yses concerning plant defensive strategies should consider how plants can safeguard themselves against severe herbivore injury by attracting predators or parasitoids (*18*). The terpenoids are reliable cues for the parasitoids because they are closely associated with herbivore damage and they are released even during the frequent pauses in eating by the caterpillars (Fig. 2). We do not yet know whether the induced reaction is limited to the damaged sites, or whether it is systemic as has been shown in other studies (*19, 20*).

Our results indicate an active release of chemicals by plants that is exploited by host-searching parasitoids. It is likely that the terpenoids and indole are involved in other types of interactions as well. They may, for example, act as oviposition deterrents for herbivorous insects searching for sites to deposit their eggs or function in communication between plants (*20, 21*). More knowledge about the injury-dependent production of airborne semiochemicals by plants may point to new possibilities for biological control of pest insects.

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16. Before release in the tunnel (*17*) each test insect was given a 20-s contact experience with a plant-host complex of BAW caterpillars on corn. Experience on host-infested leaves dramatically increases the subsequent responses by *C. marginiventris* females to host-related odors in olfactometric bioassays [T. C. J. Turlings, J. H. Tumlinson, W. J. Lewis, L. E. M Vet, *J. Insect Behav.* **2**, 217 (1988); T. C. J. Turlings, J. W. A. Scheepmaker, L. E. M. Vet, J. H. Tumlinson, W. J. Lewis, *J. Chem. Ecol.* **16**, 1577 (1990)].
17. Responses of individual *C. marginiventris* females to odors were tested in a Plexiglas flight tunnel. Dimensions of the tunnel were 60 cm by 60 cm in cross section and 2.4 m long. Air was pulled through the tunnel at 0.15 m/sec and was exhausted through a 30-cm flexible pipe with a fan. More details on the tunnel are given by F. J. Eller, J. H. Tumlinson, and W. J. Lewis [*Environ. Entomol.* **17**, 745 (1988)]. Seedlings used as odor sources were placed 20 cm apart and 30 cm from the tunnel floor approximately 80 cm upwind from the insect release point. After the females were experienced (*16*), they were released into the tunnel from a glass funnel (*10*). Their responses and choices were recorded. If a female, after three trials, had not flown all the way to a source the flight was considered incomplete.
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Increase of the Catalytic Activity of Phospholipase C-γ1 by Tyrosine Phosphorylation

SHUNZO NISHIBE,* MATTHEW I. WAHL, S. M. TERESA HERNÁNDEZ-SOTOMAYOR, NICHOLAS K. TONKS,† SUE GOO RHEE, GRAHAM CARPENTER‡

Phospholipase C-γ1 (PLC-γ1), an isozyme of the phosphoinositide-specific phospholipase C family, which occupies a central role in hormonal signal transduction pathways, is an excellent substrate for the epidermal growth factor (EGF) receptor tyrosine kinase. Epidermal growth factor elicits tyrosine phosphorylation of PLC-γ1 and phosphatidylinositol 4,5-bisphosphate hydrolysis in various cell lines. The ability of tyrosine phosphorylation to activate the catalytic activity of PLC-γ1 was tested. Tyrosine phosphorylation in intact cells or in vitro increased the catalytic activity of PLC-γ1. Also, treatment of EGF-activated PLC-γ1 with a tyrosine-specific phosphatase substantially decreased the catalytic activity of PLC-γ1. These results suggest that the EGF-stimulated formation of inositol 1,4,5-trisphosphate and diacylglycerol in intact cells results, at least in part, from catalytic activation of PLC-γ1 through tyrosine phosphorylation.

SINCE IT WAS RECOGNIZED THAT INOSITOL 1,4,5-trisphosphate (Ins 1,4,5-P_3) and diacylglycerol (DAG) are important intracellular second messengers in hormonal regulation of various cellular functions (*1, 2*), considerable effort has been invested in dissecting the molecular events that underlie receptor modulation of phosphoinositide-specific phospholipase C (PLC) activity. A variety of receptor-PLC coupling systems are modulated by bacterial toxins, aluminum fluoride, and analogs of guanosine triphosphate (GTP) reagents that are believed to modify the actions of guanine nucleotide binding proteins (G proteins). As a result, it has been suggested that these hormones modulate PLC activity by a G protein–mediated mechanism (*3*). In con-

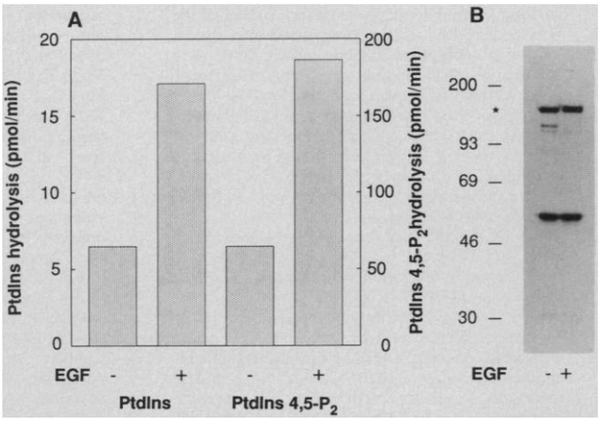

Fig. 1. Increased catalytic activity in PLC-γ1 immunoprecipitates from EGF-treated A-431 cells. PLC-γ1 was immunoprecipitated from the cytosol of A-431 cells treated without or with EGF. (**A**) PLC activity in the immunoprecipitates was measured with [^{3}H]PtdIns and [^{3}H]PtdIns 4,5-P_2 as the substrates. (**B**) The relative amount of PLC-γ1 protein present in the immunoprecipitates from (A) was estimated by immunoblot analysis with monoclonal antibodies to PLC-γ1 (anti–PLC-γ1). The star indicates the band corresponding to PLC-γ1. The lower band is anti–PLC-γ1 used in the immunoprecipitation.

trast, findings (*4*) suggest that EGF and other growth factors may modulate PLC activity by direct covalent modification of PLC-γ1. Several observations suggest that tyrosine phosphorylation of PLC-γ1 regulates its catalytic activity: (i) EGF receptor tyrosine kinase activity is essential for growth factor–stimulated phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) hydrolysis (*5*); (ii) the time course of PLC-γ1 tyrosine phosphorylation in intact cells coincides with that of EGF-induced Ins 1,4,5-P_3 formation (*6*); (iii) the stoichiometry of PLC-γ1 tyrosine phosphorylation in EGF-treated cells is high (>50%) (*7, 8*); (iv) there is a correlation between the capacity of EGF to stimulate PLC-γ1 tyrosine phosphorylation and its capacity to induce Ins 1,4,5-P_3 formation (*9, 10*); and (v) overexpression of PLC-γ1 increases the formation of Ins 1,4,5-P_3 in platelet-derived growth factor (PDGF)–treated cells (*11*). However, there has been no direct demonstration that tyrosine phosphorylation actually alters the catalytic activity of PLC-γ1. Herein, we show that phosphorylation of PLC-γ1 increased its catalytic activity.

PLC-γ1 can be immunoprecipitated from cell extracts with isozyme-specific monoclonal antibodies (*12*). Therefore, we measured the catalytic activity of PLC-γ1 in immunoprecipitates from control and EGF-treated A-431 cells (*13*), using ^{3}H-labeled PtdIns 4,5-P_2 or PtdIns as the substrate (Fig. 1A). Cells treated with EGF displayed a threefold increase in PLC activity. In similar experiments, the EGF-induced stimulation of PLC-γ1 activity varied from threefold to sixfold ($n = 6$). Immunoblot analysis of PLC-γ1 in the immunoprecipitates revealed no significant difference in the amount of the PLC-γ1 protein recovered from control and EGF-treated cells (Fig. 1B). This result indicated that EGF stimulation of A-431 cells produced an increase in PLC-γ1 catalytic activity.

Because EGF treatment of A-431 cells increases phosphorylation of PLC-γ1 on tyrosine and serine residues (*10, 14*), it was not clear that tyrosine phosphorylation per se activated this enzyme. PLC-γ1 is an effective substrate for phosphorylation by purified EGF receptor in vitro (*14, 15*); therefore, we used this approach for determining whether tyrosine phosphorylation of PLC-γ1 increased the catalytic activity of the enzyme. Immunoprecipitates of PLC-γ1 from control cells were incubated with purified EGF receptor and adenosine triphosphate (ATP), and the PtdIns 4,5-P_2 hydrolytic activity was measured (*16*). Incubation of PLC-γ1 with EGF receptor and ATP increased PLC-γ1 catalytic activity by threefold (Fig. 2A). The amount of PLC-γ1 activity detected after in vitro activation corresponded to about 70% of the PLC-γ1 activity recovered from EGF-treated cells. Neither EGF receptor nor ATP alone was capable of increasing PLC-γ1 catalytic activ-

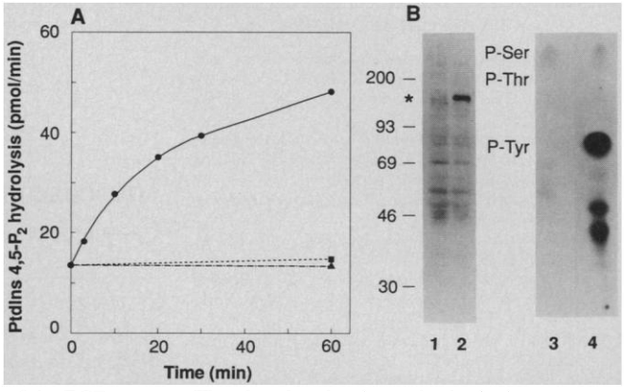

Fig. 2. Phosphorylation and activation of PLC-γ1 in vitro by the EGF receptor tyrosine kinase. (**A**) Immunoprecipitates of PLC-γ1 from nonstimulated A-431 cells were incubated with both EGF-receptor and ATP (●), EGF-receptor alone (▲), or ATP alone (■), and the PLC activity of the immunoprecipitates was measured. (**B**) PLC-γ1 immunoprecipitated from nonstimulated A-431 cells was incubated with [γ-^{32}P]ATP in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of the EGF receptor. The phosphorylated proteins in the immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography (lanes 1 and 2). Phosphoamino acid analysis of [^{32}P]PLC-γ1 from lanes 1 and 2 was performed (lanes 3 and 4). The PLC-γ1 band is denoted by a star.

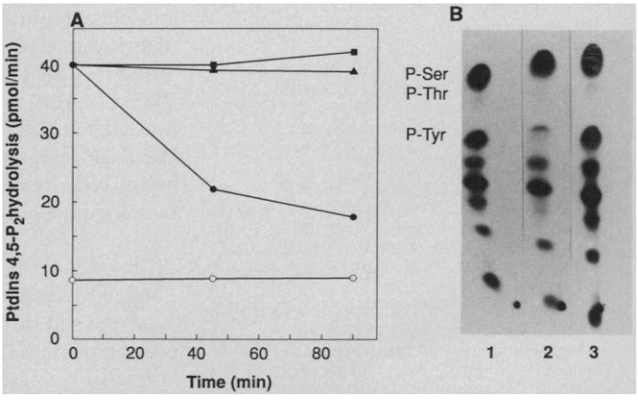

Fig. 3. Dephosphorylation and inactivation of PLC-γ1 in vitro by PTPase. (**A**) Immunoprecipitates of PLC-γ1 from EGF-treated A-431 cells were incubated with either TC-PTPase (●), CD45 (▲), or without phosphatase (■). After the indicated periods of time, PLC activity was measured with Ptd[^{3}H]Ins 4,5-P_2 as the substrate. The PLC activity in PLC-γ1 immunoprecipitates from an equivalent amount of cytosol protein from nonstimulated cells was also measured (○). (**B**) Phosphoamino acid analysis of [^{32}P]PLC-γ1 from EGF-stimulated cells, treated without or with phosphatase: lane 1, no PTPase; lane 2, TC-PTPase; and lane 3, CD45. The internal standards indicate the migration of phosphoamino acids P-Ser, phosphoserine; P-Thr, phosphothreonine; and P-Tyr, phosphotyrosine.

S. Nishibe, M. I. Wahl, S. M. T. Hernández-Sotomayor, G. Carpenter, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232–0146.
N. K. Tonks, Department of Biochemistry, University of Washington, Seattle, WA 98195.
S. G. Rhee, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, Bethesda, MD 20892.

*Current address: Second Department of Surgery, Osaka University Medical School, Osaka, 553, JAPAN.
†Current address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
‡To whom correspondence should be addressed.

ity. Nearly identical results were obtained in three similar experiments. Although there is a detectable amount of serine kinase activity in PLC-γ1 immunoprecipitates (Fig. 2B, lane 1), the purified EGF receptor preparation, which phosphorylated PLC-γ1 on tyrosine residues (Fig. 2B, lane 2), did not affect this coprecipitated serine kinase activity or the phosphoserine content of PLC-γ1. Therefore, it is unlikely that in vitro activation of PLC-γ1 by the EGF-receptor tyrosine kinase was mediated by activation of a coprecipitating serine kinase.

Finally, we sought to determine whether the activated enzyme could be deactivated by treatment with specific phosphotyrosine phosphatases (*17*) (Fig. 3). The activity of PLC-γ1 (immunoprecipitated from EGF-treated cells) was reduced by 73% after treatment with a truncated form of the 48-kD T cell protein tyrosine phosphatase (TC-PTPase) (*18*). The average inhibition ($n = 7$) of PLC-γ1 activity was 49% and could be completely blocked by tyrosine phosphatase inhibitors (100 μM vanadate and 10 μM molybdate) (*19*). No effect on PLC-γ1 catalytic activity was observed with CD45 (*20*), a tyrosine phosphatase that dephosphorylates the autophosphorylated EGF receptor more efficiently than does TC-PTPase (*19*). Phosphoamino acid analysis (Fig. 3B) of phosphatase-treated PLC-γ1 prepared from ^{32}P-labeled, EGF-treated cells showed that TC-PTPase depleted phosphotyrosine from PLC-γ1, while CD45 did not. In addition, TC-PTPase did not significantly alter the phosphoserine content of PLC-γ1 (Fig. 3B, lane 2). Neither phosphatase altered basal PLC-γ1 activity (that is, from untreated cells), nor was there evidence of proteolysis of PLC-γ1 after phosphatase treatment, as measured by immunoblotting (*19*). Furthermore, phosphatase 2A, a phosphoserine-threonine–specific phosphatase, significantly reduced the phosphoserine content of PLC-γ1, but did not decrease PLC-γ1 activity (*21*). Thus, for EGF activation of PLC-γ1, tyrosine phosphorylation appeared to be the major regulator of enzyme activity. In contrast, a mitogen-stimulated increase in both phosphotyrosine and phosphothreonine is required to increase catalytic activity of MAP-2 kinase (*22*).

The capacity of EGF to modulate cell growth and differentiation requires that the EGF receptor generate an intracellular biochemical signaling cascade. Stimulation of receptor tyrosine kinase activity is a primary event in this cascade, and identification of substrate proteins that function in the mitogenic signaling pathway has been an obvious but elusive goal. Because EGF promotes PtdIns 4,5-P_2 hydrolysis in intact cells, it has been suggested that PLC is activated by an undefined mechanism after EGF treatment. We have demonstrated reversible modulation of PLC-γ1 activity by tyrosine phosphorylation and dephosphorylation. However, it remains to be determined whether additional proteins participate in modulation of PLC-γ1 activity. Because a number of proteins immunoprecipitate with PLC-γ1 (*10, 14*), it is possible that they also influence the regulation of PLC-γ1 activity.

Formation of Ins 1,4,5-P_3 can be provoked by stimulation of tyrosine kinase–dependent receptors or G protein–dependent receptors, such as bradykinin or purinergic receptors. Thus, two distinct biochemical mechanisms are utilized for the activation of PLC activity in mammalian cells. Distinct classes of receptors may activate specific PLC isozymes by various mechanisms, or there may be multiple mechanisms for the activation of one PLC isozyme, such as PLC-γ1.

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13. After incubation in serum-free medium for 30 min, A-431 cells were treated without or with EGF at 300 ng/ml for 10 min. Cells were washed three times with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (pH 7.0, 140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4) and lysed with a Teflon Wheaton homogenizer (300 strokes, 20 strokes/min) in a homogenization buffer [20 mM Hepes, pH 7.4, 0.1 M NaCl, 1 mM EGTA, 0.4 mM Na_3VO_4, 20 mM β-glycerophosphate, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 10% glycerol]. The homogenates were centrifuged at 400,000*g* for 20 min at 4°C. The supernatants (cytosols) were incubated at 4°C overnight with 1 μg (per 200 μg of cytosolic protein) of mixed monoclonal antibodies (*12*) to PLC-γ1 plus 20 to 30 μl of 10% heat-inactivated *Staphylococcus aureus* cells coated with a rabbit antibody to mouse immunoglobulin G (IgG). The *S. aureus* cells were washed twice with cold homogenization buffer that contained 0.5% Triton X-100 and resuspended in the same buffer. Activity of PLC-γ1 in the *S. aureus* cell suspension was measured as previously described (*6, 10*) [S. H. Ryu, K. S. Cho, K. Y. Lee, P.-G. Suh, S. G. Rhee, *J. Biol. Chem* **262**, 12511 (1987), with modifications. Assay of PtdIns hydrolysis was performed in a 50-μl reaction mixture that contained 200 μM Ptd[^{3}H]Ins (20,000 cpm); 0.15% (w/v) *n*-octyl-β-D-glucopyranoside; 0.05% (v/v) Triton X-100; 3 mM $CaCl_2$; 1 mM EGTA; and 50 mM Hepes (pH 7.0). The assay of PtdIns 4,5-P_2 hydrolysis was performed in a 50-μl reaction mixture that contained 200 μM Ptd[^{3}H]Ins 4,5-P_2 (20,000 cpm); 0.15% (w/v) *n*-octyl-β-D-glucopyranoside; 0.05% (v/v) Triton X-100; 0.8 mM EGTA; 0.8 mM $CaCl_2$ (free [Ca^{2+}] = 1 μM); 35 mM NaH_2PO_4 (pH 6.8); and 70 mM KCl. The reaction mixtures were incubated for 10 to 15 min, and the reaction was terminated as described (*6*). Under these assay conditions, the PLC activities were linear with respect to time and protein concentration. The PtdIns hydrolytic activity in the PLC-γ1 immunoprecipitates (Fig. 1A) was 0.48 nmol/min/mg of cytosolic protein (nontreated A-431 cells) and 1.28 nmol/min/mg of protein (EGF-treated cells). The PtdIns 4,5-P_2 hydrolytic activities in the same samples were 3.24 nmol/min (−EGF) and 9.32 nmol/min (+EGF). Separate aliquots of PLC-γ1 immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. ^{125}I-labeled goat antibody to mouse IgG was used as the secondary antibody. Under the conditions of homogenization described above, 70 to 90% of total cell PLC-γ1 protein is recovered in the soluble fraction. The proportion of membrane-associated PLC-γ1 [G. Todderud, M. I. Wahl, S. G. Rhee, G. Carpenter, *Science* **249**, 296 (1990)] is minimized under these conditions. While the results reported are restricted to PLC-γ1 recovered from the soluble fraction, similar results demonstrating PLC-γ1 activation by EGF were obtained by immunoprecipitation of PLC-γ1 from detergent-solubilized cells (S. Nishibe and G. Carpenter, unpublished data).
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16. Immunoprecipitated PLC-γ1 from nonstimulated cells was incubated at 0°C with the EGF receptor purified from A-431 cell membranes by wheat germ lectin affinity chromatography (*15*), in a mixture that contained 20 mM Hepes (pH 7.4), 25 mM $MgCl_2$, 4 mM $MnCl_2$, and 3×10^{-7} M EGF in the presence of 80 μM ATP. After the indicated period of time, the immunoprecipitates were washed and resuspended in homogenization buffer that contained 0.5% Triton X-100, and the PLC activity in immunoprecipitates was measured with Ptd[^{3}H]Ins 4,5-P_2 as the substrate. Immunoprecipitated PLC-γ1 from nonstimulated cells was incubated at 0°C for 60 min in a buffer that contained 80 μM [γ-^{32}P]ATP (20 μCi/nmol) in the presence or absence of EGF receptor under the same condition used for the experiment shown in Fig. 2A. The immunoprecipitates were washed with buffer that contained 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM tris-HCl (pH 8.5), 150 mM NaCl, and 0.02% sodium azide. Labeled proteins were separated by SDS-PAGE and transferred to Immobilon P. After visualization by autoradiography, the bands that corresponded to PLC-γ1 were excised. Acid hydrolysis and high-voltage thin-layer electrophoresis were performed as described [M. P. Kamps and B. M. Sefton, *Anal. Biochem.* **176**, 22 (1989)]. Phosphoamino acids were separated on cellulose plates by electrophoresis (60 min, 1 kV) in a buffer that contained pyridine:acetic acid:H_2O/5:50:945, pH 3.5.
17. The PLC-γ1 immunoprecipitates from EGF-treated A-431 cells were washed twice in homogenization buffer without phosphatase inhibitors and incubated with either a truncated, 37-kD TC-PTPase (10 U/ml) or CD45 (30 μg/ml) at 30°C in a buffer that contained 20 mM Hepes (pH 7.4), 50 mM NaCl, leupeptin (5 μg/ml), aprotinin (5 μg/ml), 5% glycerol, bovine serum albumin (1 mg/ml), 5 mM EDTA, and 0.1% (v/v) β-mercaptoethanol. The 37-kD TC-PTPase has a COOH-terminal truncation created by site-directed mutagenesis [D. E. Cool, N. K. Tonks, E. G. Krebs, E. H. Fischer, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7280 (1990)]. After the indicated incubation periods, immunoprecipitates were washed with homogenization buffer plus 0.5% Triton X-100 and resuspended in the same buffer. PLC activity was measured with Ptd[^{3}H]Ins 4,5-P_2 as the substrate. For phosphoamino acid analysis of PLC-γ1, A-431 cell proteins were metabolically labeled by incubation of the cells with [^{32}P]orthophosphate (3 mCi/ml) for 3 hours at 37°C followed

by treatment with EGF (300 ng/ml, 10 min). Immunoprecipitated [^{32}P]PLC-γ1 was incubated with phosphatases for 90 min under conditions used for the experiments shown in Fig. 3A. PLC-γ1 was separated by SDS-PAGE and labeled proteins were transferred to Immobilon P. After visualization by autoradiography, phosphoamino acid analysis was performed on the bands that corresponded to PLC-γ1.
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Kinetics of Gramicidin Channel Formation in Lipid Bilayers: Transmembrane Monomer Association

ANNE M. O'CONNELL, ROGER E. KOEPPE II, OLAF S. ANDERSEN

Conducting gramicidin channels form predominantly by the transmembrane association of monomers, one from each side of a lipid bilayer. In single-channel experiments in planar bilayers the two gramicidin analogs, [Val^1]gramicidin A (gA) and [4,4,4-F$_3$-Val^1]gramicidin A (F$_3$gA), form dimeric channels that are structurally equivalent and have characteristically different conductances. When these gramicidins were added asymmetrically, one to each side of a preformed bilayer, the predominant channel type was the hybrid channel, formed between two chemically dissimilar monomers. These channels formed by the association of monomers residing in each half of the membrane. These results also indicate that the hydrophobic gramicidins are surprisingly membrane impermeant, a conclusion that was confirmed in experiments in which gA was added asymmetrically and symmetrically to preformed bilayers.

GRAMICIDIN CHANNELS ARE MEMbrane-spanning structures that serve as prototypical models for studying mechanisms of ion permeation, lipid-protein interactions, and conformational dynamics of ion-permeable channels (*1*). The primary sequence of gA is (*2*):

Formyl-L-Val–Gly-L-Ala-D-Leu-L-Ala-
D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-
D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine

The mechanism of gramicidin channel insertion into lipid bilayers is poorly understood because the membrane-spanning channels are formyl-NH-to-formyl-NH terminal dimers of β$^{6.3}$-helices, as suggested by Urry (*3*), whereas gramicidins dissolved in organic solvents exist as mixtures of parallel and antiparallel intertwined dimers and disordered monomers (*4*–*6*), depending upon solvent type and gramicidin concentration. Gramicidins therefore undergo conformational changes in their transition from dissolved molecules to molecules dispersed in water (*7*) and finally to membrane-spanning channels. Furthermore, as membrane-associated gramicidins exist in a number of (slowly interconverting) conformational states (*8*), it is not known whether the major pathway for channel formation involves the membrane insertion of (antiparallel) intertwined dimers with a subsequent formation of the β$^{6.3}$-helical dimer (*9*) or the transmembrane association of β-helical monomers residing in opposite monolayers (Fig. 1).

We addressed the channel formation problem by exploiting the following features of gramicidin channels: (i) gramicidin channels are symmetrical dimers (*10*), with a single predominant conductance state (*11*); (ii) sequence-substituted gramicidins form symmetrical channels (or homodimers) that have different conductances (*12*); and (iii) hybrid channels (or heterodimers) form between the chemically dissimilar analogs (*12, 13*). One can thus use the amplitude of individual channel events in a real-time assay to identify which molecules form each conducting event.

In 1.0 M CsCl, the conductances of gA and F$_3$gA channels differ threefold because of the -CF$_3$ substitution in F$_3$gA (*12*). When either gramicidin is added symmetrically to the aqueous solutions on both sides of a bilayer, a single characteristic channel type is observed (Fig. 2, A and B). When both gramicidins are added symmetrically, three channel types are observed (Fig. 2C), corresponding to the two homodimers and the heterodimer (*12*).

When gA and F$_3$gA are added asymmetrically to opposite sides of a preformed bilayer, all three channel types are again observed (Fig. 3, A through C). However, when the channel appearance rates are plotted versus time, the heterodimers dominate throughout all but the first few minutes (Fig. 3D). The homodimer appearance rates remain stable and may decrease slightly during the first few minutes. The dominance of heterodimers shows that under our conditions (6 to 15 pM gramicidin dispersed in water from a 15 nM solution in ethanol) most gramicidin channels result from the transmembrane association of β$^{6.3}$-helical monomers as outlined in Fig. 1A.

After 20 min, the membranes were broken and reformed in order to equilibrate both gramicidins between the monolayers. A large change in the channel appearance pattern occurred (Fig. 3C): the homodimer appearance rates increased, and the heterodimer appearance rate decreased (*15*).

Comparable results were obtained when only gA was added to one side, or to both sides, of preformed bilayers (Fig. 4). In these experiments, the gramicidin concen-

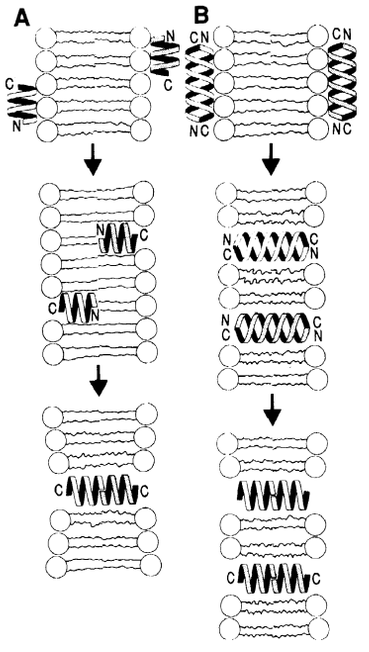

Fig. 1. Schematic representation of two gramicidin channel insertion mechanisms. (**A**) Dimerization of β$^{6.3}$-helical monomers (from each side of the membrane). Gramicidin monomers adsorb to each monolayer from the immediately adjacent aqueous solution and fold into β$^{6.3}$-helices (top), folded monomers insert into each monolayer (middle), and dimerize to form the channel (bottom). (**B**) Insertion of intertwined antiparallel dimers followed by unwinding to form β$^{6.3}$-helical dimers. Intertwined dimers adsorb to each monolayer (top), insert to span the membrane (middle), and unwind to form the channels (bottom).

A. M. O'Connell and O. S. Andersen, Department of Physiology and Biophysics, Cornell University Medical College, New York, NY 10021.
R. E. Koeppe II, Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701.