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Calcium regulates the rate of rhodopsin disactivation and the primary amplification step in visual transduction

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The kinetics of the light-induced activation of transducin as well as the subsequent disactivation process can be monitored by means of a specific light scattering transient P_A. In this communication it is demonstrated that the rate of transducin disactivation is calcium dependent, increasing when the calcium concentration is decreased. As a consequence of the accelerated recovery in low calcium, the time to the peak of the transducin activation process is shortened and the gain of the primary amplification step, i.e. the number of transducin molecules activated per bleached rhodopsin, is reduced. Experiments using hydroxylamine as an artificial quencher of rhodopsin activity suggest that calcium acts upon rhodopsin kinase and not upon the rate of the GTPase. This would indicate that calcium may control visual adaptation not only by regulating guanine cyclase activity, but also by affecting the primary step in the transduction cascade, the rhodopsin–transducin coupling

Photoreception; Transducin disactivation; Rhodopsin; Phosphorylation; Adaptation; Ca^{2+}; Light scattering

1. INTRODUCTION

Two major features of the vertebrate photoreceptor cell are sensitivity and dynamic range. Rod photoreceptor possess the ability to detect single photons [1] and they still function under conditions where thousands of photons are absorbed per rod and second [2]. Consequently, transduction must involve a high amplification (in the dark adapted rod one absorbed photon produces a photon current of 6.2×10^6 elementary charges [3]) and a mechanism that attenuates this gain in ambient light. Recent reports have suggested that calcium ions may play a key role in this process of light adaptation (reviewed in [4]), and the calcium dependence of guanylate cyclase [5,6] is currently viewed as responsible for calcium sensitivity. The intracellular calcium concentration is lowered in the light [4,7] and as a consequence the replenishment of the cGMP pool is stimulated [5,8]. However, visual transduction is comprised of a sequence of amplification steps and hence each individual gain must be attenuated if saturation is to be avoided. This calls for a mechanism that decreases the amplification of the primary amplification steps.

So far only one laboratory has reported calcium effects on early stages of phototransduction, e.g. a regulation of phosphodiesterase (PDE) activity in frog rod outer segments (ROS) [9,10]. It was found that the light sensitivity of PDE activity is decreased when the calcium concentration is lowered from 1 mM to 10 nM, and it was reported that the cellular components responsible for this regulation are easily lost: the effect is only observed with a crude suspension of freshly detached ROS. The question of whether calcium exerts its effect on rhodopsin, transducin or PDE has not been answered as yet. We have, therefore, attempted to measure the effect of calcium on the light induced activation and disactivation of transducin, using a previously established light scattering assay and

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suspensions of highly intact stacks of disks from bovine rod outer segments [11–13]. We found that there is a desensitising effect which occurs when the calcium concentration is varied in the physiological range and we therefore propose that part of the role of calcium in visual adaptation is to regulate the gain of the primary amplification step, i.e. the number of transducin molecules activated per photolysed rhodopsin.

2. MATERIALS AND METHODS

2.1. *Preparation of disk stacks*

Structurally intact disk stacks with perforated plasma membrane were prepared according to [14]. Briefly, retinas of fresh bovine eyes were isolated in Hepes-Ringer, passed through a nylon mesh after vortexing and spun at 30 000–60 000 × *g* for 20 min on a discontinuous sucrose gradient (31%, w/v). ROS at the interface were harvested and washed once in Ringers. The resulting pellets were resuspended in a sucrose/Ringer's medium in which 50 mM NaCl was replaced by 100 mM sucrose, at a rhodopsin concentration of about 0.1 mM. Small aliquots were frozen quickly in liquid nitrogen and thawed immediately prior to an experiment. In our hands this yields ROS with mostly intact disk stacks but perforated plasma membrane.

2.2. *Activation and disactivation of transducin*

Transducin activation and disactivation could be followed in real time using a well characterised light scattering assay [11–13]. As we have previously shown, the transient interaction between bleached rhodopsin and transducin results in a light scattering transient P_A, which is suppressed when transducin is permanently preactivated (using GTP-γ-S and low light) prior to a testflash and which is unaffected when PDE is preactivated by exogenous G_α-GTP-γ-S. P_A signals show complete recovery and the kinetics of this recovery follows transducin deactivation without measurable delay. The latter was derived from the fact that the degree of recovery determines the amplitude of a subsequent flash and that identical signals can be obtained more than one hundred times, provided the flash frequency was low enough to allow complete recovery in between flashes.

2.3. *Light scattering measurements*

Light scattering measurements were carried out in a home-made multi-angle flash photolysis apparatus (MAFPA). Its design and performance have been described elsewhere [15].

At the beginning of each experiment a fresh ROS sample (20 μl) was resuspended in 2 ml of the particular measuring buffer, containing 100 mM NaCl, 10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM GTP, 0.2 mM Bapta. This yields a free calcium concentration in the 10 nM range [16]. Higher concentrations were obtained by titrating with $CaCl_2$ according to [16]. The suspension was thermally equilibrated in the monitoring (IR) beam of the MAFPA [15] and then 5 min were waited until the ATP-dependent dark reactions due to rod disks [17] were completed. Subsequently one conditioning flash (10^{-3} bleach) was applied, which caused the irreversible loss of a small (5–10%) fraction of the total transducin pool, probably from damaged ROS fragments, into the medium. This loss, which could be shown by gel electrophoreses (not shown), was accompanied by a small 'loss of signal' [18]. All subsequent flashes, which gave rise to the signals displayed in this communication, stimulated no further transducin loss (no loss signal, no transducin on the gel), indicating that the functional transducin pool stayed membrane associated.

As a control it was tested whether any of the other light-induced light scattering signals obtained from our ROS preparation [15,17] was affected by calcium in such a way that it would interfere with our actual measurements. These signals are usually too small to show up at the low bleaches used in this study, and it was confirmed that at calcium concentrations between 10 nM and 1 mM this was still the case.

All chemicals were the highest grade available from Sigma.

3. RESULTS AND DISCUSSION

The gain of the primary amplification step, i.e. the number of transducin molecules activated by a single bleached rhodopsin, can be determined from a light titration of the P_A signal, provided the signal amplitude always reflects the number of activated transducin molecules. Evidence for the validity of this assumption has been given elsewhere [11–13]. Briefly it rests on the following observations: (i) P_A signals are homogeneous, i.e. both their rise and decay exhibit an identical angular dependence. Moreover, there are no signs of additional light scattering changes occurring in the time domain of P_A, provided the bleaching level is below 10^{-3}.

(ii) There is a maximal amplitude of P_A, $A_{max(0)}$, which cannot be exceeded. At any time t during the recovery of one signal, the maximally obtained response amplitude ($A_{max(t)}$) to a subsequent flash is determined by the degree of recovery at that time, i.e.:

$$A_{max(t)} + A_{(t)} = \text{const.} = A_{max(0)}.$$

(iii) The amplitude of P_A increases linearly with the amount of activated transducin. In the absence of GTP, where 1 rhodopsin can only interact with 1 transducin molecule, a very similar signal is observed, the so called P signal [15]. It has the same angular dependence as P_A, indicating that both signals share a common structural origin, and their maximal amplitude is also the same [11,14]. This would seem to suggest that the light scattering assay cannot distinguish between an activated transducin molecule, separated from its activator

rhodopsin, and a transducin molecule still associated with its activator.

The major difference between the two signals (besides the fact that P signals do not recover within minutes) lies in their saturation behaviour. Both signals increase in size linearly with increasing flash energy, however, saturation sets in much earlier in the presence of GTP. In this case, where a high amplification is involved, the light titration (see also fig.2a) is best described by a Poisson distribution, whereas in the case of the P signal, where a 1:1 stoichiometry is obeyed, a hyperbolical relationship results [11,14,15]. This is exactly what one would expect if there were a linear relationship between signal amplitude and degree of activation.

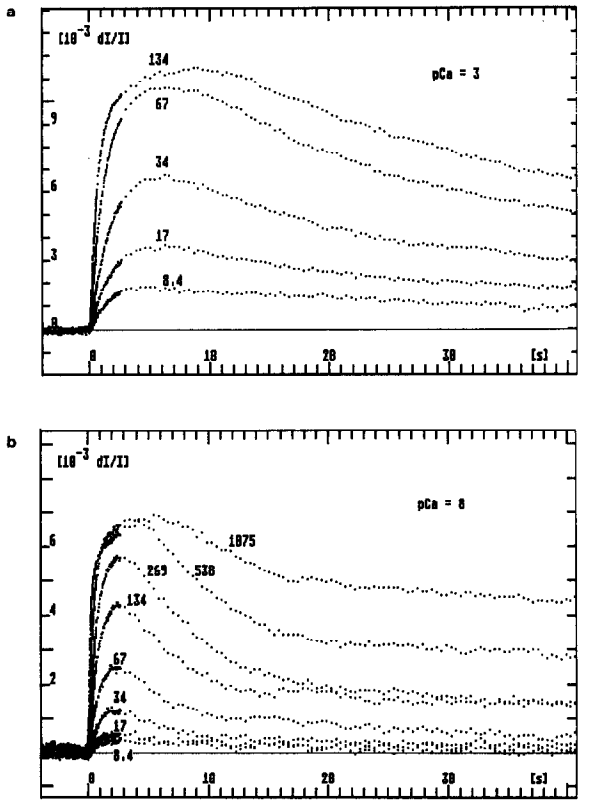

Fig.1. Light titration of P_A signals at two different calcium concentrations: (a) 10^{-3} M calcium, (b) 10^{-8} M calcium. All signals were obtained from one ROS sample. The temperature was 20°C and the scattering angle 24°. Flashes were applied every 3 min. Their energy was incremented by a factor of two, starting at a bleach of 8.4×10^{-6}. Identical results were obtained when the bleaching protocol was changed, i.e. when it was started with the brighter flashes. Similarly, it had no effect when the ROS were first exposed to low calcium and then to high calcium and vice versa.

3.1. *One bleached rhodopsin can activate all transducin molecules on a particular disk surface*

A light titration of P_A signals in the presence of 1 mM free calcium is shown in fig.1a, and signals obtained at 10 nM free calcium are depicted in fig.1b. Fig.2a shows the corresponding dose response curves, along with the curves measured at intermediate calcium concentrations. The drawn lines represent the best fit, based on a Poisson distribution and assuming that the action of one bleached rhodopsin was restricted to a small compartment of the system, containing between 26000 (pCa = 3) and 7200 (pCa = 8) rhodopsin molecules. The value of 26000 is close to the number of rhodopsin molecules per disk surface, implying that at high calcium a single rhodopsin can activate all transducin molecules on a particular disk surface. Since there are less than 10 rhodopsin molecules per transducin in our preparation [14], a gain of the primary amplification of about 3000 is implied.

3.2. *The gain of the primary amplification in visual transduction is calcium-dependent*

In fig.2b the data of fig.2a are redrawn to demonstrate the relationship between pCa and the flash energy required for a 63% maximal response. The steepest relative change occurs between pCa = 6 and pCa = 7, i.e. in the physiological concentration range.

The observed decrease in response amplitude with increasing pCa is due to an increased rate of recovery, as can be seen from fig.3a. The P_A responses to a constant stimulus are shown at different calcium concentrations. The speedier recovery at low calcium shifts the time to the peak to lower values and consequently attenuates the amplitude.

3.3. *Calcium regulates the speed of rhodopsin disactivation, most likely by affecting rhodopsin kinase*

We have previously shown that the time course of the recovery of P_A reflects rhodopsin disactivation due to phosphorylation, and this effect, rather than GTPase activity, is rate limiting under our

usual measuring conditions [12]. The calcium effects reported in this communication would therefore suggest a calcium dependence of kinase activity. The results shown in fig.3b lend further support to this notion.

When P_A signals were recorded in the absence of ATP, i.e. under conditions where kinase activity is suppressed, an artificial quencher of rhodopsin activity was required for a speedy signal recovery. Hydroxylamine is such a quencher, and in its presence rhodopsin disactivation occurs so fast that the GTPase activity of the activated G-protein becomes (at least partially) rate limiting [12]. Under such conditions the rate of recovery became independent of the calcium concentration (fig.3b). The maximal signal amplitude was unaffected, however, as well as the Poisson-shape of the light titration. Moreover, as in the case of the native signal, the maximal signal amplitude evoked by a second flash was solely determined by the degree of recovery after the first one (not shown). This clearly indicates that hydroxylamine, even at the high concentrations used, had no detrimental effect.

When ATP was present in the reaction medium together with NH_2OH (fig.3b), two quenching mechanisms were working in parallel: rhodopsin phosphorylation and the NH_2OH-mediated removal of metarhodopsin II. Under these conditions an even faster recovery was observed, and this further ATP-dependent acceleration was calcium dependent again. When the hydroxylamine concentration was reduced, i.e. when the relative contribution of phosphorylation to the

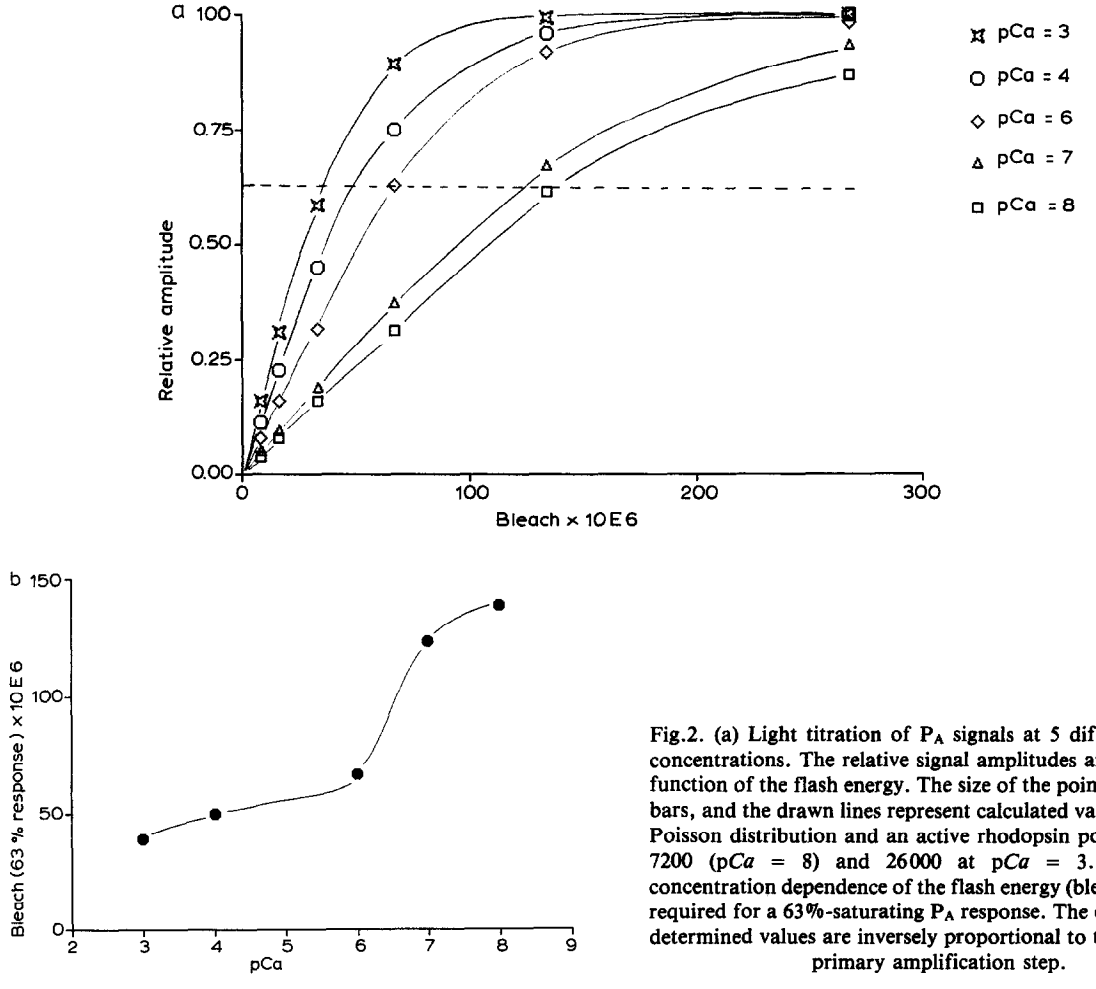

Fig.2. (a) Light titration of P_A signals at 5 different calcium concentrations. The relative signal amplitudes are plotted as a function of the flash energy. The size of the points reflect error bars, and the drawn lines represent calculated values, assuming Poisson distribution and an active rhodopsin pool of between 7200 (p*Ca* = 8) and 26000 at p*Ca* = 3. (b) Calcium concentration dependence of the flash energy (bleaching power) required for a 63%-saturating P_A response. The experimentally determined values are inversely proportional to the gain of the primary amplification step.

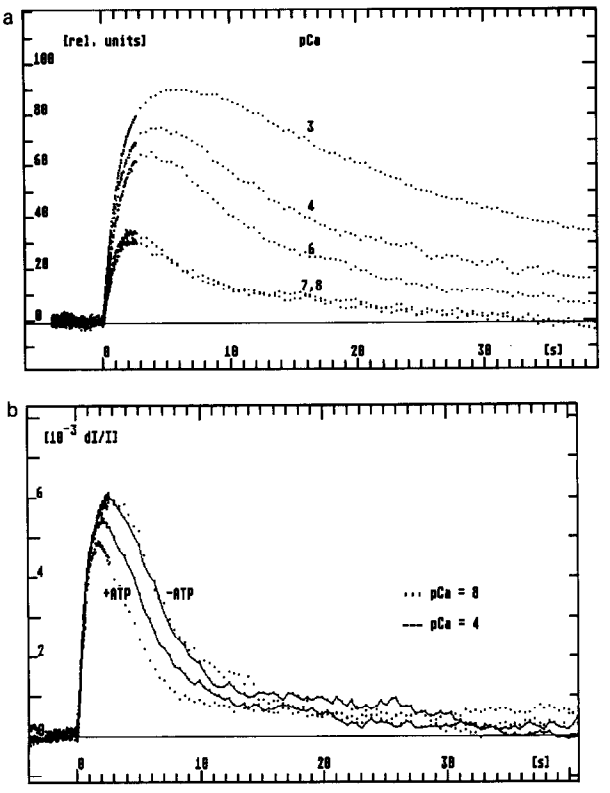

Fig.3. (a) Calcium concentration dependence of P_A signals. Each flash bleached a fraction of 6.7×10^{-5} of the total rhodopsin. Signal amplitudes were normalised such that the maximally obtainable amplitude (at a given p*Ca*) was set to 100%. (b) Calcium effect on P_A signal recovery in the presence of 100 mM hydroxylamine, with and without ATP. Each flash bleached 6.7×10^{-5} of the total rhodopsin. When the signal recovery was mostly rate limited by GTPase activity, i.e. in the absence of ATP, no calcium effect was observed. In the presence of ATP, however, a small, but distinct calcium effect became visible, indicating that phosphorylation can work in tandem with the artificial quench of rhodopsin activity due to hydroxylamine and that this kinase contribution is calcium dependent.

quench was increased, the calcium effect became more pronounced (not shown), and in the absence of hydroxylamine (fig.3a) it was maximal. It appears therefore that rhodopsin phosphorylation is regulated by calcium.

4. CONCLUDING REMARKS

Our data demonstrate that the primary amplification step of visual transduction, i.e. the coupling between rhodopsin and transducin, is calcium dependent. Lowering the calcium concentration, which occurs in vivo in light adapted rods, appears to stimulate rhodopsin kinase, thereby increasing the rate of transducin disactivation and decreasing the number of transducin molecules activated by one rhodopsin. Our observation can explain the finding of Kawamura et al. [10] that calcium ions affect light-stimulated PDE activities and it can define the site of action for calcium ions: it is the rhodopsin disactivation process.

While the concentration range over which we could measure sensitivity to calcium extends far further than that encountered in vivo, the steepest relative change occurs exactly in the physiological concentration range. The calcium effect on PDE activity, reported by Kawamura et al. [10], was similar, but smaller (they found a factor of two when the calcium concentration was varied between 1 mM and 1 nM) and it did not survive purification of the ROS. The larger effect we observe has a two-fold rate change when calcium is just varied from 0.1 μM to 1 μM, and it is stable, i.e. it survives the purification of the ROS.

The effect we describe is rather small. It is quite conceivable, however, that in a more intact system, which has retained all cell constituents, the same regulation occurs, only to a much higher extent. Preliminary experiments with supplemented soluble ROS proteins, including a 48 kDa protein [13], indicate that a calcium effect can also be observed under conditions where transducin disactivation occurs within seconds, i.e. in a physiological time domain.

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