duction to the formation of endosperm, (ii) the addition of a second female nucleus to the second fertilization event, and (iii) the reduction of the female gametophyte to the characteristic seven-celled, eight-nucleate angiosperm embryo sac form.

It is presently unresolved whether the addition of a second female nucleus to the second fertilization event occurred before or after the evolution of an embryo-nourishing tissue. However, it is likely that the extreme reduction of the female gametophyte (which in all nonflowering seed plants is responsible for the nourishment of the embryo) to the embryo sac form was predicated upon the prior evolution of a novel embryo-nourishing tissue. With the evolution of a process of double fertilization in the ancestors of flowering plants, and the subsequent modification of the second fusion product into a unique nonembryo nutritive tissue (endosperm), the pattern of sexual reproduction that is characteristic of angiosperms was established.

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9. The two sperm nuclei involved in double fertilization are likely to be from a single pollen tube, and hence genetically identical; the egg nucleus and ventral canal nucleus are derivatives of the mitotic division of the central cell nucleus. Thus, each fertilization product is genetically identical.
10. Instances were observed where fewer or more than eight free nuclei resulted from double fertilization in *E. trifurca*.
11. In most nonflowering seed plants, initial free nuclear development of the zygote is followed by cellularization to yield a single multicellular embryo. In *Ephedra*, however, a free nuclear to cellular pattern of embryogeny results in the production of multiple unicellular embryos and probably represents a modification of the sympleisiomorphic condition of embryo development.
12. In ovules where all the egg cells (typically three) are fertilized, 24 or more proembryos may theoretically be produced, only one of which will ultimately survive during maturation of the seed.
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16. No figures (photographic or camera-lucida) were published as evidence of the proposed initial division of a single zygote nucleus.
17. In six cases, a zygote nucleus was situated at the base of the egg cell, and two additional nuclei, the second sperm nucleus and ventral canal nucleus, remained separate within the apical portion of the egg cell. In six additional archegonia, the second sperm nucleus and ventral canal nucleus were in contact, but did not appear to be in the process of fusing. Thus, the second fertilization event, although almost always initiated, may not always be completed in *E. trifurca*.
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20. These findings are consistent with an hypothesis concerning the evolutionary origin and history of endosperm first proposed in 1900 [E. Sargant, *Ann. Bot.* **14**, 689 (1900)]. She suggested that subsequent to the establishment of a process of double fertilization in the ancestors of flowering plants, the second fertilization product originally yielded a supernumerary diploid embryo. Sargant hypothesized that endosperm later evolved through a modification of supernumerary embryo development into an aberrant non-embryo (endosperm) tissue.
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G_z-Mediated Hormonal Inhibition of Cyclic AMP Accumulation

YUNG H. WONG, BRUCE R. CONKLIN, HENRY R. BOURNE*

Hormones inhibit synthesis of adenosine 3′,5′-monophosphate (cAMP) in most cells via receptors coupled to pertussis toxin (PTX)–sensitive guanine nucleotide-binding (G) proteins. Mutationally activated α subunits of G_{i2} (α_{i2}) constitutively inhibit cAMP accumulation when transfected into cells. Cells have now been transfected with mutant α subunits of four other G proteins—G_z, a PTX-insensitive G protein of unknown function, and G_{i1}, G_{i3}, and G_o, which are PTX-sensitive. Mutant α_z, α_{i1}, and α_{i3} inhibited cAMP accumulation but α_o did not. Moreover, expression of wild-type α_z produced cells in which PTX did not block hormonal inhibition of cAMP accumulation. Thus, G_z can trigger an effector pathway in response to hormone receptors that ordinarily interact with PTX-sensitive G_i proteins.

HETEROTRIMERIC G PROTEINS receive biological signals from receptors for hormones and neurotransmitters and transduce them into regulation of effector enzymes and ion channels. Each G protein sorts signaling information in a distinctive pattern. Structural features of the α subunit allow it to receive incoming information from a limited subset of hormone receptors and to convey that information to a specific subset of effectors (*1*). The rapidly increasing number of new G proteins makes it desirable to identify the receptors and effector pathways associated with each.

Point mutations that replace key conserved amino acids in the α subunits of G_s (α_s) (*2*) and G_{i2} (α_{i2}) (*3, 4*) create G proteins that constitutively activate their downstream effector pathways—stimulation and inhibition of cAMP synthesis, respectively. Because these mutations replace amino acids that are conserved in all known α chains, cognate mutations in other α subunits should provide a general approach to determine whether an individual G protein can trigger a specific effector pathway. This approach is independent of receptor activation and receptor–G protein coupling. Thus, expression of mutationally activated α_{i2} in cultured cells constitutively inhibits cAMP accumulation (*4*), confirming one of the putative functions of G_{i2}. We have now extended this strategy to test whether four other G protein α chains inhibit cAMP accumulation.

Mutant α_{i1} and α_{i3} would be expected to mimic α_{i2} because these proteins resemble one another in primary structure (~85% identical amino acid sequences) and because all three members of the α_i family can open atrial potassium channels (*5*). The greater structural difference between α_o and the α_i subunits (~70% identical sequences) suggested that α_o (*6*) would not inhibit cAMP accumulation. The signaling function of G_z

Departments of Pharmacology and Medicine and the Cardiovascular Research Institute, University of California, San Francisco, CA 94143.

*To whom correspondence should be addressed.

(7) is unknown; α_z is less similar to α_i (~60% identity) than is α_o and, unlike α_{i1-3} and α_o, α_z does not serve as a substrate for PTX (*8*). Thus, mutant α_z might not inhibit cAMP accumulation.

Rat cDNAs encoding the α subunits of G_{i1}, G_{i3}, G_o (*9*), and G_z (7) were mutated to substitute a leucine codon (L) for a conserved glutamine codon (Q), cognate to glutamine 227 of α_s. The resulting mutant constructs were designated Q204L for α_{i1} and α_{i3}, and Q205L for α_o and α_z. The same substitution at the equivalent position inhibits the guanosine triphosphatase (GTPase) activity of α_s (*2, 10*) and constitutively activates effector regulation by both G_s (*2, 10*) and G_{i2} (*4*). Wild-type (WT) and mutant G protein α chains were stably expressed in NIH 3T3 mouse fibroblasts by retroviral infection (*11*) or transiently expressed in human embryo kidney 293 cells (*12*).

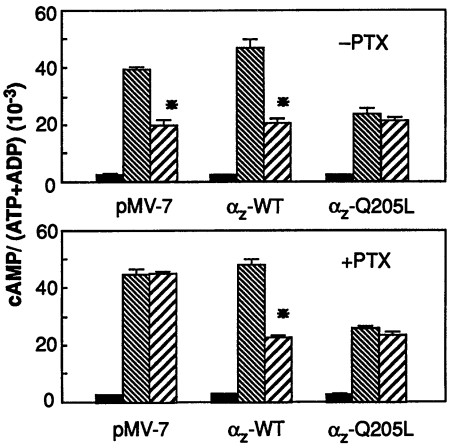

Fig. 1. Effects of PTX on LPA-induced inhibition of cAMP accumulation in NIH 3T3 fibroblasts. G418-selected pools of NIH 3T3 cells infected with vector (pMV-7) or vector containing α_z-WT or α_z-Q205L were labeled with [^{3}H]adenine for 20 hours in the absence or presence of PTX (100 ng/ml). Cyclic AMP accumulation was measured as described (*4*). Forskolin was used at 50 μM and LPA at 100 μM. The results represent the mean ± SEM of three independent experiments in triplicate determinations. On bar graph, black indicates basal; shaded, forskolin; and striped, forskolin and LPA. *LPA significantly reduced the forskolin-stimulated activity; paired Bonferroni *t* test, $P < 0.05$.

Prostaglandin-E_1 (PGE_1) and forskolin increase the concentration of cAMP in control (vector-infected) NIH 3T3 cells by approximately 4- and 20-fold, respectively, and lysophosphatidic acid (LPA) inhibits these responses by 30 to 50%; PTX blocks the inhibitory effect of LPA (Fig. 1) (*4*). Both the stimulatory and inhibitory responses are unchanged in G418-selected pools of NIH 3T3 cells infected with WT α_{i1}, α_{i3}, or α_o (Table 1). Expression of the exogenous α chains was verified by immunoblotting with specific antisera (*13*).

As reported in studies with mutant α subunits of G_{i2} (α_{i2}-Q205L and α_{i2}-R179C) (*4*), expression of either α_{i1}-Q204L or α_{i3}-Q204L constitutively inhibited PGE_1- and forskolin-stimulated cAMP accumulation (Table 1). In these cells, like NIH 3T3 cells expressing mutant α_{i2} (*4*), LPA caused little or no further inhibition of cAMP accumulation (Table 1), suggesting that the mutant α_i subunits had already maximally activated the inhibitory pathway. Basal cAMP (cAMP in unstimulated cells), however, was not changed in cells expressing mutant α_{i1} or α_{i3}. This may represent a limitation of the assay we used, because LPA also failed to lower basal cAMP.

In contrast to the α_i subunits, α_o-Q205L did not inhibit cAMP accumulation in NIH 3T3 cells; although PGE_1-stimulated cAMP accumulation was slightly reduced, responses to forskolin and LPA were fully retained (Table 1). Studies have also shown that purified bovine brain α_i, but not α_o, inhibits adenylyl cyclase in biochemical reconstitution systems that use resolved components (*14*).

Surprisingly, expression of α_z-Q205L inhibited cAMP accumulation. Agonist-stimulated cAMP accumulation was attenuated, and the inhibitory response to LPA was lost (Table 1). Because G_z lacks the cysteine residue that is adenosine diphosphate–ribosylated by PTX in α_i and α_o (*7, 8*), ligands for receptors that activate α_z should inhibit cAMP accumulation in a PTX-insensitive fashion. To determine whether LPA receptors in NIH 3T3 cells can activate G_z, we assessed the effect of LPA on forskolin-stimulated cAMP accumulation in vector- or α_z-WT–infected cells that had been treated with PTX. PTX prevented LPA from inhibiting cAMP accumulation in vector-infected cells (Fig. 1), indicating that LPA acts by an endogenous PTX substrate, probably a G_i protein. In cells expressing α_z-WT, however, PTX did not abolish the inhibitory effect of LPA (Fig. 1). This result shows that the LPA receptor can interact with α_z and confirms the conclusion, drawn from effects of mutant α_z, that G_z can inhibit cAMP accumulation. To rule out the possibility that α_z-WT produced its effect by preventing PTX-catalyzed modification of endogenous α_i, we prepared particulate extracts from PTX-treated cells expressing recombinant α_z-WT; treatment of these extracts with PTX and ^{32}P-labeled nicotinamide adenine dinucleotide revealed no residual PTX substrate proteins (*15*).

We have previously used human embryonic kidney 293 cells transiently coexpressing (*12*) a luteinizing hormone receptor (LHR) (*16*), an α_2-adrenoceptor (α_2-AR) (*17*), and WT or mutant α_{i2} to investigate G protein–mediated inhibition of cAMP synthesis (*4*). In such cells, human chorionic

Table 1. Intracellular cAMP accumulation in G418-selected pools of NIH 3T3 infectants. NIH 3T3 fibroblasts, infected either with vector alone (pMV-7) or with vectors containing the indicated cDNA, were labeled with [^{3}H]adenine (2 μCi/ml for 20 to 24 hours). We estimated intracellular [^{3}H]cAMP content in response to various drugs in the presence of 1 mM 1-methyl-3-isobutylxanthine (IBMX) by determining the ratios of cAMP to total ATP and ADP pools (*4*). Where indicated, cells were treated with PGE_1 (50 μM), forskolin (50 μM), or LPA (100 μM) for 30 min at 37°C. Values represent the mean ± SEM of three to five independent experiments in triplicate determinations.

Vector	cAMP/(ATP + ADP) (10^{-3})				
	Basal	PGE_1*	PGE_1 + LPA†	Forskolin*	Forskolin + LPA†
pMV-7	2.0 ± 0.1	7.7 ± 0.3	4.8 ± 0.1	50 ± 5.7	33 ± 4.3
α_{i1}-WT	1.7 ± 0.2	6.8 ± 0.4	4.2 ± 0.3	48 ± 2.2	27 ± 3.2
α_{i1}-Q204L	1.5 ± 0.1	3.4 ± 0.4‡	3.3 ± 0.4§	28 ± 2.8‡	27 ± 2.0§
α_{i3}-WT	1.7 ± 0.2	6.9 ± 0.3	4.5 ± 0.1	42 ± 4.7	26 ± 3.3
α_{i3}-Q204L	1.9 ± 0.1	3.2 ± 0.3‡	2.5 ± 0.3§	23 ± 3.2‡	20 ± 1.9§
α_o-WT	2.0 ± 0.1	6.2 ± 0.6	3.7 ± 0.5	44 ± 5.0	30 ± 3.8
α_o-Q205L	1.9 ± 0.1	4.6 ± 0.5‡	3.8 ± 0.3§	47 ± 3.8	33 ± 2.7
α_z-WT	1.8 ± 0.2	7.9 ± 0.7	4.7 ± 0.6	46 ± 2.8	23 ± 5.3
α_z-Q205L	2.1 ± 0.1	3.7 ± 0.5‡	3.7 ± 0.4§	24 ± 1.9‡	21 ± 3.3§

*Stimulatory responses to PGE_1 or forskolin were significantly higher than the corresponding basal values; paired *t* test with Bonferroni correction, $P < 0.05$. †Unless otherwise stated, responses to PGE_1 or forskolin were significantly inhibited in the presence of LPA; paired *t* test with Bonferroni correction, $P < 0.05$. ‡Significantly different from vector-infected cells; *t* test with Bonferroni correction, $P < 0.05$. §Inhibitory responses produced by LPA were statistically insignificant; *t* test.

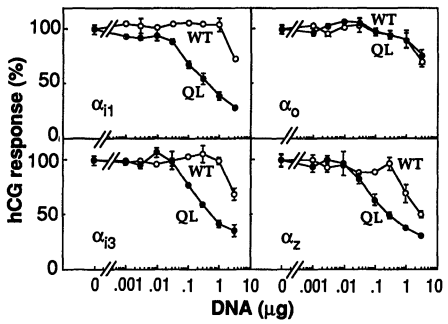

Fig. 2. Cyclic AMP accumulation in transfected 293 cells. Inhibition of the stimulatory effect of hCG (5 ng/ml) by wild-type and mutant α subunits in cells cotransfected with DNA-encoding LHR (0.5 μg of DNA per 10^6 cells) and the indicated amounts of DNA containing α_{i1}-WT, α_{i1}-Q204L, α_{i3}-WT, α_{i3}-Q204L, α_o-WT, α_o-Q205L, α_z-WT, or α_z-Q205L. Transfected cells were labeled with [^{3}H]adenine for 24 hours and cAMP accumulation was assayed (48 hours after transfection), in the presence of 1 mM IBMX and hCG (5 ng/ml), as described (*4*). Results are expressed as a percentage of the hCG-stimulated activity as compared to that measured in cells transfected with LHR alone. Actual values for cAMP (as a ratio, $\times 10^{-3}$, of radiolabeled cAMP to cAMP + ATP) (*4*) were 0.6 to 1.0 and 16 to 20 in the absence and presence, respectively, of hCG. The data represent triplicate determinations in one experiment; two additional experiments gave similar results.

gonadotropin (hCG) stimulates and UK-14304 inhibits cAMP accumulation by activating the LHR and α_2-AR, respectively; coexpression of mutationally activated α_{i2} reduces hCG-stimulated cAMP accumulation (*4*). In transient studies, inhibition of basal cAMP accumulation is difficult, if not impossible, to detect because only a small subset of the cell population expresses the recombinant proteins. Thus, hCG stimulation of the coexpressed LHR serves to target the cell population that expresses recombinant receptors and G protein subunits.

The effects of WT and mutant α subunits in 293 cells (Figs. 2 and 3) precisely parallel those found (Table 1 and Fig. 1) in NIH 3T3 cells. Specifically, mutationally activated α_{i1}, α_{i3}, and α_z inhibited hCG-stimulated cAMP accumulation, whereas mutant α_o did not (Fig. 2). The extent of inhibition depended on the amount of DNA transfected, and the maximal inhibition (~60%) was similar to that produced by mutant α_{i2} (*4*). The specificity of the inhibitory responses was shown by the failure of transfection with WT α-subunit DNAs (Fig. 2) and bovine prolactin DNA (*18*) to inhibit hCG-stimulated cAMP accumulation. At high doses of DNA (>0.3 μg per transfection), α_z-WT weakly inhibited cAMP accumulation. The fact that this inhibitory effect was more pronounced than that produced by other WT α subunits (Fig. 2) may reflect the remarkably slow rate of GTP hydrolysis reported for recombinant α_z in vitro, a rate (k_{cat} = 0.05 min^{-1}) much slower than observed with other α chains (*8*). Why is α_z activated by a mutation that presumably makes this already sluggish GTPase even slower? We cannot rule out the possibility that α_z in vivo hydrolyzes GTP at a much faster rate, which might be inhibited by mutation. Such a speculation is not necessary, however, because the rate at which GTP replaces guanosine diphosphate (GDP) bound to recombinant α_z ($k_{diss.GDP}$ = 0.001 min^{-1} at 5 mM Mg^{2+}) (*8*) is even slower than the reported rate of GTP hydrolysis. If both rates apply to α_z in vivo, most of the WT α_z in cells would be GDP-bound and hence inactive; consequently, a mutation that reduces $k_{cat.GTP}$ 100-fold, as reported for α_s-Q227L (*10*), would substantially increase the GTP-bound fraction of α_z and stimulate the appropriate effector pathway.

By treating the transfected 293 cells with PTX to inactivate endogenous α_i, we tested the ability of α_z-WT to respond to stimulation by well-defined recombinant receptors (Fig. 3). In cells coexpressing the LHR with either the α_2-AR, the human dopaminergic D_2 receptor (D_2-R) (*19*), or the rat adenosine A_1 receptor (A_1-R) (*20*), the hCG-stimulated cAMP accumulation was inhibited by the inhibitory agonists UK-14304, dopamine, or (+)-N^6-(2-phenylisopropyl)-adenosine (PIA), respectively (Fig. 3). In each case the inhibition was PTX-sensitive and was not observed in cells transfected with the LHR alone. Coexpression of α_z-WT with any one of the inhibitory receptors and the LHR, however, produced cells in which PTX treatment had no effect on inhibition of cAMP accumulation by the corresponding inhibitory agonist (Fig. 3). These results indicate that receptors of the class that ordinarily activates G_i proteins can also activate G_z. This conclusion is in agreement with a recent report that a receptor of this class, the human muscarinic m_2-acetylcholine receptor, could promote binding of guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S) to G_z (*21*).

The greater expression of α_z in brain compared to other tissues (*8, 22*) suggests that PTX-insensitive inhibition of cAMP synthesis should be found in neural cells and membranes. However, we know of only one report of PTX-insensitive hormonal inhibition of cAMP accumulation, and that was observed in rat aortic smooth muscle rather than neural tissues (*23*).

Although the mechanism by which α_z inhibits cAMP accumulation is unknown, comparison of α-chain amino acid sequences (*24*) indicates that α_z resembles the α_i subfamily (which includes α_o and α_t) more closely than it resembles other α chains such as α_q and α_s. This resemblance probably indicates a close evolutionary relationship between α_z and α_i proteins, which may account for the overlap in signaling function. It remains to be shown whether G_z inhibits cAMP accumulation directly or indirectly through other signaling pathways (*25*). G_i proteins are also reported to regulate other effectors, including phospholipases A_2 (*26*) and C (*27*) and potassium (*5*) and sodium (*28*) channels; G_z might also substitute for G_i in regulating these effectors.

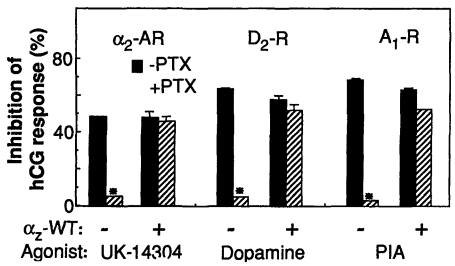

Fig. 3. PTX-insensitive, receptor-mediated inhibition of hCG-stimulated cAMP accumulation in α_z-WT–transfected 293 cells. The 293 cells (10^6 cells in 60-mm culture dishes) were cotransfected with 0.5 μg of DNA encoding the LHR and one of three other receptor DNAs: 0.1 μg of α_2-AR in pCMV4, 1 μg of D_2-R in pcDNA1, or 1 μg of A_1-R in CDM6XL. Each set of receptor DNAs was cotransfected with or without 0.3 μg of α_z-WT. The total amount of DNA used per transfection was made up to 2 μg with bovine prolactin DNA. Transfected cells were assayed for cAMP accumulation as described in the legend to Table 1, in the presence of 1 mM IBMX and hCG (5 ng/ml) with or without inhibitory agonists; 10 nM UK-14304, 1 μM dopamine, and 10 μM PIA were used as the inhibitory agonist for α_2-AR, D_2-R, and A_1-R, respectively. Results are expressed as percent inhibition of the hCG-stimulated activity in the presence of added drugs, compared to that measured in the presence of hCG alone. Actual values for cAMP (as a ratio, $\times 10^{-3}$, of radiolabeled cAMP to cAMP + ATP) (*4*) were 0.7 to 1.0 and 14 to 19 in the absence and presence, respectively, of hCG. The data represent triplicate determinations in a single experiment; two independent experiments yielded similar results. *PTX significantly reduced the ability of inhibitory agonists to inhibit hCG-stimulated activity; paired Bonferroni *t* test, $P < 0.05$.

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12. The 293 cells were maintained in minimum essential medium (Earle's) containing fetal calf serum (10%). Wild-type and mutant α-subunit cDNAs were subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen) and transfected into the 293 cells (10^6 cells on a 60-mm culture dish) in the presence of DEAE-dextran (400 μg/ml) and chloroquine (100 μM) for 2 hours. Cells were then shocked with phosphate-buffered saline (PBS) containing dimethyl sulfoxide (DMSO) (10%) for 2 min, washed once with PBS, and incubated in growth medium for 20 to 24 hours before reseeding in 12-well plates.
13. Expression of the exogenous α_{i1}, α_o, and α_z was verified by immunoblotting with antisera AS/7 (Du Pont Biotechnology Systems), GC/2 (Du Pont Biotechnology Systems), and P-961 (*8*), respectively. Antiserum to the α_{i3} peptide sequence AGSAQQGVMTSQLA (residues 111 to 124) was used to detect expression of α_{i3}. The procedure for protein immunoblotting was as described (*4*). All four proteins were expressed at readily detectable levels (~2-fold over endogenous α chains) (Y. H. Wong and H. R. Bourne, unpublished data). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; G, Gly; L, Leu; M, Met; Q, Gln; S, Ser; T, Thr; V, Val.
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Quantum Conversion and Image Detection by a Bacteriorhodopsin-Based Artificial Photoreceptor

TSUTOMU MIYASAKA,* KOICHI KOYAMA, ISAMU ITOH

A thin film (50 to 500 angstroms thick) comprising fragments of bacteriorhodopsin (bR)-containing purple membrane was formed on an SnO_2 conductive electrode by the Langmuir-Blodgett method. The film was put into contact with a thin, aqueous electrolyte gel to construct an electrochemical sandwich-type photocell with a junction structure SnO_2/bR/electrolyte/Au electrode. Under visible light irradiation, the bR-based photocell produced an efficient rectified photocurrent that proved to have unique differential responsivity to light intensity, which is characteristic of in vivo biological photoreceptors. An artificial photoreceptor comprising a pixel network of the bR photocell was fabricated in an attempt to study image-detecting and processing abilities of bR.

BACTERIORHODOPSIN (bR) IS A PROtein existing in the halobacterial purple membrane (PM), which is extremely stable as a visual pigment analog in vitro against light. Photochemistry of in vitro bR has recently stimulated extensive study of its potential application to the design of molecular electronic devices (*1*) and optical computers and memories (*2, 3*). The photovoltaic behavior of in vitro bR has been of particular interest in the field of bioelectronics and has been well elucidated with the use of dry PM films electrodeposited on conductive electrodes (*4–6*). In a manner similar to other photoconductive dyes in open-circuit voltaic cells (*7*), in vitro bR in dry films produces a steady-state photovoltage accompanied by an initial transient component. The photovoltage and concomitant photocurrent (normally very small) result from the displacement of charge in the bR molecule initiated by rapid photoisomerization of the chromophore retinal. Under direct contact between bR and electrode, the photoelectric response time of bR can thus reach the order of 10^{-11} s (*6*). However, the photoelectric behavior of dry PM films in contact with electrodes is inevitably affected significantly by the presence of water or ambient humidity (*4, 5*), reflecting the direct effect of water on the proton-pumping reaction cycle of bR.

In this study we attempted to construct a bR-incorporated electrochemical photocell with bR molecules in direct contact with both a conductive electrode as well as an aqueous medium of electrolyte. This photocell, having an ultrathin wet film of PM at the interface of electrode and electrolyte, can convert incident quanta to electric current at high efficiency with excellent linearity of output current against light intensity. As a light sensor, the bR photocell was found to have differential responsivity to light intensity, which is characteristic of in vivo biological photoreceptors. As an extended application, we fabricated an image-sensing photoreceptor based on this differential sensing type bR photocell. The bR photoreceptor, which is the first example of an artificial photoreceptor (image sensor) with immobilized biological materials, proved capable of detecting and processing an optical image in a manner similar to that of visual functions.

Purple membrane was purified from the S-9 strain of *Halobacterium halobium* by the method of Oesterhelt and Stoeckenius (*8*). PM was suspended in pure water and emulsified by mixing with *N,N*-dimethylformamide (DMF) and *n*-hexane to yield an opaque dispersion. The dispersion was carefully applied on a surface of pure water in a Langmuir film balance to form a film consisting of a two-dimensional array of PM

Ashigara Research Laboratories, Fuji Photo Film Company, Limited, Minamiashigara, Kanagawa 250-01, Japan.

*To whom correspondence should be addressed.