the outside of the thylakoid membrane. The potential of this sign would accelerate the reaction between the two charged species $ZH^+$ and $Q^-$ located on opposite side of the membrane, thereby eliciting luminescence.

The protonation model above presents an explanation for the observed synergism of triggered luminescence when simultaneous treatment of pH transition and KCl addition with valinomycin are applied to chloroplasts. It is possible that the addition of KCl generates potential which further accelerates the recombination reaction of $Q^-$ and $ZH^+$ caused by the pH transition under a suboptimal pH gradient.

If both the proton gradient and membrane potential are envisaged as reducing the activation energy for the back reaction (triggered luminescence), the rate of luminescence production $L$ may be expressed [15,16] by

$$L = \phi' J = \phi'(ZH^+ \cdot Q^-)\, k' \nu \exp\left[-\left(E_{ac} - \Delta\psi - 2.303 \frac{RT}{F} \Delta pH\right) \Big/ kT\right] \tag{3}$$

where $J$ is the rate of chlorophyll singlet formation, $\phi'$ is the fluorescence yield of chlorophyll involved in luminescence, $ZH^+ \cdot Q^-$ is the precursor for triggered luminescence, $k'$ is a constant containing entropy terms, $\nu$ is a frequency factor, $E_{ac}$ is the activation energy for the back reaction, $\Delta\psi$ is the membrane potential due to KCl pulse, $\Delta$pH is the pH gradient across the thylakoid membranes created by acid-base transition and $k$ is the Boltzmann factor. The synergistic effect described above might qualitatively be accounted for by the exponential relationship between the membrane potential and pH gradient and the rate of luminescence.

When the proton gradient per se is large, the triggered luminescence by pH alone may predominantly consumes a large part of $Q^-$ and $ZH^+$, or the sum of membrane potential by KCl and protom-motive force in Eqn. 3 may exceeds the activation energy for the triggered luminescence. Experimental errors which may be caused by the limitations of mixing time (0.3 s) in the present paper should also be considered. More detailed analyses of the synergistic effect discussed in this paper remain for future study.

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