which is polymerized is calculated from the inhibition of DNase activity by lysates prepared in the absence and presence of guanidine.

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[24] Chemoattractant-Induced Membrane Phenomena of Phagocytes

By CHARLES D. SMITH and RALPH SNYDERMAN

Introduction

Cellular activation by exogenous stimuli requires that extracellular signals be translated into intracellular signals. Therefore, events occurring at the plasma membrane of the cell play important roles in stimulus–response coupling. Exposure of phagocytes to chemoattractants initiates a number of biological responses including directed migration, secretion of hydrolytic enzymes, and production of cytotoxic oxygen species.[1] These processes are activated by second messengers which are produced upon interaction of chemoattractants with their receptors.

This chapter is divided into three sections. The first describes methods for the isolation and subcellular fractionation of human polymorphonuclear leukocytes (PMNs).[1a] The second deals with methods for studying chemoattractant-induced changes in phospholipid metabolism. The third

[1] R. Snyderman and E. J. Goetzl, *Science* **213**, 830 (1981).

[1a] Abbreviations: PMNs, polymorphonuclear leukocytes; LSM, Lymphocyte Separation Medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HHBSS, Hanks' balanced salt solution in HEPES; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; IP, inositol phosphate; IP_2, inositol bisphosphate; PT, *Bordetella pertussis* toxin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate).

deals with investigation of the role of a guanine nucleotide regulatory (N) protein in phagocyte activation by chemoattractants. Methods described herein have been used predominantly for studying responses of human PMNs to the oligopeptide chemoattractant fMet-Leu-Phe but are also applicable to other chemoattractants.

Preparation of Human PMNs and Their Subcellular Fractions

Preparation of Human PMNs (see also this series, Vol. 108 [9])

Heparinized blood (10 units of heparin/ml; Upjohn) from healthy donors is mixed with an equal volume of 3% dextran T500 (Pharmacia Fine Chemicals) in 0.9% NaCl. Erythrocytes are allowed to settle, and the leukocyte-rich plasma is transferred to a 50-ml conical centrifuge tube. This cell suspension is gently underlayed with a 15-ml cushion of Lymphocyte Separation Medium (LSM, Bionetics Laboratory Products) and centrifuged at 400 *g* for 40 min at room temperature. Leukocytes are resolved into two fractions: the mononuclear layer, which bands at the serum–LSM interface and which contains monocytes and lymphocytes, and the pellet, which contains PMNs and residual erythrocytes. For the isolation of PMNs, the serum and LSM layers are withdrawn by aspiration. Erythrocytes in the pellet are removed by 3 cycles of hypotonic lysis, each of which consists of resuspending the cells in 5 ml of ice-cold 0.2% NaCl for 15 sec followed by addition of 5 ml of cold 1.6% NaCl to restore isotonicity and centrifugation at 600 *g* for 10 min at 4° The isolation PMNs (approximately 10^9 cells/450 ml of blood which are >95% pure) are typically resuspended in Hanks' balanced salt solution (Gibco) containing 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 4.2 m*M* $NaHCO_3$, pH 7.4 (HHBSS).

Isolation of Plasma Membranes

Isolated PMNs are pelleted by centrifugation at 600 *g* for 10 min at 4° and resuspended to a concentration of 2×10^7 cells/ml of relaxation buffer (100 m*M* KCl, 3 m*M* NaCl, 3.5 m*M* $MgCl_2$, and 10 m*M* HEPES, pH 7.3). Aliquots of 15 ml are placed in a Cell Disruption Bomb (Parr Instrument Co.) and pressurized to 500 psi with nitrogen. After 20 min, the cell lysate is collected and combined with 300 μl of 100 m*M* neutralized ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) to give a final concentration of 2 m*M* EGTA. The lysate is layered over a 15-ml cushion of relaxation buffer containing 41% sucrose in a polycarbonate ultracentrifuge tube (e.g., for a Beckman SW28 rotor) and centrifuged at

90,000 *g* for 60 min.[2] Plasma membranes band at the sucrose interface and are pelleted by centrifugation at 100,000 *g* for 45 min. The membranes are then resuspended in 10 m*M* HEPES, pH 7.0, containing 0.25 *M* sucrose (sucrose/HEPES) and used immediately or frozen at −80°.

An alternate procedure which allows simultaneous isolation of specific granules and azurophilic granules involves fractionation of the PMN lysate on self-forming gradients of Percoll[3] (Pharmacia). The cell cavitate is collected with EGTA as described above and centrifuged at 600 *g* for 10 min at 4° to pellet unbroken cells and nuclei. The resulting supernatant is placed in a 45-ml centrifuge tube (e.g., for a Sorvall SS34 rotor) over a 30-ml gradient of Percoll [15 ml of 54% Percoll in relaxation buffer (density 1.050 g/ml) overlayed on 15 ml of 88% Percoll in relaxation buffer (density 1.120 g/ml)]. Following centrifugation at 40,000 *g* for 25 min at 4°, the cell lysate is resolved into three prominent fractions. The least dense band contains plasma membranes (enriched 16-fold in alkaline phosphatase activity), the intermediate band contains specific granules (enriched 7-fold in vitamin B_{12}-binding protein), and the dense fraction contains azurophilic granules (enriched 5-fold in myeloperoxidase). These fractions are recovered and centrifuged at 100,000 *g* for 45 min at 4°. The organelles collect at the top of the Percoll pellet and can be recovered with a Pasteur pipet. The membranes are then diluted with sucrose/HEPES and centrifuged again at 100,000 *g* for 45 min at 4°. The membranes are recovered as above and are resuspended in sucrose/HEPES and frozen at −80°.

Measurement of Chemoattractant-Induced Phospholipid Metabolism

Modification of the phospholipid composition of plasma membranes is a rapid response to many hormonal stimuli, including chemoattractants. Alterations of phospholipid metabolism can have pronounced effects on the activities of membrane-associated enzymes or produce second messengers which act to regulate cell responses. Methods used in our laboratory for measuring chemoattractant-induced changes in phospholipid metabolism are described below.

Phospholipid Methylation

Hormone-induced changes in methylation of membrane phospholipids, e.g., conversion of phosphatidylethanolamine to phosphatidylcho-

[2] T. Maeda, K. Balakrishnan, and S. Q. Mehdi, *Biochim. Biophys. Acta* **731**, 115 (1983).
[3] N. Borregaard, J. M. Heiple, E. R. Simons, and R. A. Clark, *J. Cell Biol.* **97**, 52 (1983).

line, may alter the structure of the membrane sufficiently to provide a mechanism for regulating membrane function. Lipid methylation can be measured as the incorporation of ^3H from *S*-adenosyl-L-[*methyl*-^3H]methionine, which is synthesized by incubating cells with L-[*methyl*-^3H]methionine, into phospholipids.[4] PMNs (5×10^6 cells in 1 ml of HHBSS) are incubated at 37° for 15 min before the addition of 10 μCi of L-[*methyl*-^3H]methionine (New England Nuclear) and various stimuli, e.g., fMet-Leu-Phe. The cells are incubated up to an additional 60 min, pelleted, resuspended in 0.1 ml of HHBSS, and dissolved in 0.5 ml of chloroform:methanol (2:1, v/v). The tubes are then vortexed and centrifuged at 2000 *g* for 5 min. The aqueous upper phase is then removed by aspiration, and 0.1 ml of the organic phase is spotted onto a thin-layer cellulose plate containing a fluorescent indicator (Eastman chromogram), along with 10 μg of phosphatidylcholine. The plates are developed with chloroform:methanol:water (75:18:2, v/v/v) to separate the phospholipids from free L-[*methyl*-^3H]methionine. The phospholipids are visualized under ultraviolet light, scraped into scintillation vials, and mixed with 10 ml of Lefkofluor scintillation fluid (Research Products International Corp.) for determination of radioactivity. Incubation of guinea pig macrophages with chemotactic peptides results in a dose-dependent suppression of phosphatidylethanolamine methylation.[4]

Phosphoinositide Metabolism

Occupancy of many receptors results in the elevation of intracellular Ca^{2+} concentrations and activation of protein kinase C. These effects are mediated by inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol, respectively, which are produced by receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C.[5] Using radiolabeled PMNs (described below), chemoattractant-stimulated polyphosphoinositide hydrolysis can be measured as the decrease in radioactivity in phosphatidylinositol 4-phosphate (PIP) and PIP_2 or the increase in radioactivity in inositol phosphates. Radioactivity in PIP and PIP_2 is measured by liquid scintillation counting after isolation of these lipids by affinity chromatography using immobilized neomycin.[6] Inositol phosphates are analyzed by anion-exchange chromatography followed by liquid scintillation spectrometry.[7]

[4] M. C. Pike, N. M. Kredich, and R. Snyderman, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 2922 (1979).
[5] M. J. Berridge and R. E. Irvine, *Nature (London)* **312,** 315 (1984).
[6] J. Schacht, this series, Vol. 72, p. 626.
[7] C. P. Downes and R. H. Michell, *Biochem. J.* **198,** 133 (1981).

PMN Labeling with $^{32}PO_4$. PMNs are isolated as described above and resuspended to 2×10^7 cells/ml of phosphate-free HHBSS (137 m*M* NaCl, 5.4 m*M* KCl, 1 m*M* $CaCl_2$, 4.3 m*M* $NaHCO_3$, 0.4 m*M* $MgCl_2$, 0.4 m*M* $MgSO_4$, 10 m*M* glucose, and 10 m*M* HEPES, pH 7.4). Neutralized ortho[^{32}P]phosphoric acid (New England Nuclear) is added to 40 μCi/ml, and the suspensions are incubated at 37° for 90–120 min. The cells are washed with HHBSS 3 times and then resuspended in HHBSS to 10^8 cells/ml, divided into 0.18 ml aliquots, and kept on ice. After 1–5 min of preincubation at 37°, the ^{32}P-labeled cells are stimulated by exposure to a chemoattractant for 15–300 sec. Reactions are terminated by the addition of 2 ml of chloroform:methanol (1:1, v/v), and [^{32}P]PIP and [^{32}P]PIP_2 levels are quantitated as described below.

PMN Labeling with myo-[2-^{3}H]Inositol. Isolated PMNs are resuspended to 2×10^7 cells/ml of Medium 199 (Hazelton Dutchland Corp.) containing 2% fetal calf serum. *myo*-[2-^{3}H]Inositol (New England Nuclear) is then added to 40 μCi/ml, and the cells are incubated for 18–24 hr at 37°. The cells are harvested, washed twice with HHBSS, resuspended to 10^8 cells/ml of HHBSS containing 2 m*M* LiCl, and divided into 0.18 ml aliquots on ice. Cells are stimulated as above, and the levels of [^{3}H]PIP, [^{3}H]PIP_2, and [^{3}H]inositol phosphates are determined as described below.

Analysis of Polyphosphoinositides. Reactions (volume $\leq$200 μl) are terminated by the addition of 2 ml of chloroform:methanol (1:1, v/v). Phospholipids are extracted by the addition of 0.5 ml of 2.4 *N* HCl, thorough mixing, and centrifugation at 1000 *g* for 10 min.[6] The organic lower phase is removed, and 1 ml of chloroform is added to the aqueous phase, which is mixed and centrifuged. The lower phase is combined with the previous extract and washed twice with 2 ml of methanol:1 *N* HCl (1:1, v/v) by mixing and centrifuging as above, each time removing the aqueous phase. The resulting organic phase is combined with 2 ml of 200 m*M* ammonium acetate in absolute methanol and subjected to affinity chromatography on 0.5 ml of neomycin covalently bound to glass beads as described by Schacht.[6] All the phospholipids except PIP and PIP_2 are washed from the column with 7 ml of chloroform:methanol:water (3:6:1, v/v/v) containing 150 m*M* ammonium acetate. PIP is eluted by washing with 7 ml of chloroform:methanol:water (3:6:1, v/v/v) containing 600 m*M* ammonium acetate. PIP_2 is eluted by washing with 7 ml of chloroform:methanol:14 *N* ammonium hydroxide (3:6:1, v/v/v). The amount of ^{32}P in the phospholipids can be directly analyzed as Cerenkov radiation in a liquid scintillation spectrometer (this typically produces approximately 40% of the cpm value determined in the presence of scintillation fluid). ^{3}H-Labeled phospholipids must be first dried (e.g., by heating in the scintillation vials overnight) before fluid is added for counting.

Analysis of Inositol Phosphates. Reactions are terminated, and the samples are acidified as described above. After centrifugation, the aqueous phase is removed and combined with 15 ml of 0.1 *N* NaOH to neutralize the extract and to reduce its ionic strength. The samples are then chromatographed on a 0.5-ml column of Dowex-1 (formate) equilibrated with 50 m*M* ammonium formate.[7] The column is washed with 10 ml of 50 m*M* ammonium formate to remove neutral compounds (e.g., inositol). Inositol phosphate (IP) is eluted into a scintillation vial with 10 ml of 0.2 *M* ammonium formate/0.1 *N* formic acid. Inositol bisphosphate (IP_2) is eluted with 10 ml of 0.4 *M* ammonium formate/0.1 *N* formic acid. IP_3 is eluted with 10 ml of 1.0 *M* ammonium formate/0.1 *N* formic acid. The eluants are then mixed with Lefkofluor scintillation fluid for determination of radioactivity. These chromatographic procedures do not resolve possible isomers of inositol phosphates (e.g., inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate); however, these may be separated using high-pressure liquid chromatography.[8]

Role of an N Protein in Receptor-Induced Polyphosphoinositide Breakdown

N proteins play pivotal roles in regulating the activity of adenylate cyclase and hence cAMP levels in cells. Accumulating evidence suggests that N proteins may play similar roles in coupling phospholipase C activation to receptors which stimulate polyphosphoinositide breakdown.[9] We have used two approaches to investigate N protein involvement in phospholipase activation by chemoattractants. The first involves use of *Bordetella pertussis* toxin (PT),[10] and the second involves direct activation of the phospholipase in isolated plasma membranes.[11,12]

PT as a Probe for N Protein Involvement

Bacterial toxins have provided powerful tools for analyzing the involvement of N proteins in regulating adenylate cyclase activity. For example, cholera toxin catalyzes the transfer of the ADP-ribose moiety of NAD to the α subunit of N_s. This modification results in the persistent activation of

[8] R. F. Irvine, E. E. Anggard, A. J. Letcher, and C. P. Downes, *Biochem. J.* **229,** 505 (1985).
[9] C. D. Smith, M. W. Verghese, and R. Snyderman, *in* "Sensing and Response in Microorganisms" (M. Eisenbach and M. Balaban, eds.), p. 215. Elsevier, New York, 1985.
[10] M. W. Verghese, C. D. Smith, and R. Snyderman, *Biochem. Biophys. Res. Commun.* **127,** 450 (1985).
[11] C. D. Smith, B. C. Lane, I. Kusaka, M. W. Verghese, and R. Snyderman, *J. Biol. Chem.* **260,** 5875 (1985).
[12] C. D. Smith, C. Cox, and R. Snyderman, *Science* **232,** 97 (1987).

the N protein and therefore of adenylate cyclase. In contrast, PT catalyzes the ADP-ribosylation and consequent inactivation of N_i and transducin.[13] Incubation of human PMNs with PT inhibits chemoattractant-induced cellular responses by inhibiting the activation of phospholipase C-mediated hydrolysis of PIP_2.[10,11]

Incubation Conditions. Pertussis toxin (List Biological Products Campbell, CA 95008) is incubated with unlabeled, $^{32}PO_4$-labeled, or [^{3}H]inositol-labeled PMNs (2×10^7 cells/ml of HHBSS) at concentrations of 0.1–1 μg/ml of HHBSS for up to 4 hr at 37°. The cells are pelleted, washed twice with HHBSS, and then assayed for chemoattractant-stimulated PIP_2 breakdown or production of inositol phosphates. By varying the dose of PT and the time of incubation, conditions can be defined which will cause inhibition of N protein function without inhibiting nonreceptor-mediated mechanisms.[10] For example, treating PMNs with 1 μg of PT/ml for 90 min at 37° produces an 84% inhibition of [^{3}H]IP_3 production induced by 10^{-6} *M* fMet-Leu-Phe, without inhibiting concanavalin A (100 μg/ml)-induced [^{3}H]IP_3 production, phorbol ester-induced superoxide production, or Ca^{2+} ionophore-stimulated secretion. Increasing the incubation time with PT to 4 hr produces even more complete inhibition of [^{3}H]IP_3 production induced by chemoattractants. For an accurate reflection of the effects of PT on receptor-mediated activation of phospholipase C, it is essential that the conditions used do not cause general cytotoxicity.

Identification of a PT-Sensitive N Protein. PT-sensitive N proteins in isolated membranes can be demonstrated by *in vitro* ribosylation using [*adenylate*-^{32}P]NAD and product analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. For studies with PMNs, plasma membranes (approximately 25 μg of protein) are incubated with 25 m*M* Tris–HCl, 4 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10 m*M* nicotinamide, 10 m*M* thymidine, 0.5 m*M* ADP, 10 μ*M* NAD with 1 μCi of [^{32}P]NAD (New England Nuclear), and 2 μg of PT in a volume of 50 μl for 30 min at 30°. The samples are then dissolved in an equal volume of electrophoresis sample buffer (125 m*M* Tris–HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromphenol blue, pH 6.8) and analyzed by SDS–PAGE using a 10% polyacrylamide gel. The gels are dried and exposed to X-ray film for autoradiography. We have observed a PT-specific ADP-ribosylation product in PMN plasma membranes which migrates with an apparent molecular weight of 41,000. This protein therefore appears to be similar to the α subunit of N_i. Ribosylation of this protein by PT treatment of intact cells is indicated by a reduction in label incorporated into this protein during *in vitro* ribosylation of membranes from the PT-treated cells.

[13] A. G. Gilman, *Cell* **36**, 577 (1984).

N Protein-Mediated PIP_2 Breakdown in Isolated Plasma Membranes

Purified preparations of plasma membranes provide the most definitive system for analyzing N protein involvement in phospholipase C activation since possible regulatory compounds (e.g., Ca^{2+} and guanine nucleotides) can be directly added. Under physiological conditions, N proteins are activated through interaction with occupied receptors. This interaction promotes the dissociation of GDP bound to the N protein, thereby freeing it to bind GTP. Binding of GTP, or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), activates the N protein until hydrolysis of the terminal phosphate of GTP regenerates the inactive GDP–N protein complex.[13] Therefore, activation of the N protein, and subsequently its effector enzyme, requires both hormone and GTP. Nonhydrolyzable analogs of GTP, e.g., GTPγS, directly bind to and activate N proteins. Therefore, receptor-mediated reactions which result in the activation of N proteins can often be mimicked by GTPγS.

An efficient method for producing radiolabeled PIP_2 in isolated membranes involves phosphorylation of the inositol phospholipids with [γ-^{32}P]ATP.[11] Isolated plasma membranes (10–25 μg of protein) in 25 μl of sucrose/HEPES (see above) are incubated for 60 sec at 37° with an equal volume of 100 m*M* HEPES, 10 m*M* $MgCl_2$, 1 m*M* spermine (Sigma), and 200 m*M* [γ-^{32}P]ATP (New England Nuclear) (800–1000 cpm/pmol), pH 7.0. With PMN plasma membranes, this results in the synthesis of phosphatidic acid, PIP, and PIP_2 which contain approximately 40, 50, and 30 pmol of ^{32}P/mg of protein, respectively.[11] These ^{32}P-labeled phospholipids can be conveniently analyzed by affinity chromatography using immobilized neomycin as described above. Appearance of ^{32}P-labeled PIP and PIP_2 demonstrates that the isolated plasma membranes possess functional phosphatidylinositol kinase and PIP kinase. The amount of ^{32}P-labeled PIP_2 reaches a maximum after 60 sec and remains stable for at least an additional 2 min. Therefore the change in [^{32}P]PIP_2 levels between 1 and 2 min is compared in samples incubated with $CaCl_2$ (0.1–1000 μM) and guanine nucleotides.

In the PMN plasma membrane system, PIP_2 hydrolysis can be activated by exposing the labeled membranes to $\geq 100\ \mu M$ $CaCl_2$, which directly activates phospholipase C. Addition of GTP causes a decrease in the Ca^{2+} requirement so that PIP_2 hydrolysis can be activated with an EC_{50} (concentration required for half-maximal PIP_2 breakdown) of approximately 4 μM Ca^{2+}. Most importantly, addition of a combination of fMet-Leu-Phe (10^{-7} *M*) and GTP (10 μM) further reduces the Ca^{2+} dependence so that PIP_2 hydrolysis occurs with as low as 0.1 μM Ca^{2+}.[12] In contrast to those from control cells, plasma membranes isolated from PT-treated

PMNs do not demonstrate stimulation of PIP_2 breakdown upon treatment with fMet-Leu-Phe plus GTP. GTPγS, either alone or in the presence of fMet-Leu-Phe, also activates the hydrolysis of PIP_2 at low concentrations of Ca^{2+}.[12] Therefore, the use of the ^{32}P-labeled plasma membrane system has provided strong support for the proposal that the fMet-Leu-Phe receptor is coupled to phospholipase C through an N protein. Similar systems should be generally applicable in studying the coupling of other receptors to PIP_2 breakdown.

[25] Biochemical Changes in Leukocytes in Response to Chemoattractants

By MARGRITH W. VERGHESE and RALPH SNYDERMAN

Introduction

Activation of phagocytic leukocytes by chemoattractants and most other inflammatory stimuli is associated with rapid increases in intracellular $[Ca^{2+}]$.[1,2] Optimal stimulation of the secretory and respiratory burst responses of these cells to chemoattractants requires Ca^{2+} in the extracellular medium.[3] Chemoattractants stimulate the release of the Ca^{2+} mobilizer inositol 1,4,5-trisphosphate (IP_3)[3a] and 1,2-diacylglycerol in leukocytes as a consequence of phospholipase C (PLC) activation and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2).[4] The products resulting from PIP_2 hydrolysis stimulate Ca^{2+}/phospholipid- and/or calmodulin-dependent protein kinases which apparently leads to cellular activation.[5,6] Doses of chemoattractants which stimulate the respiratory burst and lysosomal

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[2] H. M. Korschak, K. Vienne, L. E. Rutherford, C. Wilkenfeld, M. C. Finkelstein, and G. Weissmann, *J. Biol. Chem.* **259,** 4076 (1984).

[3] J. E. Smolen and G. Weissmann, *Biochim. Biophys. Acta* **720,** 172 (1982).

[3a] Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; EGTA, (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DMSO, dimethyl sulfoxide; IBMX, isobutylmethylxanthine; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; HHBSS, Hanks' balanced salt solution with HEPES; TCA, trichloroacetic acid.

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[6] M. J. Berridge and R. F. Irvine, *Nature (London)* **312,** 315 (1984).