9E10 monoclonal antibody (4 μg/ml) (Boehringer, Mannheim, Germany) and phycoerythrin-conjugated goat antibodies to mouse immunoglobulin G (16 μg/ml) (Dako, Glostrup, Denmark). Fixed cells were analyzed for green and red fluorescence with an Epics Elite flow cytometer (Coultronics).
29. HeLa-P4 cells were cotransfected by calcium phosphate precipitation with a hygromycin-resistance vector (SV-hygro) and either Rc/CMV–CCR5 or Rc/CMV–US28 (1:20 ratio). Cell clones were selected in the presence of hygromycin B (150 μg/ml).
30. We performed the experiments essentially as described (*2*). Simian COS cells or HeLa cells were cotransfected with Rc/CMV vectors containing the CXCR4, CCR5, or US28 ORFs (cloned downstream of the T7 promoter) and Rc/CMV–CD4, and were infected with vT7pol. Cocultures were then performed with HeLa–Env/ADA or HeLa–Env/LAI cells transiently transfected with a T7-*lacZ* construct.
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Activation of the G Protein Gq/11 Through Tyrosine Phosphorylation of the α Subunit

Hisashi Umemori, Takafumi Inoue, Shoen Kume, Naohiro Sekiyama, Motoshi Nagao, Hiroshi Itoh, Shigetada Nakanishi, Katsuhiko Mikoshiba, Tadashi Yamamoto

Various receptors coupled to the heterotrimeric guanine nucleotide-binding protein Gq/11 stimulate formation of inositol-1,4,5-trisphosphate (IP_3). Activation of these receptors also induces protein tyrosine phosphorylation. Formation of IP_3 in response to stimulated receptors that couple to Gq/11 was blocked by protein tyrosine kinase inhibitors. These inhibitors appeared to act before activation of Gq/11. Moreover, stimulation of receptors coupled to Gq/11 induced phosphorylation on a tyrosine residue (Tyr^{356}) of the $G\alpha_{q/11}$ subunit, and this tyrosine phosphorylation event was essential for Gq/11 activation. Tyrosine phosphorylation of $G\alpha_{q/11}$ induced changes in its interaction with receptors. Therefore, tyrosine phosphorylation of $G\alpha_{q/11}$ appears to regulate the activation of Gq/11 protein.

IP_3 is a second messenger that controls many cellular processes by causing release of Ca^{2+} from intracellular stores. Formation of IP_3 is stimulated by heterotrimeric guanine nucleotide-binding protein (G protein)–coupled receptors (GPCRs) and by receptors linked by tyrosine kinases either directly or indirectly. The formation of IP_3 activated by GPCRs is catalyzed by phospholipase Cβ (PLCβ) (*1*). G proteins are composed of three polypeptides denoted α, β, and γ. The α subunits, which bind and hydrolyze guanosine triphosphate (GTP) (*2*, *3*), are divided into four classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and Gα12. The Gα subunits that regulate PLCβ belong to the Gq class ($G\alpha_q$, Gα11, Gα14, Gα15/16) (*4*). When an agonist binds to a GPCR, the receptor-linked G protein dissociates into a Gα subunit and a Gβγ dimer, each of which can activate target effectors. However, the precise in vivo mechanisms of receptor-mediated G protein activation remain to be elucidated (*2*, *3*). Several Gq or G11 (Gq/11)–coupled receptors induce tyrosine phosphorylation of cellular proteins (*5*). Here, we examined the role of tyrosine phosphorylation events in IP_3-Ca^{2+} signaling through Gq/11-coupled receptors.

The metabotropic glutamate receptor 1α (mGluR1α) is a Gq/11-coupled receptor (*6*). Application of glutamate to Chinese hamster ovary (CHO) cells expressing mGluR1α (*7*) increased tyrosine phosphorylation of cellular proteins within 1 min (Fig. 1A) (*8*). The tyrosine phosphorylation event was almost completely suppressed by genistein, a protein tyrosine kinase (PTK) inhibitor (*9*, *10*), and was enhanced by vanadate-H_2O_2, a protein tyrosine phosphatase (PTP) inhibitor (*10*) (Fig. 1B). The mGluR1α itself was immunoprecipitated with antibody to phosphotyrosine (anti-PY) only from cells treated with glutamate (Fig. 1C).

We examined the possible role of PTKs in Ca^{2+} mobilization (*11*). Addition of glutamate (100 μM) to the mGluR1α-expressing CHO cells increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). However, when cells were incubated in the presence of genistein, no increase in $[Ca^{2+}]_i$ was observed [median inhibitory concentration (IC_{50}) ≈ 10 μM], even in cells treated with a high concentration of glutamate (1 mM; Fig. 2A). After genistein was washed out, the Ca^{2+} response was recovered. To demonstrate that the inhibition of Ca^{2+} release by PTK inhibitors is not a cell type–specific event, we recorded Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes injected with mGluR1α mRNA (*12*). Current elicited by glutamate was blocked by any of three PTK inhibitors: genistein, tyrphostin AG213 (*9*), and AG60 (*13*) (Fig. 2B). The currents were restored after the drugs were removed. Thus, the stimulation-induced increase in $[Ca^{2+}]_i$ in these cells apparently requires PTK activity.

To determine whether the PTK inhibitors act before or after IP_3 formation, we examined the formation of IP_3 (*14*) in the presence or absence of PTK inhibitors. Genistein and tyrphostin AG213 almost completely abolished glutamate-stimulated IP_3 formation in mGluR1α-expressing CHO cells (Fig. 2C). The effect of genistein was concentration-dependent (IC_{50} ≈ 10 μM) (*15*). Daidzein, an analog of genistein that lacks PTK inhibitory activity (*10*), had no effect on IP_3 formation. A protein kinase C (PKC) inhibitor, H7,

H. Umemori and T. Yamamoto, Department of Oncology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.
T. Inoue, S. Kume, K. Mikoshiba, Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.
N. Sekiyama and S. Nakanishi, Department of Biological Sciences, Kyoto University Faculty of Medicine, Kyoto 606, Japan.
M. Nagao and H. Itoh, Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226, Japan.

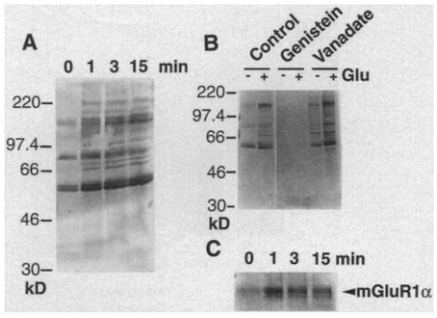

Fig. 1. Tyrosine phosphorylation initiated through mGluR1α. (**A**) Time course of glutamate-induced tyrosine phosphorylation. CHO cells expressing mGluR1α were stimulated with 100 μM glutamate for the indicated time, and cell lysates were subjected to immunoblotting with anti-PY. Positions of prestained molecular size markers (Amersham) are indicated. (**B**) Effects of a PTK inhibitor and a PTP inhibitor on tyrosine phosphorylation. Cells were incubated with 0.1% dimethyl sulfoxide (control), 100 μM genistein, or 1 mM vanadate with 3 mM H_2O_2 (vanadate) for 10 min and then stimulated with glutamate for 3 min. (**C**) Tyrosine phosphorylation involves mGluR1α. Cells were stimulated with glutamate for the indicated time. The cell lysates were immunoprecipitated with anti-PY, and the immunoprecipitates were subjected to immunoblotting with anti-mGluR1α.

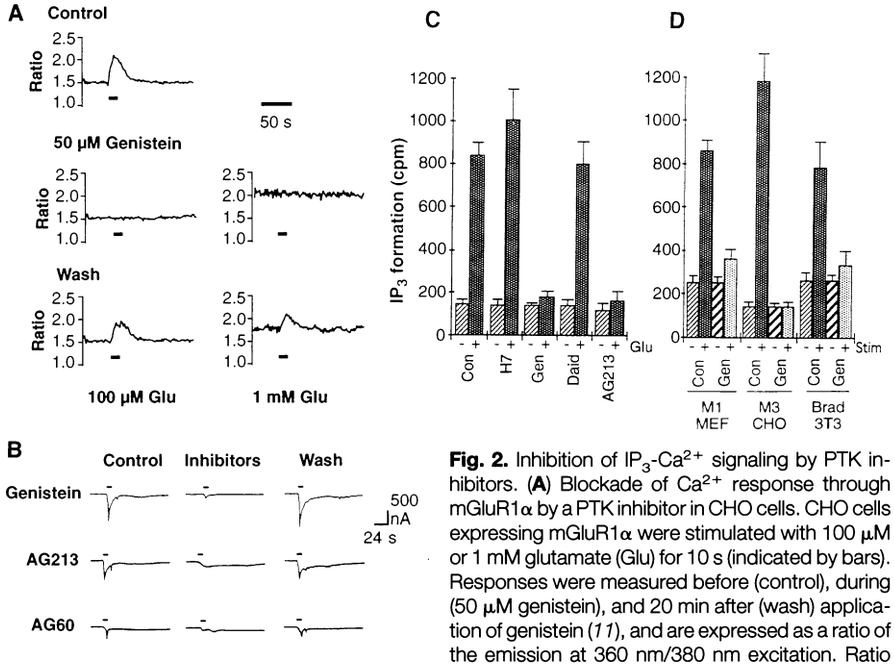

Fig. 2. Inhibition of IP_3-Ca^{2+} signaling by PTK inhibitors. (**A**) Blockade of Ca^{2+} response through mGluR1α by a PTK inhibitor in CHO cells. CHO cells expressing mGluR1α were stimulated with 100 μM or 1 mM glutamate (Glu) for 10 s (indicated by bars). Responses were measured before (control), during (50 μM genistein), and 20 min after (wash) application of genistein (*11*), and are expressed as a ratio of the emission at 360 nm/380 nm excitation. Ratio values from five cells were averaged. (**B**) Inhibition of Ca^{2+}-induced Cl^- currents through mGluR1α by PTK inhibitors in *Xenopus* oocytes. Typical traces elicited by application of 100 μM glutamate for 10 s (horizontal bars) on oocytes injected with mGluR1α transcripts recorded before (control), during (inhibitors), and 10 min after (wash) application of each inhibitor (100 μM genistein, 50 μM AG213, or 100 μM AG60) are shown. Holding potential, −60 mV. (**C**) Inhibition of IP_3 formation through mGluR1α by PTK inhibitors in CHO cells. The cells were stimulated with 100 μM glutamate (Glu); the formation of IP_3 is expressed as counts per minute (cpm) per well. Drug concentrations used were 300 μM H7, 100 μM genistein (Gen), 100 μM daidzein (Daid), or 50 μM AG213. All experiments were done in duplicate or triplicate, at least five times. The values represent means ± SD of all results. (**D**) Inhibition by genistein (100 μM) of IP_3 formation stimulated through M1 mAChR in MEF cells, M3 mAChR in CHO cells, or bradykinin receptor (Brad) in Swiss 3T3 cells. The cells were stimulated (Stim) with 100 μM carbachol or 100 nM bradykinin in the presence (Gen) or absence (Con) of genistein.

increased the formation of IP_3, which is consistent with the finding that PKC suppresses PLCβ activity (*16*). Therefore, PTK activity is specifically required for IP_3 formation through mGluR1α. We also analyzed IP_3-Ca^{2+} signaling in mouse embryo fibroblast (MEF) cells expressing M1 muscarinic acetylcholine receptor (mAChR) (*17*), CHO cells expressing M3 mAChR, or Swiss 3T3 cells that naturally express bradykinin receptor (*18*). Genistein also inhibited IP_3 formation in response to the activation of M1, M3, or bradykinin receptors (Fig. 2D).

To determine whether the PTK inhibitors act before or after G protein activation, we constructed a GTPase-deficient, constitutively active form of the Gα11 subunit [Gα11-Q209L (*19*)] and expressed the active form in MEF cells (*17*). In the absence of inhibitors, this active form of Gα11 resulted in an amount of IP_3 formation ~2.5 times that elicited by wild-type Gα11 (Fig. 3). Incubation with 100 μM genistein had little effect on the increase in IP_3 formation brought about by the active form of Gα11 (Fig. 3). Thus, PTK inhibitors act before G protein activation.

The $G\alpha_s$ subunit is phosphorylated on Tyr^{37} and Tyr^{377} by Src PTK in vitro (*20*). Tyr^{377} is the fourth amino acid from the COOH-terminus of $G\alpha_s$. $G\alpha_q$ and Gα11 also have a tyrosine residue at the corresponding position (Tyr^{356}) (*4*). Therefore, we examined whether the $G\alpha_{q/11}$ subunits were phosphorylated on tyrosine upon stimulation of Gq/11-coupled receptors. Stimulation by carbachol of MEF cells expressing M1 mAChR (*8*, *17*) induced tyrosine phosphorylation of both $G\alpha_q$ and Gα11 (Fig. 4A); ~2 to 5% of $G\alpha_{q/11}$ was phosphorylated on tyrosine (*15*). We prepared Gα11 with a $Tyr^{356} \rightarrow$ Phe (Y356F) mutation (*19*); when Gα11-Y356F was expressed with M1 mAChR in MEF cells (*8*, *17*), no increase in tyrosine phosphorylation was observed after agonist stimulation (Fig. 4B). Thus, stimulation of Gq/11-coupled receptors appears to induce tyrosine phosphorylation on Tyr^{356} of $G\alpha_{q/11}$.

We examined whether tyrosine phosphorylation of the $G\alpha_{q/11}$ subunit is involved in the formation of IP_3. When M1 mAChR was expressed with Gα11-Y356F, carbachol-induced IP_3 formation was reduced (33.6 ± 3%) relative to that in cells expressed with wild-type Gα11 or transfected with empty vector (Fig. 4C) (*14*, *17*). The mechanism for the effect of Gα11-Y356F is still unknown, and Gα11-Y356F could fail to interact with the receptor. However, because Gα14, whose corresponding residue is phenylalanine, can interact with receptors (*4*, *21*), it is likely that Gα11-Y356F also can interact with the receptor. Therefore, tyrosine phosphorylation on Tyr^{356} of Gα11 appears to be essential for GPCR-mediated formation of IP_3. We also exam-

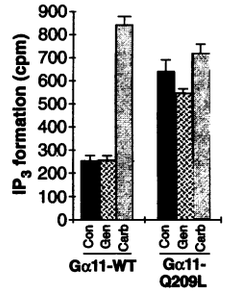

Fig. 3. IP_3 formation in MEF cells expressing wild-type (Gα11-WT) or a constitutively active form (Gα11-Q209L) of Gα11. The cells were incubated with 100 μM genistein or stimulated with 100 μM carbachol (Carb); the formation of IP_3 is expressed as counts per minute per well. All experiments were done in duplicate or triplicate, at least three times. The values represent means ± SD of all results.

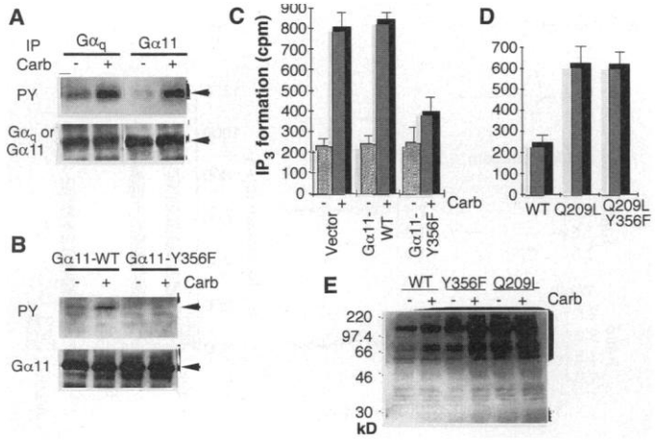

Fig. 4. Role of tyrosine phosphorylation on Tyr^{356} of the Gα11 subunit in M1 mAChR–mediated activation of G11. (**A**) Tyrosine phosphorylation of $Gα_q$ and Gα11 in response to M1 mAChR stimulation. M1 mAChR was expressed in MEF cells, which were stimulated with 100 μM carbachol for 3 min. $Gα_q$ and Gα11 were immunoprecipitated from the cell lysates (*8*) and subjected to immunoblotting with anti-PY or antibodies to the respective proteins. (**B**) Tyrosine phosphorylation on Tyr^{356} of Gα11 after M1 mAChR stimulation. Wild-type (Gα11-WT) or mutated (Gα11-Y356F) Gα11 were expressed with M1 mAChR in MEF cells and stimulated with 100 μM carbachol for 3 min. Gα11 was immunoprecipitated and subjected to immunoblotting with anti-PY or anti-Gα11. The amount of expression of the transfected Gα11 was at least 10 times that of the endogenous Gα11 (*15*). (**C**) Role of phosphorylation on Tyr^{356} of Gα11 for M1 mAChR-mediated formation of IP_3. M1 mAChR was expressed with empty vector (pCMV5), Gα11-WT, or Gα11-Y356F in MEF cells, and the formation of IP_3 was measured. All experiments were done in duplicate or triplicate, at least three times. The values represent means ± SD of all results. (**D**) Phosphorylation on Tyr^{356} of Gα11 is not essential for the activity of constitutively active Gα11-Q209L. Wild type (WT), Q209L-mutated, or Q209L- and Y356F-mutated Gα11 were expressed in MEF cells, and the formation of IP_3 was measured. (**E**) Tyrosine phosphorylation of cellular proteins occurs independently of Gq/11 activity. Wild type (WT), Y356F-mutated, or Q209L-mutated Gα11 were expressed with M1 mAChR in MEF cells and stimulated with 100 μM carbachol for 3 min. The same amounts of lysates were tested for phosphotyrosine. Positions of prestained molecular size markers are indicated.

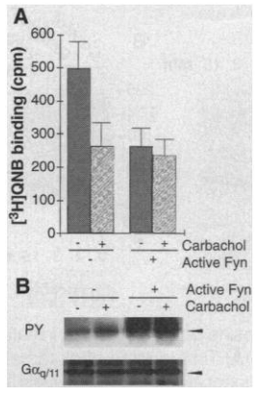

Fig. 5. Effect of tyrosine phosphorylation of the $Gα_{q/11}$ subunit on interaction with M1 mAChRs. (**A**) $Gα_{q/11}$-associated [^{3}H]QNB binding activity. Empty vector (–) or active Fyn (+) was expressed with M1 mAChR in MEF cells, which were stimulated with 100 μM carbachol for 3 min. $Gα_{q/11}$ was immunoprecipitated with anti-$Gα_{q/11}$ and assayed for [^{3}H]QNB binding activity. All experiments were done in duplicate or triplicate, at least five times. The values represent means ± SD of all results. Similar results were obtained when we used anti-$Gα_q$ and anti-Gα11 for immunoprecipitation (*15*). (**B**) Tyrosine phosphorylation of the $Gα_{q/11}$ subunits. Immunoprecipitated $Gα_{q/11}$ was blotted with anti-PY or anti-$Gα_{q/11}$.

ined the effect of Y356F mutation on IP_3 formation stimulated by the constitutively active form of Gα11 (Gα11-Q209L). The Y356F mutation did not affect the Gα11-Q209L–mediated increase in IP_3 formation (*14*, *17*) (Fig. 4D), suggesting that phosphorylation on Tyr^{356} of Gα11 is not required for the activity of the constitutively active form of Gα11. Thus, phosphorylation on Tyr^{356} of $Gα_{q/11}$ is apparently essential for the activation of Gq/11 by agonist stimulation.

Tyrosine phosphorylation events are induced after G protein activation (*22*). To demonstrate that tyrosine phosphorylation events occur before activation of Gq/11 as well, we examined the carbachol-dependent tyrosine phosphorylation of cellular proteins in the presence of various Gα11 mutants in M1 mAChR–expressing MEF cells (*8*, *17*). Carbachol-dependent tyrosine phosphorylation of cellular proteins was observed in the presence of either the wild-type or Y356F-mutated Gα11 (Fig. 4E). Because phosphorylation occurred comparably even in the presence of Gα11-Y356F, tyrosine phosphorylation of cellular proteins occurs independently of phosphorylation of Tyr^{356} of Gα11, which suggests that tyrosine phosphorylation of some proteins may precede the activation of G11. In cells expressing Gα11-Q209L, tyrosine phosphorylation was increased without agonist stimulation (Fig. 4E), suggesting that tyrosine phosphorylation is enhanced as a consequence of activation of Gq/11 as well (*22*).

We examined receptor–G protein interactions after agonist stimulation or tyrosine phosphorylation of the $Gα_{q/11}$ subunit. To do so, we expressed M1 mAChRs with or without a constitutively active form of the PTK Fyn (active Fyn) in MEF cells (*17*, *23*), stimulated them with carbachol, and measured the $Gα_{q/11}$-associated binding activity of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), an M1 mAChR antagonist (*24*–*26*). The [^{3}H]QNB binding activity associated with $Gα_{q/11}$ from carbachol-stimulated cells was ~50% of that from unstimulated cells (Fig. 5A). Thus, agonist binding results in receptor–G protein dissociation or conformational changes that prevent the antibody to $Gα_{q/11}$ from accessing its epitope (*25*). Active Fyn increased tyrosine phosphorylation of $Gα_{q/11}$ without changing the amount of M1 mAChR expression (*15*) (Fig. 5B). When active Fyn was expressed in the cells, the $Gα_{q/11}$-associated [^{3}H]QNB binding activity was reduced even without carbachol stimulation (Fig. 5A). These results suggest that tyrosine phosphorylation of the $Gα_{q/11}$ subunit regulates the interaction between receptors and Gq/11. Taken together, our results demonstrate that tyrosine phosphorylation of the $Gα_{q/11}$ subunit by PTKs contributes to GPCR-mediated activation of Gq/11.

In vitro, the Gq class of proteins can activate PLCβ by agonist stimulation when reconstituted in lipid vesicles with purified receptors (*27*). Tyrosine-phosphorylated $Gα_{q/11}$ is more active in stimulating PLCβ in vitro (*28*). Thus, PTKs may act to facilitate receptor-mediated G protein activation. In vivo, receptor–G protein coupling and G protein activation may be more tightly regulated than in vitro (*2*, *21*, *29*). Such regulation may be controlled at least in part through tyrosine phosphorylation of $Gα_{q/11}$.

Many Gα subunits have tyrosine residues near their COOH-termini (*4*), and the $Gα_i$ and $Gα_s$ families of proteins can be tyrosine-phosphorylated in vitro (*30*). Their activities may also be regulated by tyrosine phosphorylation. We suggest that tyrosine phosphorylation of the Gα subunits is one mechanism by which receptor–G protein coupling and G protein activation are regulated in vivo.

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8. CHO cells expressing mGluR1α or MEF cells transfected with M1 mAChR were incubated, 48 hours after transfection, in phosphate-buffered saline (PBS) for 1 hour and stimulated with glutamate or carbachol, respectively. The inhibitors were applied 10 min before the application of agonists. CHO cells were lysed in tris-NP40-EDTA (TNE) buffer [H. Umemori, S. Sato, T. Yagi, S. Aizawa, T. Yamamoto, *Nature* **367**, 572 (1994)]. MEF cells were lysed in RIPA buffer [H. Umemori *et al.*, *Mol. Brain Res.* **16**, 303 (1992)]. Equal amounts of lysates were subjected to immunoblotting with anti-PY (RC20, Transduction Laboratories); subjected to immunoprecipitation with anti-PY (4G10, Upstate Biotechnology Inc.) followed by immunoblotting with anti-mGluR1α [R. Shigemoto, T. Abe, S. Nomura, S. Nakanishi, T. Hirano, *Neuron* **12**, 1245 (1994)]; or subjected to immunoprecipitation with anti-$G\alpha_q$, anti-Gα11 [non–cross-reactive with each other (*15*)], or antibody that recognized both $G\alpha_q$ and Gα11 ($G\alpha_{q/11}$) (Santa Cruz) followed by immunoblotting with RC20 or anti-$G\alpha_{q/11}$.
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19. Wild-type Gα11 cDNA was isolated by the polymerase chain reaction (PCR) after reverse transcription of RNA from mouse S49 lymphoma cells, and its sequence was confirmed by sequencing. Mutations in the Gα11 cDNA were introduced by PCR mutagenesis with wild-type cDNA as a template. The PCR primer pairs used were 5′-TAGCAAGCTTCATATGACTCTGGAGTCCATGATGGC-3′ and 5′CAATGGATCCACTTCCTGCGCTCTGACCTCAGGGCCTCCC-3′ for the Q209L mutation [N.-X. Qian, S. Winitz, G. L. Johnson, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4077 (1993)] and 5′-ACCTTCTAGAAGACAAGATC-3′ and 5′-CATGCCCGGGTCACACCAGGTTGAACTCCTTCAG-3′ for the Y356F mutation. The PCR fragments were inserted into the corresponding region of the wild-type Gα11 cDNA. Wild-type and mutated Gα11 cDNAs were subcloned into the Hind III site of pCMV5 [S. Andersson, D. N. Davis, H. Dählbach, H. Jörnvall, D. M. Russell, *J. Biol. Chem.* **264**, 8222 (1989)]. Mutations were confirmed by sequencing.
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26. The [^{3}H]QNB binding assay was done as described (*24, 25*). $G\alpha_{q/11}$ was immunoprecipitated from the cell lysate (*25*), washed with binding buffer (*25*), and then incubated with 1 nM [^{3}H]QNB for 90 min at 30°C. In a typical experiment, total binding obtained from cell lysates was 2500 counts per minute.
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TECHNICAL COMMENTS

Estimating Chaos in an Insect Population

R. F. Costantino *et al.* (*1*) state that their laboratory data of the population dynamics of the flour beetle *Tribolium castaneum* show convincing evidence of transitions to chaos. Their methodology was similar to earlier studies (*2*) that assessed the population dynamics of a time series by fitting some mechanistic or empirical model and then inspecting realizations from the deterministic skeleton of the fitted model. However, Ellner and Turchin (*3*) argued powerfully that such an approach was flawed because it did not allow for a random component in the dynamics and might lead to the misidentification of series dynamics.

Ellner and Turchin identify three sources of variation that might influence the sensitivity of the system to initial conditions—endogenous dynamics, exogenous dynamics, and measurement error—and ask how fluctuations can be categorized as stochastic or dynamic if the methodology assumes the absence of noise. They presented methods for calculation of the Lyapunov exponent that allow for dynamic noise; these methods have now been supplemented by associated randomization tests that indicate the variability of Lyapunov exponents under two population dynamic hypotheses (*4*). While this new methodology cannot disentangle the relative contributions of measurement error (which is usually assumed to be small) from exogenous dynamics, it does identify the effects of the exogenous dynamics, which is usually the aim of the exercise.

The estimates of the Lyapunov exponents given by Costantino *et al.* must be shown to be robust to the presence of noise [that the authors themselves estimate in their variance-covariance matrix sum (Σ)] if a valid characterization of the *Tribolium* dynamics is to be obtained. We urge Costantino *et al.* to provide such estimates for the stochastic version of their model and then to compare their data with such output, rather than to use estimates from the deterministic skeleton.

Joe N. Perry
Ian P. Woiwod
Rothamsted Experimental Station, Institute of Arable Crops Research, Harpenden Herts, AL5 2JQ United Kingdom
E-mail: joe.perry@bbsrc.ac.uk
Robert H. Smith
University of Leicester, Leicester, United Kingdom
David Morse
University of Kent at Canterbury, United Kingdom

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Response: We agree with Perry *et al.* that more study is needed of nonlinear dynamics in the presence of noise. We have computed the Lyapunov exponents (LE) for both the deterministic and stochastic versions of our model (Table 1) by using our published estimates for the model parameters and variance-covariance matrix. If one accepts a positive stochastic LE as a hallmark of chaos, then these results demonstrate that our statements about chaos are "robust to the presence of noise."

We remain unconvinced, however, that the stochastic LE (*2*) advocated by Perry *et al.* should be viewed as an objective hallmark of chaos. Consider, for instance, a population model in which population size, N_t, obeys a stochastic Ricker (discrete time logistic) model

$$N_t = N_{t-1} \exp(r - aN_{t-1} + \sigma Z_t)$$