Membrane-permeable Calmodulin Inhibitors (e.g. W-7/W-13) Bind to Membranes, Changing the Electrostatic Surface Potential

**DUAL EFFECT OF W-13 ON EPIDERMAL GROWTH FACTOR RECEPTOR ACTIVATION**

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Membrane-permeable calmodulin inhibitors, such as the naphthalenesulfonamide derivatives W-7/W-13, trifluoperazine, and calmidazolium, are used widely to investigate the role of calcium/calmodulin (Ca²⁺/CaM) in living cells. If two chemically different inhibitors (e.g. W-7 and trifluoperazine) produce similar effects, investigators often assume the effects are due to CaM inhibition. Zeta potential measurements, however, show that these amphipathic weak bases bind to phospholipid vesicles at the same concentrations as they inhibit Ca²⁺/CaM; this suggests that they also bind to the inner leaflet of the plasma membrane, reducing its negative electrostatic surface potential. This change will cause electrostatically bound clusters of basic residues on peripheral (e.g. Src and K-Ras4B) and integral (e.g. epidermal growth factor receptor (EGFR)) proteins to translocate from the membrane to the cytoplasm. We measured inhibitor-mediated translocation of a simple basic peptide corresponding to the calmodulin-binding juxtamembrane region of the EGFR on model membranes; W-7/W-13 causes translocation of this peptide from membrane to solution, suggesting that caution must be exercised when interpreting the results obtained with these inhibitors in living cells. We present evidence that they exert dual effects on autoprophosphorylation of EGFR; W-13 inhibits epidermal growth factor-dependent EGFR autophosphorylation under different experimental conditions, but in the absence of epidermal growth factor, W-13 stimulates autophosphorylation of the receptor in four different cell types. Our interpretation is that the former effect is due to W-13 inhibition of Ca²⁺/CaM, but the latter results could be due to binding of W-13 to the plasma membrane.

The ubiquitous second messenger Ca²⁺ (1, 2) exerts many of its signaling effects by binding to calmodulin (CaM); Ca²⁺/calmodulin (Ca²⁺/CaM) in turn binds to and modulates the function of >100 target proteins (3–6). Many investigators have used membrane-permeable calmodulin inhibitors (e.g. trifluoperazine (TFP), calmidazolium (CDZ), and the naphthalenesulfonamide derivatives W-7/W-13) to sort out the multiple potential roles of calmodulin in living cells; a PubMed search for W-7 alone turns up 1,700 publications. Frequently, investigators reason that if two or more chemically distinct inhibitors (e.g. W-7 and TFP) produce similar effects on a cell, they can assume that the effects are due to specific inhibition of calmodulin. If, however, these amphipathic weak bases bind to Ca²⁺/CaM mainly through nonspecific hydrophobic and electrostatic interactions, they also will bind to the inner leaflet of the plasma membrane at about the same concentration at which they inhibit calmodulin. This binding reduces the net negative charge on the inner leaflet, which is due mainly to the monovalent acidic lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (PS). Decreasing the net negative charge on the inner leaflet of the plasma membrane can have several biological effects. For example, it could reduce the electrostatically mediated (7–10) membrane binding of clusters of basic residues on numerous peripheral (e.g. K-Ras4B, Src, myristoylated alanine-rich C kinase substrate (MARCKS), and gravin) and integral membrane proteins.**

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proteins (e.g. receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR)).

We investigated whether commonly used membrane-permeable calmodulin inhibitors bind to phospholipid vesicles formed with the same mole fraction of PS present in the inner leaflet of a typical mammalian plasma membrane. They do. For example, ~10 μM W-7 or W-13 reduces the negative zeta potential of a phospholipid vesicle significantly and induces membrane-bound basic peptide translocation from membrane to solution, as determined by both centrifugation and fluorescence correlation spectroscopy (FCS) measurements; the same inhibitor concentration binds to and inhibits Ca²⁺/CaM.

Our results suggest that these inhibitors bind both to Ca²⁺/CaM and to membranes in cells. We investigated the dual effects of Ca²⁺/CaM inhibitors on an important cellular process, EGFR activation. Exposing cells to EGF produces receptor dimerization (11) and a transient increase in the cytoplasmic free concentration of Ca²⁺, [Ca²⁺], and consequently in Ca²⁺/CaM (12–17). We hypothesize that this transient increase in Ca²⁺/CaM could be important in EGFR activation, because Ca²⁺/CaM binds both to the EGFR (18–20) and to peptides corresponding to its cytoplasmic juxtamembrane (JM; residues 645–660) region with a sufficiently high affinity (Kd ~ 10 nm for the peptide) (21) to suggest that this interaction could be biologically relevant. A recent report provides important evidence that ligand-mediated activation of EGFR involves dimer formation between two kinase domains, which stimulates rearrangement of the activation loop within the kinase domain by an allosteric mechanism (22–24). Ca²⁺/CaM, for example, could facilitate formation of the kinase domain dimers by enhancing desorption of the JM plus kinase domains from the cytoplasmic leaflet (7, 25). Previous published (20, 21) and unpublished work showed that treating cells with appropriate concentrations of W-7, W-13, and the less potent W-12 inhibits the initial transient phase of EGF-stimulated intermolecular EGFR autophosphorylation (hereafter referred to as autophosphorylation) in five different cell types. We report new experiments that provide additional evidence that W-13 inhibits this phase of EGF-mediated autophosphorylation by inhibiting Ca²⁺/CaM in cells exposed to a calcium ionophore. These results support the hypothesis that Ca²⁺/CaM is involved in the initial phase of ligand-mediated EGFR activation in several cell types. Introducing a mutation that impedes Ca²⁺/CaM binding to the EGFR JM domain abrogates W-13-mediated inhibition of EGFR autophosphorylation, which suggests that Ca²⁺/CaM interacts directly with the EGFR.

W-7 and W-13, however, exert an opposing effect that appears not to be restricted to their ability to inhibit Ca²⁺/CaM; measurements on four different cell types show that W-13 stimulates autophosphorylation of EGFR in the absence of EGF, confirming and extending earlier work (21, 26, 27). We discuss how the membrane binding of W-7 and W-13 could produce this effect.

**EXPERIMENTAL PROCEDURES**

**Electrostatic Potentials Adjacent to Membranes and Determination of the Zeta Potential**—Fixed negative charges on the inner leaflet of a typical mammalian plasma membrane are due mainly to the presence of monovalent acidic lipids, such as PS, which accounts for 20–35% of these phospholipids (28). Most lipid species are neutral (e.g. cholesterol) or zwitterionic (e.g. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (PC) and phosphatidylethanolamine); positive lipids are rare or absent. The fixed charges on PS (red circles at left in Fig. 1A) attract ions of opposite charge, K⁺ (blue circles), from the aqueous phase; these counterions form an ion atmosphere, as shown in Fig. 1A. Helmholtz in 1879 (29) first recognized that the fixed charges and counterions “form an electrical double layer... that has an extraordinarily small, but not disappearing thickness”; double layer theory is discussed in detail in an excellent modern physical chemical text (30) and reviews of membrane electrostatics (e.g. Refs. 31 and 32 and references therein). The essential features are summarized very briefly below and in Fig. 1.

The thickness of the ion atmosphere adjacent to the inner leaflet is about 1 nm = 10 Å. The counterions do not stick to the membrane surface for the same reason the gas molecules in the earth’s atmosphere do not fall to the ground; they diffuse away from a region of high concentration. Gouy and Chapman used the Poisson equation to describe the electrostatic attraction of the counterions for the charged surface and the Boltzmann equation to describe the statistical distribution of the counter- and co-ions in the aqueous diffuse double layer; Fig. 1, B and C, illustrates two key predictions of the Gouy-Chapman theory.

When the surface potential, ψ(0), is small, it is proportional to both the surface charge density, σ (number of fixed charges per unit area of surface), and the Debye length,

$$\psi(0) = \sigma / \varepsilon_r \varepsilon_0 \kappa$$

(Eq. 1)

where $\varepsilon_r$ is the dielectric constant or relative permittivity (~80), $\varepsilon_0$ is the permittivity of free space, and 1/κ is the Debye length (~1 nm for 0.1 M salt; ~10 nm for 0.001 M salt). Note that this limiting form of the Gouy equation also describes the potential difference between two capacitor plates with charge density $\sigma$ separated by a distance 1/κ (see Fig. 1C). Equation 1 and the simple capacitor model illustrate the following two important features of double layer theory. The surface potential increases with increasing density of fixed charge (i.e. fraction of acidic lipids increases) and/or Debye length (i.e. salt concentration decreases). Fig. 1B shows the dependence of potential on distance. When the potential in the aqueous phase is small, it falls with distance $x$ from the surface according to Equation 2.

$$\psi(x) = \psi(0) \exp(-\kappa x)$$

(Eq. 2)

Numerous experimental studies have shown that this theory provides a surprisingly accurate description of the potential adjacent to a phospholipid bilayer membrane (32).

The Helmholtz-Smoluchowski equation gives the relationship between the measured electrophoretic mobility, $\mu$ (velocity of a multilamellar vesicle in a unit electric field), and the zeta potential, $\zeta$.

$$\zeta = \mu \eta / (\varepsilon_r \varepsilon_0)$$

(Eq. 3)

where $\eta$ is the viscosity of the aqueous solution, $\varepsilon_r$ is the dielectric constant (~80) and $\varepsilon_0$ is the permittivity of free space. By definition, $\zeta$ is the potential at the hydrodynamic plane of shear,
which experiments suggest is located about 0.2 nm from the surface (see Fig. 1B) in a 100 mM salt solution (32). The experimental aspects of determining the electrophoretic mobility are considered in detail elsewhere (33). For the experiments shown in Figs. 3 and 4, we added the inhibitor in excess concentration relative to the lipids ([lipid] < 1 μM), so the binding of inhibitor to the vesicles does not significantly decrease its free concentration in solution.

Reagents—Fig. 2 shows the chemical structures of five commonly used membrane-permeable calmodulin inhibitors. The naphthalenesulfonamide derivatives (W-7, W-12, and W-13), trifluoperazine (TFP), and calmidazolium (CDZ) are all amphipathic weak bases. (As discussed in the supplemental materials, the neutral form of these weak bases equilibrates between the bathing solution and cytoplasm.) W-7, W-13, and W-12 were purchased from both Sigma and Calbiochem. Similar results (zeta potential and peptide binding to large unilamellar vesicles (LUVs)) were obtained with both samples. CDZ, TFP, and rhodamine green were purchased from Sigma. The phospholipids PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (PG) and PS were from Avanti Polar Lipids (Alabaster, AL). Peptides corresponding to the JM region of EGFR (EGFR-(645–660)), with and without a Cys residue at the N terminus, were purchased from American Peptide Co. (Sunnyvale, CA). Alexa-488 maleimide was purchased from Invitrogen. Radioactively labeled [dioleoyl-1-14C]L-[H9251]-dioleoylphosphatidylcholine and [ethyl-1,2-3H]-N-ethylmaleimide were from PerkinElmer Life Sciences.

Cell culture media and sera were purchased from Invitrogen or Biological Industries (Beit Haemek, Israel). Mouse monoclonal anti-phosphotyrosine RC20 antibody conjugated to horseradish peroxidase was from Transduction Laboratories/BD Biosciences. A23187, ionomycin, and KN-93 were from Calbiochem. GF109203X and Fast Green FCF were from Sigma. The enhanced chemiluminescent Luminol (ECL™) reagents were from Amersham Biosciences. Polyvinylidene difluoride membranes were from Pall Gelman Laboratory (Ann Arbor, MI), and Immobilon-P filters were from Millipore Corp. (Billerica, MA).

Vesicle Preparation—We used 100-nm diameter LUVs for the FCS and centrifugation-binding experiments, as described in detail elsewhere (34). Briefly, we added a mixture of solutions of PC and PS in chloroform to a 50-ml round-bottom flask, which was then immersed in a 30–35 °C water bath and attached to a rotary evaporator. The flask was rotated without vacuum for 5 min to warm the flask and solution. We then evaporated most of the solvent rapidly by applying the maximum vacuum that does not boil the chloroform. The flask was kept under full vacuum for 30 min to remove all traces of chloroform. A solution containing 100 mM KCl, 10 mM MOPS, pH 7.0) was added to form multilamellar vesicles (MLVs), followed by five cycles of rapid freezing and thawing. We formed LUVs by extruding the MLVs through 100-nm diameter polycarbonate filters. A solution containing 176 mM sucrose, 10 mM MOPS, pH 7.0, was used to obtain sucrose-loaded LUVs for centrifugation binding measurements. The solution bathing the LUVs was then exchanged for 100 mM KCl, 10 mM MOPS, pH 7.) We used MLVs, which typically had a

![FIGURE 1. The electrostatic potential adjacent to the inner leaflet of a plasma membrane or a phospholipid bilayer. A, schematic diagram of a surface with fixed negative charges (e.g. PS (red circles on surface)) that attract ions of opposite charge (e.g. K⁺ (blue circles in solution)); this forms an ionic atmosphere or “diffuse double layer” in the adjacent aqueous phase. B, the potential adjacent to the surface falls approximately exponentially with distance. The space constant is the Debye length, 1/k, which is about 10 Å in a 100 mM salt solution. The potential is drawn according to the Gouy-Chapman theory for a surface with a charge density of one electronic charge per 300 Å². Experiments show that the zeta potential, ζ, of a phospholipid vesicle is the potential 2 Å from the surface, as indicated in the diagram. C, capacitor model of the diffuse double layer. If all of the counterions are located a Debye length from the surface, the fixed charges on the membranes and the counterions in the aqueous phase constitute a parallel plate capacitor, which is a good model of the double layer. Note that decreasing the net number of charges per unit area on the membrane by 50% (e.g. by adsorption of a positively charged weak base, such as W-7) also decreases the potential at the surface by 50%.](image-url)
diameter of 10–20 μm for electrophoretic mobility/zenet potential measurements.

Peptide Labeling and Purification—Labeled or unlabeled peptides used for experiments were determined to be >95% pure by HPLC and MALDI-TOF mass spectroscopy. We used a protocol modified from Ref. 36 to label the peptides with the thiol-reactive fluorescent probes. In brief, we mixed 1 ml of 1 mM peptide in 10 mM K2HPO4/KH2PO4, pH 7.0, with the probe dissolved in N,N-dimethyl formamide (probe/peptide molar ratio 1:1) for 1 h. We purified the labeled peptide using HPLC and checked that it has the correct molecular weight using MALDI-TOF mass spectrometry (Proteomics Center, State University of New York at Stony Brook). We labeled the percentage of peptide bound by measuring the radioactivity of the peptide in the supernatant and in the pellet.

Peptide Labeling and Purification—N7XHERc fibroblasts, a stable transfected cell line expressing the wild type human EGFR, was donated by Axel Ullrich from the Max-Planck-Institut für Biochemie (Martinsried, Germany), and R11 fibroblasts, a stable transfected cell line expressing a human EGFR mutant with an insertion of a highly acidic 23-amino acid sequence between Arg447 and His448, which divides the CaM-binding domain into two segments (42), was a kind gift of Andrey Sorokin from the Medical College of Wisconsin (Milwaukee, WI). N7XHERc, N7XHERc/654A, R11, EGFR-T17 (stably transfected mouse fibroblasts overexpressing human EGFR), and green monkey kidney COS-1 cells (expressing 4 × 105 EGFR/cell) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 5 mM pyruvate, 2 mM l-glutamine, and 40 μg/ml gentamicin in a humidified atmosphere of 5% (v/v) CO2 at 37°C. Mouse NIH3T3 (clone WT8) fibroblasts (expressing 4 × 105 EGFRs/cell) (43) were grown as above except that 10% (v/v) donor calf serum was used. Porcine aortic endothelial (PAE/EGFR-GFP) cells, a stable transfected cell line expressing a human EGFR-green fluorescent protein (GFP) chimera (2 × 105 EGFR-GFP/cell) (44), were grown in F-12 medium with the supplements indicated above. Cells were counted using a Neubauer’s chamber after detachment from the culture dishes and seeded in 6-well plates (1.4 × 105 cells/well) in the media indicated above and grown to 90% confluence for 24 h. Thereafter, the cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH2PO4, and 10 mM Na2HPO4 at pH 7.4) and maintained overnight in a serum-free medium before performing the experiments.

FCS measures the residence time, τD, of molecules diffusing through a small, optically defined, open probe volume using a digital temporal correlator to compute the autocorrelation function G(τ), the measured variable for FCS experiments, as described in the supplemental materials. Fig. 6, C and D, shows the fluorescence time traces and the autocorrelation functions calculated from those time traces, respectively, using the same color coding. We can extract the characteristic residence time, τD, of a molecule (i.e., the average time the molecule takes to diffuse in and out of the ~300-nm diameter probe volume) by fitting the autocorrelation functions to the appropriate equation. Because the LUV-bound peptides diffuse more slowly than free peptides, the autocorrelation function decays on a longer time scale, and the correlation time is ~25-fold longer. It is easy to distinguish the bound and free species when τD(bound) is >τD(ung). The residence time calculation principle is similar to the FCS principle of FCS and how this technique can be used to measure the binding of fluorescently labeled EGF-R (~450–660 nm) to negatively charged phospholipid vesicles (35). Fig. 6B shows a schematic diagram of free and LUV-bound peptides undergoing Brownian motion in the plane of the medium. The fluorescence signal from this probe volume fluctuates as the molecules diffuse in and out of it. This fluorescence fluctuation is recorded with an avalanche photodiode detector with good temporal resolution and then analyzed by a digital temporal correlator to compute the autocorrelation function G(τ), the measured variable for FCS experiments, as described in the supplemental materials. Fig. 6, C and D, shows the fluorescence time traces and the autocorrelation functions calculated from those time traces, respectively, using the same color coding. We can extract the characteristic residence time, τD, of a molecule (i.e., the average time the molecule takes to diffuse in and out of the ~300-nm diameter probe volume) by fitting the autocorrelation functions to the appropriate equation. Because the LUV-bound peptides diffuse more slowly than free peptides, the autocorrelation function decays on a longer time scale, and the correlation time is ~25-fold longer. It is easy to distinguish the bound and free species when τD(bound) is >τD(ung).
containing 2 ml of medium. Depending on the experiment (see legends to Figs. 7 and S1), the medium also contained a calcium/calmodulin-activated kinase II inhibitor, a protein kinase C inhibitor, the CaM inhibitor W-13, and a Ca$^{2+}$ ionophore. We added 1 ml of ice-cold 30% trichloroacetic acid to stop the reaction and prepared a total cell lysate using Laemmli sample buffer, boiling the sample for 5 min as described previously (20). We measured EGFR-independent EGFR autophosphorylation after adding W-13 to serum-starved cells (see legend to Fig. 8), preparing the cell lysate as described previously (26).

Electrophoresis and Western Blot Analysis—Slab gel linear gradient (5–20% (w/v) polyacrylamide and 0.1% (w/v) SDS at pH 8.3) electrophoresis was performed according to Laemmli (45) at 12 mA overnight. Alternatively, we used 8% (w/v) polyacrylamide gels. Proteins were electrotransferred from the gel to a polyvinylidene difluoride/Immobilon-P membrane for 2–3 h at 300 mA in a medium containing 48 mM Tris-base, 36.6 mM glycine, 0.04% (w/v) SDS, and 20% (v/v) methanol; fixed with 0.2% glutaraldehyde in 25 mM Tris-HCl (pH 8), 150 mM NaCl, and 2.7 mM KCl (TNK buffer) for 45 min; and transiently stained with Fast Green FCF to ascertain the regularity of the transfer procedure. The membrane was blocked with 5% (w/v) bovine serum albumin in 0.1% (w/v) Tween 20, 100 mM Tris-HCl (pH 8.8), 500 mM NaCl, and 0.25 mM KCl at pH 8.8 (T-TBS medium) and probed overnight with a $1:5000$ dilution of the anti-phosphotyrosine antibody conjugated to horseradish peroxidase (RC20). The phosphotyrosine-containing 170-kDa EGFR band and 205-kDa EGFR-GFP bands were visualized following development with ECL reagents according to the manufacturer’s instructions and exposure of x-ray films for appropriate periods of time.

When required, the intensity of the phosphorylated EGFR band was quantified with a computer-assisted scanning densitometer using the NIH Image 1.60 program. Corrections were made for small changes in the amount of protein present in the electrophoretic tracks as detected by Fast Green FCF staining followed by densitometric reading. To avoid any exposure time differences between gels loaded with samples corresponding to experiments performed in parallel, we exposed a single film to the different polyvinylidene difluoride membranes. Protein concentration was determined by the method of Lowry (46) using bovine serum albumin as a standard.

RESULTS

W-7/W-13/W-12 Bind Strongly to Phospholipid Membranes, Reducing the Magnitude of the Negative Zeta Potential—Fig. 1 shows how the acidic lipid PS produces a negative electrostatic potential in the aqueous phase adjacent to a phospholipid bilayer. The zeta potential, $\zeta$, is the electrostatic potential close to the surface of the membrane, as shown in Fig. 1B; $\zeta = -45$ mV (left points in Fig. 3A) for the 2:1 PC/PS vesicles used for these measurements. Fig. 3A shows the effect of exposing the vesicles to W-7, W-13, or W-12. Adding 10–100 $\mu$M W-7/W-13 (the concentration range typically used in cell biology experiments) markedly reduces the magnitude of the zeta potential. For example, 10 or 30 $\mu$M W-7/W-13 reduces $\zeta$ by $\sim 15$ or $\sim 20$ mV, respectively. W-12, which is about 10-fold less potent as a CaM inhibitor (47, 48), decreases the magnitude of the zeta potential about 10-fold less effectively.

Fig. 3B illustrates the principle of the measurements; the schematic diagram shows an MLV moving in an electric field, with the length of the arrow indicating the velocity. We calculate $\zeta$ from the measured velocity/field, $\mu$, using Equation 3. Fig. 3C shows that positively charged amphipathic weak bases, such as W-7, bind to the membrane surface and reduce its net negative charge. This decreases the magnitude of $\zeta$, slowing the vesicle’s velocity measured in a unit field; Equation 1 and the simple capacitor model of a diffuse double layer shown in Fig. 1C illustrate this effect. For example, adding $30 \mu$M W-7/W-13 decreases the mobility/zeta potential $\sim 50\%$ (Fig. 3A); this implies a 50% reduction in the charge density on the surface of the vesicle or half as many positively charged W-7 adsorbed to the membrane as negatively charged PS in the outer leaflet. We note in passing that the velocity of the MLVs in an electric field is independent of their radius and depends only on the charge density on the outer leaflet, so only the inhibitors bound to this leaflet are illustrated in Fig. 3C. The neutral form of the weak base, B, crosses the membrane rapidly and recombines with a proton to form HB$^+$, which also binds to the inner leaflet of the MLV, or the inner leaflet of the plasma membrane in a cell.

TFP and CDZ Also Bind to Phospholipid Membranes—TFP binds to 2:1 PC/PS vesicles with $\sim 3$-fold higher affinity than W-7/W-13, as shown by comparing Figs. 4 and 3 (e.g., adding 30 $\mu$M TFP or 100 $\mu$M W-7/W-13 produces a similar effect on $\zeta$). Note that TFP, like W-7 (data not shown), binds with equal affinity to 2:1 PC/PS and PC/PG vesicles (black and white diamonds in Fig. 4). This suggests that the acidic lipids PS and PG enhance the binding of TFP/W-7 to membranes through non-specific electrostatic effects rather than through specific chemical interactions. Electrophoretic mobility experiments allow us to determine the relative importance of the electrostatic and hydrophobic components of the binding. We measured the binding of TFP to electrically neutral PC vesicles, which do not move in an electric field (i.e., $\zeta = 0$ mV) (Fig. 4, gray diamond on the left); exposing the PC vesicles to TFP produces a positive zeta potential (gray diamonds in Fig. 4). Gouy-Chapman-Stern theory (32) can describe quantitatively the hydrophobic binding of amphipathic weak acids or bases to PC vesicles (see Fig. 6 in Ref. 49) and their effect on the zeta potential. The only adjustable parameter is the molar partition coefficient, $K$, of TFP onto the surface of PC membranes ($K = 10^4$ M$^{-1}$; see Ref. 50 for a detailed theoretical description of TFP binding to PC). This is equivalent to assuming $K_f = 10^{-4}$ M for TFP binding to an individual lipid or that TFP partitions hydrophobically with an energy of $\sim 6$ kcal/mol onto the surface. Earlier work (50) showed an apparent $K_f = 10^{-3}$ M described the effect of TFP on the zeta potential of the membranes containing 30% acidic lipid; the 10-fold increase in affinity for these negatively charged membranes is due to the Boltzmann effect illustrated in Fig. 1. In other words, nonspecific electrostatics concentrates the monovalent cationic TFP by 10-fold adjacent to the surface of the PC/PS membranes, where TFP then absorbs hydrophobically. Thus, the overall binding energy to 2:1 PC/PS vesicles, 7.5 kcal/mol, comprises contributions from both nonspecific electrostatic (1.5 kcal/mol) and hydrophobic (6

Calmodulin Inhibitors Bind Membranes

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importantly, TFP binds to 2:1 PC/PS membranes and Ca\(^{2+}\)/H\(_{11001}\)/CaM with about the same affinity: K\(_{d}\) of 10\(^{-9}\) M (Fig. 4) and 5\(^{-10}\) M (51, 52), respectively. CDZ binds to membranes with such high affinity that technical limitations preclude accurate zeta potential measurements for [CDZ] \(< 1 \mu M\). Adding 1 \(\mu M\) CDZ reverses the charge on a 2:1 PC/PS vesicle, changing \(\zeta\) from -45 to +17 \pm 2 (\(n = 8\)) mV; 2 \(\mu M\) CDZ changes the zeta potential to +19 \pm 1.5 (\(n = 16\)) mV, as shown in Fig. 4 (triangles). Note that 1 \(\mu M\) CDZ exerts a larger effect on the zeta potential of 2:1 PC/PS vesicles than 100 \(\mu M\) TFP (Fig. 4). Application of the Gouy-Chapman-Stern theory (32, 49) to these data shows that the affinity of CDZ for these membranes is 100-fold stronger than that of W-7/W-13/TFP, an observation that is qualitatively consistent with the former’s 1000-fold higher affinity for Ca\(^{2+}\)/CaM (51).

Calmodulin Inhibitors Also Reduce Binding of Basic Peptides to Membranes—if 30 \(\mu M\) W-7 decreases the magnitude of the potential at the surface of the plasma membrane by ~20 mV, as it does for 2:1 PC/PS membranes (Fig. 3A), the Boltzmann relation predicts that it should greatly decrease the electrostatic binding of basic clusters on numerous peripheral and intrinsic kcal/mol) interactions. Importantly, TFP binds to 2:1 PC/PS membranes and Ca\(^{2+}\)/CaM with about the same affinity: K\(_{d}\) of ~10 \(\mu M\) (Fig. 4) and ~5 \(\mu M\) (51, 52), respectively. CDZ binds to membranes with such high affinity that technical limitations preclude accurate zeta potential measurements for [CDZ] < 1 \(\mu M\). Adding 1 \(\mu M\) CDZ reverses the charge on a 2:1 PC/PS vesicle, changing \(\zeta\) from -45 to +17 \pm 2 (\(n = 8\)) mV; 2 \(\mu M\) CDZ changes the zeta potential to +19 \pm 1.5 (\(n = 16\)) mV, as shown in Fig. 4 (triangles). Note that 1 \(\mu M\) CDZ exerts a larger effect on the zeta potential of 2:1 PC/PS vesicles than 100 \(\mu M\) TFP (Fig. 4). Application of the Gouy-Chapman-Stern theory (32, 49) to these data shows that the affinity of CDZ for these membranes is >100-fold stronger than that of W-7/W-13/TFP, an observation that is qualitatively consistent with the former’s ~1000-fold higher affinity for Ca\(^{2+}\)/CaM (51).

Calmodulin Inhibitors Bind Membranes—If 30 \(\mu M\) W-7 decreases the magnitude of the potential at the surface of the plasma membrane by ~20 mV, as it does for 2:1 PC/PS membranes (Fig. 3A), the Boltzmann relation predicts that it should greatly decrease the electrostatic binding of basic clusters on numerous peripheral and intrinsic
membrane proteins (e.g. Src, K-Ras4B, MARCKS, gravin, and the cytoplasmic JM region of the EGFR, respectively). We illustrate this effect by measuring the effect of W-7 on the binding of EGFR-(645–660), a +8 valent basic/hydrophobic peptide (sequence RRHIVVRKKTLRLLQ) to 2:1 PC/PS membranes. This peptide corresponds to the putative Ca\(^{2+}\)/CaM and membrane-binding JM region of the EGFR.

We first analyze the expected result qualitatively, assuming (incorrectly) that EGFR-(645–660) is a point charge of valence \(z = +8\) that does not perturb the potential. The Boltzmann relation predicts that the \(\Delta \psi = 20\) mV decrease in the magnitude of the surface potential by 30 \(\mu M\) W-7/W-13 should decrease in the local concentration of the peptide at the membrane solution interface \(>100\)-fold (\(\exp(-z\epsilon \Delta \psi/kT) = 10^{10^{3}}\)) and thus decrease the binding \(>100\)-fold. Measurements of peptide binding versus lipid concentration do indeed show that 30 \(\mu M\) W-7/W-13 decreases the molar partition coefficient (reciprocal of the lipid concentration required to bind 50% of the peptide) of EGFR-(645–660) 100-fold, from \(10^{6}\) to \(10^{4}\) M\(^{-1}\) (data not shown).

Fig. 5 shows how the membrane binding of EGFR-(645–660) depends on the concentration of W-7/W-13/W-12 for a lipid concentration that initially binds most of the peptide. Note that 10 \(\mu M\) W-7/W-13 decreases the binding markedly; only \(\sim 20\%\) of the peptide remains bound to the vesicles. (As expected, 10 \(\mu M\) W-7 exerted a similarly large effect on the membrane binding of a different basic/hydrophobic peptide corresponding to the effector domain of MARCKS; data not shown.) W-12 decreases the binding of the peptide \(\sim 10\)-fold less effectively than W-7/W-13, consistent with its smaller effect on the zeta potential (Fig. 3A). We confirmed the results of experiments shown in Fig. 5, which used a centrifugation technique to separate free radioactively labeled peptide from that bound to LUVs, by performing FCS experiments with a fluorescently labeled peptide; the results, shown in Fig. 6, are essentially the same.

Fig. 6A shows how W-7/W-13/W-12 affect membrane binding of Alexa 488 EGFR-(645–660); 10 \(\mu M\) W-7 or W-13 decreases the binding markedly, from \(\sim 90\) to \(\sim 15\%\); W-12 is \(\sim 10\)-fold less effective. Fig. 6B illustrates the principle of these FCS binding measurements; Rusu et al. (35) discuss the biophysical aspects of these measurements in more detail. The schematic diagram depicts peptides as gray rectangles with orange circles and LUVs as black donuts. The free peptides diffuse through the optically defined observation volume (green, diameter \(\sim 0.3\) \(\mu m\)) of the FCS instrument more rapidly than peptides bound to the relatively large LUVs. This diffusion produces temporal fluctuations in the fluorescence signal, evident in the time traces (Fig. 6C). The temporal autocorrelation functions, \(G(t)\) values (Fig. 6D), are calculated from these time traces. Fitting the autocorrelation functions to a two-component diffusion equation (see Equation 6 in supplemental materials) yields the percentage of peptide bound to the LUVs. The experiments show that W-13 alters EGFR-(645–660) binding to LUVs; 92% peptide is bound in 0 \(\mu M\) W-13 (red), 60% peptide is bound in 3 \(\mu M\) W-13 (green), and 2% peptide is bound in 30 \(\mu M\) W-13 (blue). Fig. 6A summarizes the FCS results from experiments performed with different concentrations of W-7, W-13, and W-12.

Summary of Results Obtained with Model Membranes and Predictions about Inhibitor Effects on Cells—The membrane-permeable CaM inhibitors illustrated in Fig. 2, which all bind to membranes through hydrophobic/electrostatic interactions at about the same concentrations that they bind to Ca\(^{2+}\)/CaM, are used widely in cell biology. Extrapolating the results on model membranes to the inner leaflet of the plasma membrane suggests that these inhibitors will reduce the magnitude of the negative electrostatic surface potential (Figs. 3 and 4) and increase the electrostatic binding of clusters of positively charged residues to the membrane (Figs. 5 and 6). Although one must obviously be cautious when interpreting cell biological experiments with CaM inhibitors, we do wish to suggest that they can provide useful information about cellular processes. For example, Ca\(^{2+}\)/CaM binds to the EGFR (18, 20) and to peptides corresponding to its JM region (19, 21, 53). Does this binding contribute to the activation of the receptor? EGFr-mediated dimerization of EGFR (11) produces a transient increase in the level of free [Ca\(^{2+}\)], (12–17), and thus Ca\(^{2+}\)/CaM; our working hypothesis is that this transient elevation in Ca\(^{2+}\)/CaM contributes to the observed transient pulse of EGFR autophosphorylation observed in many different cell types (20, 54, 55). The observation that calmodulin inhibitors suppress the initial transient pulse of EGFR autophosphorylation in several cell types is consistent with the hypothesis (20, 21).

To further test our hypothesis, we exposed cells to a calcium ionophore before adding EGF; the ionophore should increase the steady-state level of [Ca\(^{2+}\)], and thus Ca\(^{2+}\)/CaM. The hypothesis makes two obvious predictions about the effect of this treatment: (i) adding EGF should produce a consistently
high level of EGFR autophosphorylation, and (ii) W-13 should decrease autophosphorylation at all times after the addition of EGF.

Effect of W-13 on EGF-dependent Autophosphorylation of EGFR—The increase in \([\text{Ca}^{2+}/\text{H}^{100}]\), that results from exposing a cell to the \(\text{Ca}^{2+}\) ionophore A23187 will have many effects. For example, it will activate both protein kinase C and calcium/calmodulin-activated kinase II, both of which inhibit EGFR autophosphorylation (56–62); thus, we added inhibitors of these EGFR regulatory protein kinases before exposing cells to the \(\text{Ca}^{2+}\) ionophore. Fig. 7A illustrates the results from a control experiment in N7XHERc cells treated with KN-93 and GF109203X, inhibitors of calcium/calmodulin-activated kinase II and protein kinase C, respectively. In the absence of W-13 (open symbols), adding EGF produces rapid EGFR autophosphorylation that reaches a maximum after \(\sim\)2 min and then

![Graph showing the effect of W-13 on EGF-dependent autophosphorylation of EGFR.](image)

**FIGURE 6. FCS measurements of the effect of calmodulin inhibitors on the membrane binding of EGFR-(645–660).** A, binding of Alexa 488-labeled EGFR-(645–660) to 20 \(\mu\text{M}\) 1:1 PC/PS LUVs in presence of different concentrations of W-7 (open squares), W-13 (filled circles), and W-12 (open circles) measured using FCS. The peptide concentration is 2–4 nm in solutions containing 100 mM KCl, 1 mM MOPS buffer at pH 7. Each point on the graph is an average of more than six measurements ± S.D., when larger than the size of the symbol. Note that exposure of the vesicles to 10 \(\mu\text{M}\) W-7 or W-13 reduces EGFR-(645–660) binding from 90 to 15%. W-12 reduces binding about 10-fold less effectively. B, schematic diagram of the optically defined confocal open volume (green) of an FCS instrument. Fluorescent molecules (in our experiment, peptides with an Alexa 488 label (gray rods with orange circles) that are free or bound to LUVs (black donuts)) diffuse through this volume, producing temporal fluctuations in the fluorescence signal. C, fluorescence signal (arbitrary units) obtained from three different solutions containing 4 nm Alexa 488-EGFR-(645–660), 20 \(\mu\text{M}\) 1:1 PC/PS LUVs in different concentrations of W-13: 0 \(\mu\text{M}\) (red), 3 \(\mu\text{M}\) (green), and 30 \(\mu\text{M}\) (blue). The temporal autocorrelation function, \(G(\tau)\), is calculated from each of these time traces. D, normalized temporal autocorrelation functions, \(G(\tau)\), calculated from data presented in C and using the same color code. Fitting the autocorrelation functions (black lines) to a two-component diffusion equation (Equation 6) produces the percentage of peptide bound to LUVs; 92% peptide is bound in 0 \(\mu\text{M}\) W-13 (red), 60% in 3 \(\mu\text{M}\) W-13 (green), and 2% in 30 \(\mu\text{M}\) W-13 (blue). The data in A combine the results of similar measurements and analysis for different concentrations of W-7, W-13, and W-12.
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**FIGURE 7. Effect of W-13 (filled symbols) on EGF-dependent EGFR autophosphorylation in the absence (A) or presence (B) of a calcium ionophore, A23187.** A, serum-starved N7XHeRC cells were incubated for 1 h at 37 °C with 10 μM KN-93 (calcium/calmodulin-activated kinase II inhibitor), and then 10 μM GF109203X (protein kinase C inhibitor) was added, and the cells were maintained for an additional 1 h. Thereafter, the cells were incubated in the absence (open symbols) and presence (filled symbols) of 15 μg/ml (40 μM) W-13 for 30 min at room temperature (22 °C), followed by the addition of 10 nM EGF. The reaction was arrested with ice-cold 10% (w/v) trichloroacetic acid at the indicated times after EGF addition. B, serum-starved N7XHeRC cells were treated with 10 μM KN-93 plus 10 μM GF109203X as above and then incubated in the absence (open symbols) and presence (filled symbols) of 15 μg/ml (40 μM) W-13 for 15 min at room temperature (21–23 °C). We added 5 μg/ml (9.5 μM) A23187 (calcium ionophore) and incubated the cells for an additional 15 min before adding 10 nM EGF. The reaction was arrested at the indicated times after the addition of EGF as above. Samples in A and B were processed for SDS-PAGE and Western blot using an anti-phosphotyrosine antibody (RC20); densitometry of the 170-kDa phosphorylated EGFR band was performed as indicated under “Experimental Procedures.” The ordinates represent the mean ± S.D. of three (A) or two (B) independent experiments, relative to control experiments (100%) performed in the absence of both inhibitors and ionophore. Control cells were treated with 10 nM EGF for 1 min. Error bars are shown if larger than the symbol.

declines to a plateau after ~15 min. In the presence of W-13 (filled symbols), the initial pulse is absent, but the steady state value is unchanged, as expected from our hypothesis. These results reflect closely earlier studies on cells not treated with calcium/calmodulin-activated kinase II and protein kinase C inhibitors (see Fig. 4A of Ref. 20). The simplest interpretation of Fig. 7A is that W-13 inhibits Ca\(^{2+}\)/CaM, which is needed to produce the transient maximum in EGF-mediated EGFR autophosphorylation.

Fig. 7B shows results from experiments in cells treated with A23187 (plus the two kinase inhibitors) prior to the addition of EGF, which agree with the two predictions of our working hypothesis. First, the plateau or steady state level of EGFR autophosphorylation is higher (compare open symbols in Fig. 7, A and B). Second, adding W-13 reduces not only the initial phase of EGFR autophosphorylation but also the level observed after longer times (Fig. 7, compare A and B). This important result supports our hypothesis that Ca\(^{2+}\)/CaM enhances EGFR autophosphorylation mediated by EGFR, at least in this cell type. (We report additional control experiments with a peptide inhibitor of Ca\(^{2+}\)/CaM and experiments designed to test the hypothesis that Ca\(^{2+}\)/CaM acts directly on the EGFR in the supplemental materials.)

**W-13 Stimulates EGFR Phosphorylation in the Absence of EGF**—We previously reported W-13 and W-7 stimulate EGFR activation in COS-1 cells in the absence of EGF or other ligand (21, 27); we investigated whether this effect is more general by measuring EGFR tyrosine autophosphorylation after W-13 treatment in four different cell lines. Cultures of NIH3T3 (wt8), COS-1, and N7XHeRC cells expressing human EGFR or porcine aortic endothelial cells expressing a chimeric EGFR with GFP at its C terminus (PAE/EGFR-GFP) were starved overnight, treated with 10 μg/ml (~30 μM) W-13 for 10 min, and prepared as cell lysates for Western blot analysis with a monoclonal anti-phosphotyrosine antibody. W-13 treatment increased EGFR-phosphotyrosine levels in each cell type (Fig. 8). (The band at 170 kDa corresponds to the EGFR, and the band at 205 kDa corresponds to the chimeric receptor. PAE cells do not express detectable levels of endogenous EGFR). As discussed in more detail below, one explanation for these effects is that W-13 binds to the inner leaflet of the plasma membrane, reducing the magnitude of the negative electrostatic surface potential (e.g. Fig. 3), allowing the positively charged JM region (residues 645–660) of the EGFR to desorb from the membrane more easily (see Figs. 4 and 5) and thus mimicking the action of Ca\(^{2+}\)/CaM in EGF-stimulated cells.

**DISCUSSION**

Our most important results, shown in Fig. 7, support the hypothesis that Ca\(^{2+}\)/CaM plays a role, albeit probably a minor one, in the EGF-mediated activation of the EGFR. Our experiments on model membranes (Figs. 3 and 4) also indicate that
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The commonly used membrane-permeable Ca\(^{2+}\)/CaM inhibitors shown in Fig. 2 should all bind significantly to the inner leaflet of the plasma membrane at approximately the same concentrations at which they are used to inhibit calmodulin in cells. Thus, the common assumption that using two chemically different inhibitors (e.g. TFP and W-7) provides evidence that they are acting specifically on calmodulin is not correct. The inhibitors bind to and have concomitant effects on the plasma membrane that complicate interpretation of the cellular effects. The inhibitors shown in Fig. 2 bind to membranes and to the hydrophobic pocket of Ca\(^{2+}\)/CaM (63, 64) via nonspecific hydrophobic and electrostatic interactions (Figs. 3 and 4). Because the membrane-binding results in Figs. 3–6 may be of general interest to cell biologists, we discuss them before the EGFR results.

Membrane Binding Allows the Molecules Shown in Fig. 2 to Function Efficiently as Ca\(^{2+}\)/CaM Inhibitors in Living Cells—The membrane binding of the Ca\(^{2+}\)/CaM inhibitors documented in Figs. 3 and 4 is not without a fortuitous side effect. We argue, in the supplemental materials, that they would not be useful Ca\(^{2+}\)/CaM inhibitors in a cell if they did not bind strongly to membranes. Specifically, we argue that the plasma and intracellular membranes act as reservoirs of the calmodulin inhibitors, binding most of the inhibitor that enters the quiescent cell and rapidly releasing the inhibitor to bind Ca\(^{2+}\)/CaM when the level of [Ca\(^{2+}\)], rises in the cell.

Effects of Calmodulin Inhibitors Binding to the Inner Leaflet of the Plasma Membrane—Our observations suggest that cellular studies using high concentrations of weak base Ca\(^{2+}\)/CaM inhibitors can be complicated by a wide variety of membrane binding effects. We discuss three side effects; more are likely. (i) Many important peripheral proteins, such as Src, KRas4B, gravin, AKAP79, MARCKS, and HIVgag, require electrostatic interactions to adhere to the plasma membrane (7, 8, 65–67). These proteins all contain one or more clusters of basic residues that interact with the negative electrostatic surface potential produced by acidic lipids in the plasma membrane (Fig. 1). High inhibitor concentrations weaken these interactions, and, if the net negative charge on the inner leaflet becomes sufficiently low, these proteins will desorb from the membrane and translocate to the cytoplasm. Clusters of basic residues on intrinsic membrane proteins, such as the EGFR, should also bind less strongly to membranes when the Ca\(^{2+}\)/CaM inhibitors are present. Figs. 5 and 6 show that the inhibitors produce the expected desorption of a basic peptide from a model phospholipid membrane. We suspect that the action of these calmodulin inhibitors on membranes could be responsible for a significant fraction of their many observed effects on cells. To cite just one example of a peripheral protein, Young et al. (68) showed that 50 \(\mu\)M W-7 prevents the binding of sphingosine kinase to the plasma membrane and reasonably attributed this effect to W-7 inhibiting Ca\(^{2+}\)/CaM inhibition or plasma membrane effects or both. (ii) The positively charged HB form of the weak base inhibitors shown in Fig. 2 should produce similar effects. (iii) Because the positively charged HB\(^{+}\) form of the weak base inhibitors will bind more strongly to the inner than to the outer leaflet of the plasma membrane, because the inner leaflet contains 100% of the major acidic lipid PS and is thus more negatively charged. The resulting differential increase in lateral surface pressure on the inner leaflet will induce a "bilayer couple" effect that may cause curvature of the plasma membrane, as discussed in the classic paper by Sheetz and Singer (73) and in subsequent reports (74). They exposed erythrocytes to amphipathic weak bases, such as local anesthetics, chlorpromazine, etc., to induce the bilayer couple effect; the amphipathic weak bases in Fig. 2 should produce similar effects. (iii) Because the positively charged HB\(^{+}\) form of the weak base binds more strongly to the inner than to the outer leaflet of the plasma membrane, the magnitude of the transmembrane electric field within the membrane will decrease. Voltage-gated ion channels have voltage sensors located within the membrane that will sense this change and respond to it as to a depolarization, even in the absence of a change in the measurable poten-
tial difference between the bulk aqueous solutions outside and inside the cell (75). This could open ion channels permeable to Ca\(^{2+}\), increasing [Ca\(^{2+}\)]. High concentrations (>100 \(\mu\)M) of W-7 are known to increase [Ca\(^{2+}\)], (76, 77), but these concentrations are well above the level required to inhibit Ca\(^{2+}\)/CaM.

The inhibitors shown in Fig. 2 could produce these and numerous other effects due to binding to membranes in living cells; nevertheless, we argue below that they remain useful tools for studying the role of calmodulin in cellular processes.

**Using Inhibitors to Investigate the Role of Calmodulin in the EGF-mediated Activation of the EGFR—**The activation and trafficking of the EGFR have been reviewed elsewhere (78–81). Villalobo and co-workers (18, 20) showed that EGFR is a Ca\(^{2+}\)/CaM-binding protein. What is the physiological significance of this binding in EGF-mediated activation of EGFR? The question is important, because the EGFR activation mechanism differs from that of most other receptor tyrosine kinases. Specifically, the activation loop in the EGFR kinase domain is not phosphorylated when the receptor binds its ligand and undergoes dimerization (79). A recent report from the Kuriyan laboratory provides strong evidence that when EGF mediates EGFR dimer formation, the kinase domain of one EGFR in the dimer pair binds to and activates the other, changing the conformation of its activation loop by an allosteric mechanism (22, 23).

But other autoinhibitory mechanisms may exist for the EGFR family, as they do for other receptor tyrosine kinases (82). Factors that remove these extra autoinhibitory mechanisms may act in parallel with the allosteric activation mechanism described by Zhang et al. (22). For example, Landau et al. (83) proposed that a portion of the C-terminal domain binds directly to and inhibits the kinase domain; McLaughlin et al. (21) hypothesized that the positively charged residue 645–660 of the JM domain and the positive face of the kinase domain bind electrostatically to the plasma membrane, contributing to autoinhibition.

Our working hypothesis in this report is that Ca\(^{2+}\)/CaM releases an autoinhibitory function, probably by binding to the EGFR JM region. Stimulation by EGF produces only a transient increase in [Ca\(^{2+}\)], suggesting that Ca\(^{2+}\)/CaM should contribute to the initial pulse of EGFR autophosphorylation but not to the steady state level. Other groups have proposed alternative explanations for the initial transient peak in autophosphorylation; e.g. Kholodenko et al. (54, 55) suggested that the peak is due to binding of Src homology 2-containing proteins/adaptors to the Tyr(P) residues, providing temporary protection from the action of phosphatases. We attempted to distinguish between our working hypothesis and other models that do not involve Ca\(^{2+}\)/CaM via experiments with the calmodulin inhibitor W-13. Li et al. (20) and others (21) showed that appropriate concentrations of W-7/W-13/W-12 do indeed inhibit the initial transient maximum, but not the steady state level, of EGF-mediated EGFR autophosphorylation in different cell types (e.g. N7XHERc fibroblasts, COS1 monkey kidney cells). Fig. 7A shows similar effects in cells examined under different conditions (inhibitors for calcium/calmodulin-activated kinase II and protein kinase C present). Note that this inhibition is observed in many but not all cell types; W-7 did not affect EGF-mediated EGFR autophosphorylation kinetics in hepatocytes.5 Furthermore, Li et al. (20) noted that with cells overexpressing EGFR, such as EGFR-T17 and A431, the calmodulin inhibitor W-13 was effective only if the cells were first exposed to a calcium ionophore.

Two obvious corollaries emerge from our hypothesis that Ca\(^{2+}\)/CaM is important for the initial phase of the EGFR autophosphorylation. First, maintaining high levels of Ca\(^{2+}\) and thus Ca\(^{2+}\)/CaM (e.g. by exposing the cell to a Ca\(^{2+}\) ionophore) should increase the steady state level of EGFR autophosphorylation after EGF-mediated dimerization. Second, under these conditions, a Ca\(^{2+}\)/CaM inhibitor, such as W-13, should reduce EGFR autophosphorylation at all times after the addition of EGF, not just during the initial phase. The data in Fig. 7B are consistent with these two predictions and support the hypothesis that Ca\(^{2+}\)/CaM augments EGFR autophosphorylation. This raises the question of where Ca\(^{2+}\)/CaM is acting: directly on the EGFR or indirectly through some other Ca\(^{2+}\)/CaM dependent process? We addressed this question by testing the effect of W-13 on cells expressing an EGFR mutant in which the only known Ca\(^{2+}\)/CaM binding region on the protein, residues 645–660 in the cytoplasmic JM region, is disrupted by insertion of an acidic 23-amino acid sequence; the inhibitor does not affect autophosphorylation of this mutant (Fig. S1B).

In summary, the simplest explanation of the results in Figs. 7 and S1 is that Ca\(^{2+}\)/CaM binds to the JM region and augments the initial phase of the EGF-mediated EGFR autophosphorylation; the specific mechanism for this augmentation and its role in the more general allosteric mechanism for activation of the kinase domain described by Zhang et al. (22) remains to be determined. Two proposed models could account for these data; the basic JM region could bind to a cluster of acidic residues in the C-terminal tail region of an adjacent EGFR (84) or to acidic phospholipids in the membrane (21). Recent experiments support the claim that the JM region can bind with high affinity to the inner leaflet of the plasma membrane. Specifically, NMR and fluorescence measurements on peptides corresponding to transmembrane plus JM domains of EGFR reconstituted into phospholipid vesicles (25) show the transmembrane helix breaks at the membranesolution interface, and the extended JM region binds to the membrane if it contains acidic lipids (either 1% phosphatidylinositol 4,5-bisphosphate or >5% PS); adding Ca\(^{2+}\)/CaM can release the JM region from the membrane.

**Evidence That Calmodulin Inhibitors Can Activate EGFR in the Absence of Ligand by Binding to Membranes—**In the absence of EGF, adding W-13 to four different cell types activates EGFR (Fig. 8). The simplest interpretation of this result is that W-13 can activate EGFR autophosphorylation through an effect other than binding to Ca\(^{2+}\)/CaM, possibly through binding to the inner leaflet of the plasma membrane. There are many ways this activation might occur. In the electrostatic engine model (21), for example, either reducing the electrostatic attraction of the JM + kinase domains for the plasma membrane or binding of Ca\(^{2+}\)/CaM to the JM region should

5 J. Hoek, personal communication.
release autoinhibition, as illustrated in Refs. 7, 21, and 25. Figs. 5 and 6 in this report show that W-13 releases a simple peptide corresponding to the EGFR JM region from a phospholipid vesicle.

This model predicts that other weak bases that do not act as CaM inhibitors should also activate EGFR at the same concentration at which they bind to membranes. For example, the hydrophobic tail of sphingosine is tall and skinny in contrast to the short and broad hydrophobic regions of the inhibitors shown in Fig. 2; one expects micromolar concentrations (e.g. 1–2 μM) of sphingosine to bind more strongly to membranes than to the hydrophobic pocket of Ca2+/CaM, and experiments confirm this expectation (data not shown). As expected from the model, sphingosine activates EGFR in the absence of EGF (85) at the same concentration (2 μM) at which it binds to membranes and reverses the sign of the zeta potential (21). Furthermore, fluorescence experiments show that sphingosine causes the JM region of reconstituted transmembrane + JM EGFR peptides to desorb from bilayer membranes (25).

Conclusions—A model proposing that membrane binding of the JM and kinase domains contributes to autoinhibition of EGFR (21) predicts both of our key observations of the action of W-13 on the EGFR: its ability to inhibit EGF-mediated autophosphorylation (Fig. 7) and its ability to stimulate autophosphorylation of EGFR in the absence of EGF (Fig. 8). These dual effects are difficult to explain with other models for autoinhibition (e.g. see Landau et al. (83)). Specifically, one simple interpretation of our EGFR results is that calmodulin inhibitors decrease the EGF-mediated autophosphorylation by preventing Ca2+/CaM from removing the positively charged EGFR JM region from the negatively charged plasma membrane (see Fig. 6 of Ref. 7); slightly higher concentrations of inhibitors stimulate the autophosphorylation in the absence of EGF by decreasing the net negative charge on the membrane, which facilitates desorption of the JM + kinase region from the membrane. In both cases, activation of the kinase domain and subsequent autophosphorylation of the EGFR in its C-terminal tail region presumably requires juxtaposition of the kinase domains and repositioning of the activation loop by the allosteric mechanism described by Kuriyan and co-workers (22). Thus, the JM region of the EGFR may play its autoinhibitory role by binding to the membrane and decreasing the collision frequency of the two kinase domains in an EGFR dimer. As reviewed elsewhere (82), the JM regions of many different receptor tyrosine kinases provide an additional secondary layer of autoinhibition, which complements the primary role played by the activation loop in the kinase domain.

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REFERENCES

1. Clapham, D. E. (1995) Cell 80, 259–268
2. Berriedge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell Biol. 4, 517–529
3. Chin, D., and Means, A. R. (2000) Trends Cell Biol. 10, 322–328
4. Hoeflich, K. P., and Ikura, M. (2002) Cell 108, 739–742
5. Vetter, S. W., and Leduc, E. (2003) Eur. J. Biochem. 270, 404–414
6. Yamniss, A. P., and Vogel, H. J. (2004) Mol. Biotechnol. 27, 35–57
7. McLaughlin, S., and Murray, D. (2005) Nature 438, 605–611
8. Yeung, T., Terebuznik, M., Yu, L., SilviuS, J., Abidi, W. M., Philip, M., Levine, T., Kapus, A., and Grinstein, S. (2006) Science 313, 347–351
9. McLaughlin, S. (2006) Science 314, 1402–1403
10. Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., and Meyer, T. (2006) Science 314, 1458–1461
11. Ferguson, K. M. (2004) Biochem. Soc. Trans. 32, 742–745
12. Pandiella, A., Magni, M., Lovisolo, D., and Meldolesi, J. (1989) J. Biol. Chem. 264, 12914–12921
13. Cheyette, T. E., and Gross, D. J. (1991) Cell Regul. 2, 827–840
14. Hughes, A. R., Bird, G. S., Obie, J. F., Thastrup, O., and Putney, J. W., Jr. (1991) Mol. Pharmacol. 40, 254–262
15. Bezerrides, V. J., Ramsey, J. S., Kotchea, S., Greka, A., and Clapham, D. E. (2004) Nat Cell Biol. 6, 709–720
16. Li, W. P., Tsikas, L., Sansom, S. C., and Ma, R. (2004) J. Biol. Chem. 279, 4570–4577
17. Uyemura, T., Takagi, H., Yanagida, T., and Sako, Y. (2005) Biophys. J. 88, 3720–3730
18. Li, H., and Villalobo, A. (2002) Biochem. J. 362, 499–505
19. Martin-Nieto, J., and Villalobo, A. (1998) Biochemistry 37, 227–236
20. Li, H., Ruano, M. J., and Villalobo, A. (2004) FEBS Lett. 559, 175–180
21. McLaughlin, S., Smith, S. O., Hayman, M. J., and Murray, D. (2005) J. Gen. Physiol 126, 41–53
22. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) Cell 125, 1137–1149
23. Hubbard, S. R. (2006) Cell 125, 1029–1031
24. Pellicena, P., and Kuriyan, J. (2006) Curr. Opin. Struct. Biol. 16, 702–709
25. Sato, T., Pallavi, P., Golebiowska, U., McLaughlin, S., and Smith, S. O. (2006) Biochemistry 45, 12704–12714
26. Tebar, F., Villalonga, P., Sorkina, T., Agell, N., Sorkin, A., and Enrich, C. (2002) Mol. Biol. Cell 13, 2057–2068
27. Tebar, F., Llado, A., and Enrich, C. (2002) FEBS Lett. 517, 206–210
28. Holtthuis, J. C., and Levine, T. P. (2005) Nat. Rev. Mol. Cell Biol. 6, 209–220
29. Helmholz, H. L. (1879) Ann. Phys. Leipzig 7, 337–382
30. Dill, K., and Bromberg, S. (2003) Molecular Driving Forces, pp. 369–447, Garland Science, New York
31. McLaughlin, S. (1977) Curr. Top. Membr. Transp. 19, 71–144
32. McLaughlin, S. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 113–136
33. Caviso, D., McLaughlin, A., McLaughlin, S., and Winiski, A. (1989) Methods Enzymol. 171, 342–364
34. Wang, J., Arbusova, A., Hangay-Mihalyne, G., and McLaughlin, S. (2001) J. Biol. Chem. 276, 5012–5019
35. Rusu, L., Gembhir, A., McLaughlin, S., and Radler, J. (2004) Biophys. J. 87, 1044–1053
36. Golebiowska, U. P., Gambhir, A., Hangay-Mihalyne, G., Zaitseva, L., Raedler, J., and McLaughlin, S. (2006) Biophys. J. 91, 588–599
37. Buser, C. A., and McLaughlin, S. (1998) Methods Mol. Biol. 84, 267–281
38. Magde, D., Elson, E. L., and Webb, W. W. (1974) Biopolymers 13, 29–61
39. Maiti, S., Haupts, U., and Webb, W. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11573–11577
40. Sengupta, P., Balaji, J., and Saiti, S. (2002) Methods 27, 374–387
41. Elson, E. L. (2004) J. Biomed. Opt. 9, 857–864
42. Sorokin, A. (1995) Oncogene 11, 1531–1540
43. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) J. Biol. Chem. 271, 13377–13384
44. Carter, R. E., and Sorkin, A. (1998) J. Biol. Chem. 273, 35000–35007
45. Laemmli, U. K. (1970) Nature 227, 680–685
46. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
Calmodulin Inhibitors Bind Membranes

47. Chafouleas, J. G., Bolton, W. E., Hidaka, H., Boyd, A. E., III, and Means, A. R. (1982) Cell 28, 41–50
48. Nishikawa, M., and Hidaka, H. (1982) J. Clin. Invest. 69, 1348–1355
49. Peitzsch, R. M., and McLaughlin, S. (1993) Biochemistry 32, 10436–10443
50. McLaughlin, S., and Whitaker, M. (1988) J. Physiol 396, 189–204
51. Johnson, J. D., and Wittenauer, L. A. (1983) J. Biol. Chem. 258, 473–479
52. Anfinogenova, Y. J., Rodriguez, X., Grygorczyk, R., Adragna, N. C., Lauf, P. K., Hamet, P., and Orlov, S. N. (2001) Am. J. Physiol. 281, C1169–C1170
53. Aifa, S., Johansen, K., Nilsson, U. K., Liedberg, B., Lundstrom, I., and Svensson, S. P. (2002) Acta Physiol. Scand. 175, 1100–1104
54. Hunter, T., Ling, N., and Cooper, J. A. (1984) Nature 311, 480–483
55. Davis, R. J. (1988) J. Biol. Chem. 263, 9462–9469
56. Livneh, E., Dull, T. J., Berent, E., Prywes, R., Ullrich, A., and Schlessinger, J. (1988) Mol. Cell. Biol. 8, 2302–2308
57. Lund, K. A., Lazar, C. S., Chen, W. S., Walsh, B. J., Welsh, J. B., Herbst, J. L., Walton, G. M., Rosenfeld, M. G., Gill, G. N., and Wiley, H. S. (1990) J. Biol. Chem. 265, 20917–20923
58. Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992) J. Biol. Chem. 267, 1129–1140
59. Theroux, S. J., Latour, D. A., Stanley, K., Reden, D. L., and Davis, R. J. (1992) J. Biol. Chem. 267, 16620–16626
60. Feinmesser, R. L., Wicks, S. J., Taverner, C. J., and Chantry, A. (1999) J. Biol. Chem. 274, 16168–16173
61. Osawa, M., Swindells, M. B., Tanikawa, J., Tanaka, T., Mase, T., Furuya, T., and Ikura, M. (1998) J. Mol. Biol. 276, 165–176
62. Osawa, M., Kuwamoto, S., Iizumi, Y., Yap, K. L., Ikura, M., Shibanuma, T., Yokokura, H., Hidaka, H., and Matsushima, N. (1999) FEBS Lett. 422, 173–177
63. Cho, W., and Stahelin, R. V. (2005) Annu. Rev. Biochem. 74, 119–151
64. DiNitto, J. P., Cronin, T. C., and Lambright, D. G. (2003) Science 299, 21833–21840
65. Oke, N. M., and Gelb, M. H. (2004) J. Biol. Chem. 279, 21833–21840
66. Young, K. W., Willets, J. M., Parkinson, M. J., Bartlett, P., Spiegel, S., Nahorski, S. R., and Stahelin, R. V. (2003) Cell Calcium 33, 119–128
67. Sutherland, C. M., Moretti, P. A., Hewitt, N. M., Bagley, C. J., Vadas, M. A., and Pitson, S. M. (2006) J. Biol. Chem. 281, 11693–11701
68. Stahelin, R. V., Hwang, J. H., Kim, J. H., Park, Y. Z., Johnson, K. R., Obeid, L. M., and Cho, W. (2005) J. Biol. Chem. 280, 43030–43038
69. Chapin, S. J., Enrich, C., Aroeti, B., Havel, R. J., and Mostov, K. E. (1990) J. Biol. Chem. 265, 1336–1342
70. Apodaca, G., Enrich, C., and Mostov, K. E. (1994) J. Biol. Chem. 269, 19005–19013
71. Sheetz, M. P., and Singer, S. J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4457–4461
72. Sheetz, M. P., and Singer, S. J. (1976) J. Cell Biol. 70, 247–251
73. Chandler, W. K., Hodgkin, A. L., and Meves, H. (1965) J. Physiol. 180, 821–836
74. Watanabe, H., Takahashi, R., Tran, Q. K., Takeuchi, K., Kosuge, K., Satoh, H., Uehara, A., Terada, H., Hayashi, H., Ohno, R., and Ohashi, K. (1999) Biochem. Biophys. Res. Commun. 265, 697–702
75. Jan, C. R., Lu, C. H., Chen, Y. C., Cheng, J. S., Tseng, L. L., and Jun-Wen, W. (2000) Pharmacol. Res. 42, 323–327
76. Carpenter, G. (2000) BioEssays 22, 697–707
77. Pike, L. J., Han, X., and Gross, R. W. (2005) J. Biol. Chem. 280, 26796–26804
78. Hubbard, S. R. (2004) Nat. Rev. Mol. Cell. Biol. 5, 464–471
79. Landau, M., Fleishman, S. J., and Ben-Tal, N. (2004) Structure 12, 2265–2275
80. Aifa, S., Milad, S., Frika, F., Aniba, M. R., Svensson, S. P., and Rebai, A. (2006) FEBS Lett. 57, 5373–5379
81. Hidaka, H., Yamaki, T., Nakamura, M., Tanaka, T., Hayashi, H., and Kobayashi, R. (1980) Mol. Pharmacol. 17, 66–72