neither learning by bees of how to forage at female flowers nor male divergence in form and color will reduce natural hybridization (*22*). Reproductive isolation in these orchids apparently depends primarily on differences in fragrances (*23*) and flowering times (*5*) rather than on form.

Since it should be advantageous for male flowers to discourage subsequent visits to other males in any animal-pollinated species having unisexual flowers, similar evolutionary processes should occur in other plants.

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7. An obvious stipe persists after pollination.
8. July–August 1984 ($\chi^2 = 6.64$, $P < 0.01$); outlying plants not included were all males.
9. Some wild *C. ochraceum* plants changed sex (some plants with male flowers bore only female flowers the next year and vice versa) yielding a ratio of 30 male per 1 female inflorescence in 1985 (*5*).
10. *Euglossa* hovering within 10 cm of a flower were counted from 0900 to 1100 on 1 day for ten male and ten female inflorescences in July 1984. Four of 48 approaching males were loaded as were 2 of 34 approaching females ($\chi^2 = 0.176$, $P > 0.5$).
11. Forcible emplacement and attachment of pollinarium is characteristic of *Catasetum*, except for the *C. discolor* alliance (*1*).
12. D. H. Janzen, personal communication concerning *C. maculatum* in Costa Rica; Romero (*5*) for *C. ochraceum*; G. Carnevali, personal communication concerning *C. barbatum* in Venezuela.
13. C. H. Dodson and P. G. Frymire [*Mo. Bot. Gard. Bull.* **49**, 133 (1961)] for *C. macroglossum* in Ecuador.
14. Some *Mormodes* species have unisexual flowers (*1*).
15. The viscidium (Fig. 1E) is "violently" discharged onto the bee, thus attaching the pollinarium and anther cap (*4*).
16. The high pollen-donation success suggested for *Catasetum maculatum* in Costa Rica [D. H. Janzen, *Biotropica* (suppl.) **13**, 77 (1981)] is numerically impossible in populations with strongly male-biased sex ratios (*1, 5*).
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18. Calculated as the area within which a second viscidium would contact the first pollinarium divided by the area within which most pollinaria strike.
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Estrogen-Induced Factors of Breast Cancer Cells Partially Replace Estrogen to Promote Tumor Growth

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The hormone 17β-estradiol acts through its receptor system to induce MCF-7 human breast cancer cells to form tumors in athymic mice. In vitro studies have identified the production of estrogen-induced growth factors from MCF-7 cells that may have a role in growth control. These induced growth factors were sufficient to stimulate MCF-7 tumor growth in ovariectomized athymic mice, thus partially replacing estradiol. Growth factors may act as estrogen-induced "second messengers" in estrogen-responsive growth of human breast cancer.

BREAST CANCER, ONE OF THE MOST prevalent of all cancers, is characterized by marked hormonal control of its growth in many species. In women, a high proportion of primary breast cancers contain the estrogen receptor and require 17β-estradiol (E_2) or other estrogenic activities for tumor growth. Past and current therapies have been directed toward interruption of estrogen action by ovariectomy (or hypophysectomy) or the use of antiestrogens such as tamoxifen. However, antiestrogen resistance has limited these therapeutic approaches (*1*). Several estrogen-responsive cell lines have been isolated; MCF-7 and ZR-75-1 are among the most widely studied (*2*). In vitro, the growth of these cell lines and the release of several major proteins (including proteases such as plasminogen activator), are estrogen-stimulated (*3*). In one in vivo model system, the athymic (nude) mouse, formation of tumors by MCF-7 cells is completely dependent on estrogen stimulation (*4*). We and others have recently presented evidence that growth factor activities, some of which are estrogen-induced, are released by these cell lines into their serum-free conditioned medium (*5–7*). We have identified one of these, a 30-kilodalton transforming growth factor α (TGFα)–like activity that binds to the epidermal growth factor (EGF) receptor (*6*). Insulin-like growth factor I (IGF-I) is also released by MCF-7 cells, but its regulation by estrogen is much less marked (*6, 7*). The aim of this study was to determine if an estrogen-induced growth factor (or factors) is sufficient to replace estrogen itself in induction of tumor formation by MCF-7 cells in vivo in the nude mouse.

Initial experiments were carried out to compare growth-promoting activities of serum-free conditioned medium from MCF-7 cells (CM) with CM obtained from MCF-7 cells that had been treated with E_2 (CME_2). Acid dialysis of CME_2 samples, a typical extraction procedure in the initial isolation of many growth factor activities, allowed complete (>99.98%) removal of residual E_2. This was demonstrated by the addition of ^{125}I-labeled E_2 (10^7 dis/min; New England Nuclear) to three CME_2 samples. After concentration and acid extraction only 2×10^2 to 2×10^3 dis/min remained in the medium. Removal of E_2 was further verified by bioassay [acid-extracted CME_2 was infused into athymic nude mice as in Table 1; no stimulation of uterine wet weight was observed and E_2 was <25 pg/ml blood by radioimmunoassay (*8*)]. Acid-extracted CM preparations were used in all experiments of the current study, both in vitro and in vivo. CM from E_2-treated MCF-7 cells contained sufficient growth-promoting activity to reduce MCF-7 cell-doubling times in vitro from 40 to 21 hours (Table 1, A). In contrast, CM from untreated MCF-7 cells was only weakly growth-stimulating, reducing MCF-7 cell-doubling time to 33 hours.

We then tested the biological activity of CM and CME_2 samples for their ability to stimulate the development of MCF-7 cell implants into tumors grown in ovariectomized athymic mice. Highly concentrated extracts of CME_2 were nearly as active as E_2 itself over a 14-day treatment period in inducing tumor development (Table 1, A). The CME_2 sample induced an average of one tumor per animal (or 25% incidence, since four separate fat pad sites were implanted with MCF-7 cells). This was a significant incidence of tumor induction as compared to the CM group ($P < 0.05$ by χ^2 analyses). On histologic analysis of tumors

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induced either by E_2 itself, by CME_2 or CM, we observed that all were typical, poorly differentiated, invasive adenocarcinomas; the three groups of tumors were indistinguishable (Fig. 1). The tumor cross-sectional area after treatment with CME_2 was significantly greater than that observed in the group treated with serum-free medium alone ($P < 0.05$ by t test).

To address the target-tissue specificity of this activity, we examined the growth of uteri from nine or more E_2-implanted or CME_2 pump–implanted animals. Since only ovariectomized mice were used, their uterine wet weight provides a very sensitive indicator for circulating estrogen-like activities (*9*). After 14 days of treatment with a 0.5-mg E_2 pellet, the uterine wet weight (mean ± SD) was 78.8 ± 11.4 mg as compared to 8.4 ± 2.7 mg in animals implanted with a pump that contained saline ($P < 0.001$ by t test). In contrast, CME_2 did not induce any changes in uterine wet weight (8.36 ± 3.4 mg). Histologic analysis of uterine sections confirmed the lack of a proliferative response. In addition, neither E_2 nor its active metabolites E_1 and E_3, could be detected in the blood of the CME_2 treatment group (*8*). These results further confirm that stimulation of tumor growth by CME_2 was not due to estrogen itself (either residual in CM or synthesized in vivo from extraovarian sources). Further, they suggest that the CME_2 component active in stimulating tumor growth was not estrogen-like for all estrogen receptor–containing tissues.

We further characterized the CME_2 activity to determine its chemical stability. CME_2 samples from 2-liter volumes were prepared as in Table 1 and were exposed to (i) 2 mg of trypsin (Worthington Type 3) for 1 hour at 37°C and were then treated with soybean trypsin inhibitor (2 mg, Sigma); (ii) dithiothreitol (65 m*M*, 1 hour at 37°C); or (iii) heat (56°C, 30 minutes). A CME_2 control had only soybean trypsin inhibitor added. Each sample initially contained 20 mg protein and was dialyzed against phosphate-buffered saline (PBS) after inactivation treatment. Final sample volumes were 1 ml and were implanted in nude mice as in Table 1. After 10 days (10 ml of CME_2 equivalent per mouse per day) tumor formation was observed. In the presence of CME_2, tumor incidence was 50%; trypsin reduced tumor incidence to 7%, heating reduced the incidence to 20%, and dithiothreitol reduced the incidence to 13%. These data further confirm that the tumor-stimulating activity was unlike E_2 (which is resistant to these treatments) and suggested the possibility that it might be similar to some known polypeptide growth factors (*10*).

We next sought to characterize the kinetics and dose dependence of tumor growth in response to CME_2, the most active conditioned-medium preparation. The tumor incidence after 10 days was dose-dependent between 1 and 25 ml of CME_2 equivalents per day of infusion (Fig. 2A). In these experiments, tumor incidence reached a plateau of two per animal with the largest CME_2 doses. The size of the largest tumor

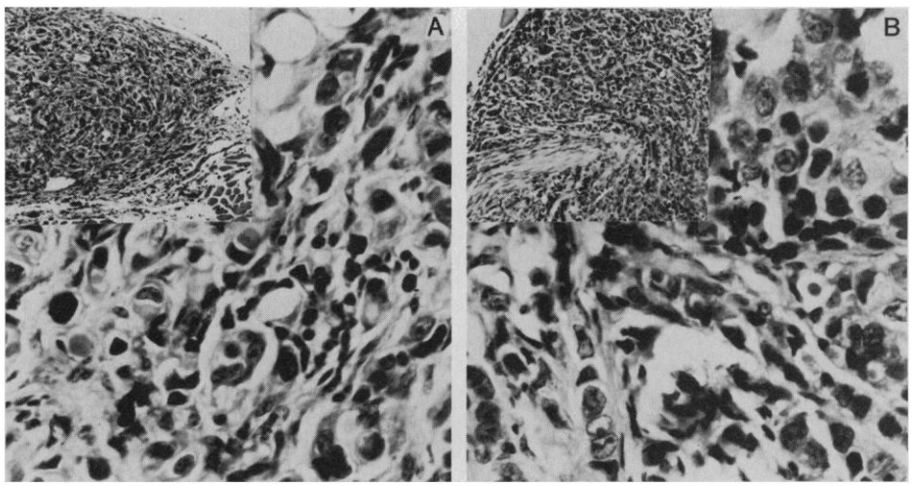

Fig. 1. Morphology of E_2- or CME_2-induced tumors. Nude mice were implanted with MCF-7 cells and (A) a 0.5-mg E_2 pellet or (B) Alzet minipumps containing concentrated extracts of CME_2 (see legend to Table 1). After 14 days, tumors were removed, formaldehyde-fixed, sectioned, stained with hematoxylin and eosin, and observed by light microscopy. Insets were photographed at ×20, while high-power views were photographed at ×135.

Table 1. Growth factors and estradiol in development of human breast cancers in athymic mice. Serum-free conditioned medium was prepared from MCF-7 cells that had been grown with (CME_2) or without (CM) 4 days of E_2-treatment ($10^{-9}M$) as described (*6*,); SFM, serum-free medium. Media were combined with protease inhibitors (*6*), concentrated by Amicon filtration (YM2 filter), and dialyzed three times against 1000-fold excess of 1*M* acetic acid. Media were then lyophilized, reconstituted in PBS, and clarified by centrifugation and passage through a Millipore filter. This resulted in a 500-fold concentration of the media. Each medium collection was obtained from cell monolayers containing equivalent amounts of DNA (*16*). Samples were placed in Alzet minipumps (size 2002) and implanted mid-dorsal subcutaneously (infusion site posterior and away from insertion incision) in athymic mice (Harlan Sprague-Dawley, Madison, WI). Unless otherwise stated, CM equivalents were infused at 10 ml/day. Five animals per group in four replicate experiments were implanted with 2×10^6 MCF-7 cells in each of the first four mammary fat pads of every animal (*8*). Tumors were scored after 14 days as palpable lumps, at least 0.2 × 0.2 cm in size (by calipers). Tumor incidence was calculated as tumors per total injection site. Interexperiment variation was not significant (ranges are presented) (*17*).

Treatment	MCF-7 doubling time in vitro* (hours)	Tumor incidence† % (range)	Tumor cross-sectional area (cm^2)	
			Maximum	Mean ± SD (total)
		A. Conditioned medium		
No treatment	40	4 (0–5)	0.08	0.06 ± 0.03 (3)
Control (SFM)	40	5 (0–7)	0.09	0.06 ± 0.02 (4)
CM	33	11 (8–14)	0.25	0.12 ± 0.07 (9)
CME_2	21	25 (21–50)	0.25	0.13 ± 0.06 (20)
E_2 (0.5-mg pellet)	20	38 (30–50)	0.25	0.16 ± 0.06 (30)
		B. Dose response‡		
Control SFM (5×)		8	0.09	0.06 ± 0.03 (2)
CM		17	0.16	0.12 ± 0.04 (4)
CM (5×)		58	0.30	0.13 ± 0.06 (14)
CME_2		54	0.30	0.15 ± 0.05 (13)
E_2		83	0.42	0.25 ± 0.1 (20)
		C. IGF-I and EGF§		
Control (saline)		2 (0–5)	0.04	0.04 ± 0 (2)
IGF-I (0.6 μg/day)		14 (10–19)	0.20	0.15 ± 0.06 (11)
EGF (1 μg/day)		29 (25–36)	0.25	0.14 ± 0.07 (23)
IGF-I + EGF		25 (24–26)	0.25	0.16 ± 0.07 (10)

*Measured during log-phase growth in SFM, 5 μl of CME_2 or $10^{-9}M$ of E_2 were used. †Tumors per total injected sites. ‡Medium samples were infused at an equivalent of 3 ml/day, with the exception of CM (5×) and SFM (5×), which were infused at an equivalent of 15 ml/day. The CM (5×) group was significantly different from the CM group in tumor incidence ($P < 0.05$ by χ^2 analyses). The CM (5×) group was significantly different from the SFM (5×) control in tumor cross-sectional area ($P < 0.05$ by t test). §IGF-I and EGF were obtained from Amgen. For IGF-I + EGF, five animals were used in each of two experiments.

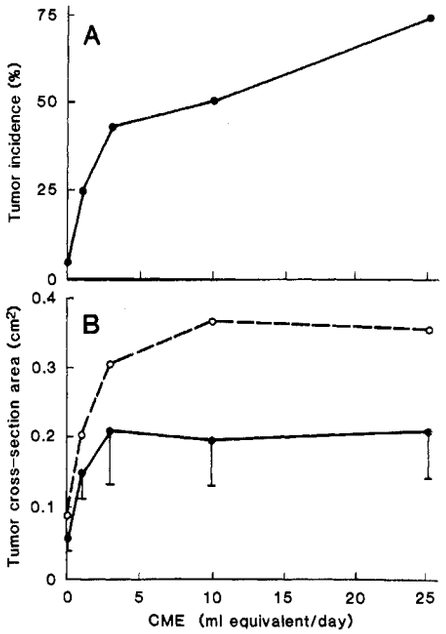

Fig. 2. Dose dependence of CME_2 activity. CME_2 was placed in Alzet minipumps and implanted with MCF-7 cells in nude mice (see legend to Table 1). Different concentrations of CME_2 resulted in dose-dependent increases in tumor incidence (A) and size (B). ○------○ indicates maximum size of tumors per group; ●——● indicates mean ± SD size of tumors per group. Tumor measurements were taken after 10 days of treatment.

in the treatment group reached a plateau at 10 ml of CME_2 equivalents per day (Fig. 2B). To determine if the observed differences between tumorigenic activities of CM and CME_2 were quantitative or qualitative in nature, we compared CME_2 to CM and a fivefold more concentrated preparation of CM (Table 1, B). We observed that CM activity was dose dependent (like CME_2 in Fig. 2) and that fivefold concentrated CM had an amount of activity similar to CME_2.

The time of CME_2 treatment (10 ml of CME_2 equivalent per day) required for maximal incidence of tumors was 9 days and tumors persisted at this incidence until 14 days (Fig. 3). After 2 weeks, tumors began to regress. Tumors were still present after 4 weeks, although the incidence was 50% of the peak value. During tumor regression, necrotic areas in central portions of the adenocarcinoma were observed in histological analyses of tumor sections. Similar regression kinetics were also observed in experiments where infusion pumps (Alzet #2001) were reimplanted every 7 days. The mechanism of this tumor regression in the nude mouse system is not understood. However, it is not simply a CME_2 dosage effect; tumors under stimulation by as much as 25 ml of CME_2 equivalent per day underwent a similar, time-dependent regression.

One possibility is that the tumors under CME_2 stimulation do not produce sufficient angiogenic stimuli (*11*) or some other locally acting or highly labile growth-factor activity in contrast to tumors under full E_2 stimulation. Alternatively, it is possible that stimulation of MCF-7 cells by E_2, but not by growth factor (or factors), directly changes expression of cell surface determinants that may be required for extensive growth or invasion. It is also possible that other host effects, such as suppression of natural killer lymphocytes, must be present with E_2 stimulation to yield full estrogen-dependent tumorigenesis (*12*).

Since the tumor-stimulating factor (or factors) present in CME_2 had some properties in common with polypeptide growth factors (acid-soluble and sensitive to trypsin and dithiothreitol), we attempted to reproduce the CME_2 effects with authentic polypeptide growth factor components. An EGF-like (or TGF-α) activity is under positive regulation by E_2 in MCF-7 cells (*6*) and an IGF-I–like activity is also produced but not as markedly regulated by E_2 (*7*). IGF-I was detected in CME_2 at 60 ng/ml and the EGF-like component was present at 100 ng/ml (EGF equivalents). We repeated the tumor-induction experiments in vivo with these concentrations of human serum IGF-I and synthetic human EGF (Table 1, C). We observed that both growth factors at the above-mentioned doses stimulated some MCF-7 tumorigenesis. EGF appeared to be the stronger of the two growth factors, giving a tumor incidence and size in the same range as E_2 itself. The EGF-treated group was significantly different from the control in terms of tumor incidence ($P < 0.05$ by χ^2 analyses) and mean cross-sectional area ($P < 0.05$ by t test). Again, growth factor–induced tumors were of indistinguishable morphology compared to E_2-induced tumors at the light microscopic level. EGF-induced and IGF-I–induced tumors had similar induction and regression kinetics to CME_2. These findings are consistent with a role for EGF-like components of CME_2 in tumorigenic stimulation by the conditioned medium. IGF-I effects were weaker than those of EGF; its role at present is uncertain.

These data provide evidence that MCF-7 cells under E_2 stimulation release activities capable of replacing E_2 in vivo as a tumorigenic stimulus in the nude mouse. While early tumor development was indistinguishable under E_2 and CME_2 stimulation, CME_2-induced tumors were not capable of growth beyond approximately 0.5 cm in diameter. As a beginning in the analysis of the active components of CME_2, we have identified EGF (TGFα)-like and IGF-I–like

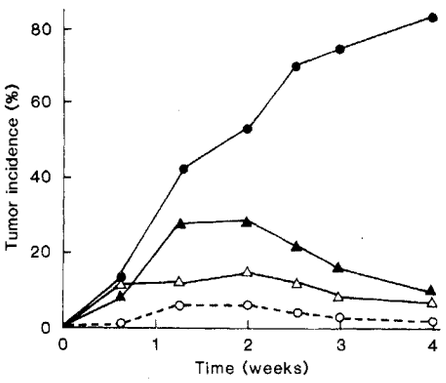

Fig. 3. Time dependence of CME_2 activity. CME_2 (▲) or CM (△) was placed in Alzet minipumps and implanted along with MCF-7 cells in nude mice (see legend to Table 1). Other groups of animals were treated with unconditioned negative control medium (○) or with a 0.5-mg E_2 pellet (●) to stimulate MCF-7 tumor formation. After 14 days, fresh pumps and E_2 pellets were reimplanted. Tumor incidence was observed over a 4-week period. Tumors were scored as palpable lumps greater than 0.2 × 0.2 cm. Twenty or more animals were used for each group.

activities. We have previously noted that enhanced secretion of IGF-I and EGF (TGFα)-related growth factors is associated with the estrogen-independent tumorigenic state of MCF-7 cells stably transfected with the v-ras^H oncogene (*13, 14*). We have further observed that these growth factor activities are constitutively secreted at high levels by some estrogen-independent human breast cancer cell lines (*6*). These data complement and extend previous data obtained in vitro, demonstrating that MCF-7 cells are growth-stimulated by insulin, IGF-I, and EGF (*15*).

MCF-7 cells under E_2 stimulation may also secrete proteases and other active growth factor–like components, such as angiogenic activities, into their surrounding local environment. These other components may be required to act with growth factor activities to promote full tumorigenesis. The possibility that E_2 may exert at least some of its tumorigenesis effects via secreted polypeptide growth factor mediators [in particular, an EGF (TGFα)-like component] suggests new strategies for arrest of tumor growth by interrupting growth factor action.

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Blockade of Visual Excitation and Adaptation in *Limulus* Photoreceptor by GDP-β-S

ALAN FEIN

Light causes both depolarization and adaptation to light in *Limulus* ventral photoreceptors. Both visual excitation and adaptation were blocked by guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S), a metabolically stable analog of guanosine 5′-diphosphate (GDP). However, GDP-β-S did not block the excitation caused by injection of inositol 1,4,5-trisphosphate into the cell. These results suggest a molecular cascade of visual excitation and adaptation: Light isomerizes the visual pigment rhodopsin, which in turn activates a guanyl nucleotide–binding protein. The binding protein then stimulates production of inositol 1,4,5-trisphosphate, which causes release of calcium from the endoplasmic reticulum.

BECAUSE OF THEIR LARGE SIZE, THE ventral photoreceptors of *Limulus* have been a favorite preparation for the investigation of the molecular events underlying phototransduction in microvillar photoreceptors. Independent lines of evidence suggest that both a guanine nucleotide–binding protein (N) and a phospholipase C may be involved in phototransduction in these cells. (i) Agents that activate guanine nucleotide–binding proteins excite ventral photoreceptors by depolarizing them in a manner similar to light (*1–4*). These findings led to the suggestion that illumination of ventral photoreceptors isomerizes the visual pigment rhodopsin, which in turn activates N and thereby leads to membrane depolarization (*2, 4*). (ii) The intracellular messenger inositol 1,4,5-trisphosphate (IP_3), which is released from phosphatidylinositol 4,5-bisphosphate (PIP_2) by the enzyme phospholipase C (*5*), can excite and adapt ventral photoreceptors when injected intracellularly (*6, 7*). Thus, phospholipase C may play a role in visual transduction.

A growing body of evidence suggests that receptor-mediated production of IP_3 involves a guanine nucleotide–binding protein (*5, 8*). In enzyme cascades such as hormonal activation of adenylate cyclase and light-triggered hydrolysis of cyclic guanosine monophosphate, information flows from the receptor to N and then to the enzyme that is regulated (*9, 10*). Application of these ideas to phototransduction in ventral photoreceptors suggests a model for the visual cascade. Photoisomerization of rhodopsin activates N, which in turn activates phospholipase C, thereby causing the production of IP_3. One would predict that block of an early step of the cascade would leave later stages unaffected. Thus, blockade

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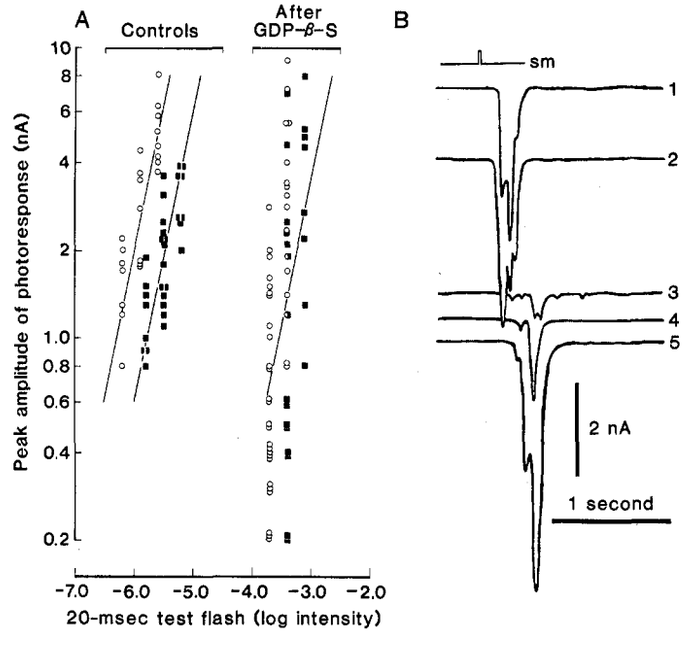

Fig. 1. Voltage-clamp recordings of light-induced current in ventral photoreceptors before and after injection of GDP-β-S. The methods for preparation, intracellular recording, voltage clamping, and pressure injection have been described (*25, 26*). Cells were impaled with two microelectrodes, a current-passing electrode filled with 2.5*M* KCl and a voltage-measuring electrode filled with 20 m*M* GDP-β-S, 100 m*M* potassium aspartate, 10 m*M* Hepes, *p*H 7.0. In some experiments 0.001% Triton X-100 was added to the GDP-β-S solution to facilitate pressure injection (*16*). GDP-β-S was from Boehringer Mannheim (Indianapolis). (A) A log-log plot of the peak amplitude of the light-induced current as a function of light intensity for two different cells (closed squares, open circles). Light intensities are relative to the maximum available from the light source. The solid lines have a slope of 1. The injected volume of GDP-β-S–containing solution was between 1% and 10% of the cell volume (*26*) giving a final concentration of 0.2 to 2 m*M* in the cell. For 16 cells injected with GDP-β-S, the range of desensitization was between 2 and 4 log units with a mean of 2.6 log units. When nine cells were injected with 20 m*M* GDP (three of the nine also received 0.001% Triton X-100), they showed no loss in sensitivity. The amount of desensitization with GDP-β-S is similar to that observed previously (*3*). Records 1 and 2 of (B) are responses to a 20-msec flash of log intensity −5.6 obtained before injection of GDP-β-S. After injection, records 3, 4, and 5 were obtained with a flash of log intensity −2.9. The flash is indicated by the stimulus monitor (sm). The light-induced currents are plotted downward in (B) with the inward currents negative. The data in (B) are from a different cell than those in (A).