Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5867–5870, October 1982
Biochemistry

Platelet-derived growth factor binds specifically to receptors on vascular smooth muscle cells and the binding becomes nondissociable

(growth factor endocystosis)

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Communicated by D. Mark Hegsted, July 7, 1982

ABSTRACT **Radioiodinated platelet-derived growth factor (^{125}I-PDGF) was used in studies of PDGF binding sites on vascular smooth muscle cells. There was an excellent correlation between the ability of ^{125}I-PDGF to stimulate cell proliferation and to bind specifically to cultured vascular smooth muscle cells. The half-maximal concentration for both processes was 0.1 nM. There were 50,000 binding sites per cell. Reduced PDGF, prepared by treatment of PDGF with 20 mM dithiothreitol, had neither the ability to bind to smooth muscle cells nor to stimulate cellular proliferation. Epidermal growth factor, nerve growth factor, fibroblast growth factor, and histone B did not compete for the binding sites at a concentration of 10 nM. ^{125}I-PDGF binding was slowly reversible at 4°C and was rapidly and totally reversible after a 1-min incubation at 37°C. After continued incubation at 37°C, the binding became irreversible. The half-time for formation of the nondissociable state of ^{125}I-PDGF binding was ≈5 min at 37°C. The nondissociable state of binding was not formed at 4°C even after 1 hr of incubation. These data suggest that the sites we labeled are the PDGF receptors that mediate PDGF's mitogenic action and that a nondissociable state of PDGF binding is formed at 37°C. It is likely that nondissociable PDGF represents internalized ligand or binding to sites that are converted to a high-affinity state after the ligand binds.**

Platelet-derived growth factor (PDGF) is a potent stimulant of the growth of vascular smooth muscle and other mesenchymal cells *in vitro*. Presumably, this process is important *in vivo* in the proliferation of smooth muscle cells found in atherosclerotic lesions (1). Although the mechanism of action of PDGF is unproven, it is thought to involve internalization of receptor-bound PDGF, activation of protein kinase, or release of an unknown intracellular mediator after the binding of PDGF to its receptor. A prerequisite to an investigation of these phenomena is to develop methods for identifying PDGF receptor sites. We report here that PDGF binds to specific sites on cultured vascular smooth muscle cells. The binding sites we have identified have a high affinity for PDGF (half-maximal concentration of 0.1 nM), which is higher than the affinity of binding sites on 3T3 cells recently reported by Heldin *et al.* (half-maximal concentration of 1 nM) (2). We demonstrate an excellent correlation of mitogenic activity and binding, indicating that these sites are probably the physiologically relevant receptors for PDGF. The kinetics of binding suggest that receptor-bound PDGF rapidly becomes nondissociable at 37°C probably because of internalization of PDGF or transition of the receptor to a higher affinity state.

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METHODS

PDGF. Human PDGF was purified by a modification of a published procedure (3). In brief, platelet extracts were purified by the following sequential steps: ion-exchange chromatography (CM-Sephadex; stepwise elution with 1 M NaCl), boiling, adsorption on Blue Sepharose CL-6B, gradient elution from CM-Sephadex, and gel filtration on Bio-Gel P-150. Usually these procedures yielded PDGF that was electrophoretically homogeneous, although some preparations required further purification by HPLC with a Waters μBondapack alkylphenyl column equilibrated with 0.1% trifluoroacetic acid and an elution gradient from 0 to 70% acetonitrile. The homogeneous M_r 35,000 material was biologically active at 1 ng/ml.

^{125}I-Labeled PDGF (^{125}I-PDGF). Purified PDGF (10 μg) was radioiodinated by the iodogen method (4). PDGF in 0.3 M phosphate buffer (pH 7.3) was added to a glass tube that had been coated with 5 μg of iodogen (Pierce) and was incubated with 1 mCi (1 Ci = 3.7×10^{10} becquerels) of Na^{125}I for 10 min at 4°C. The reaction was terminated by removal of the soluble material, which was separated from free iodide by filtration with Sephadex G-25 that had been equilibrated with 1 M acetic acid/0.3% human serum albumin. The high M_r peak contained predominantly a M_r 35,000 species and a minor contaminant of M_r 17,000, which could be removed by HPLC as described above. The specific activity of ^{125}I-PDGF was 20,000 cpm/ng. The material was stable over 4 mo when stored frozen in 1 M acetic acid/0.3% human serum albumin.

Biological Activity of PDGF. Mitogenic activity of PDGF was estimated by the stimulation of DNA synthesis ([^{3}H]-thymidine uptake) in confluent BALB/3T3 clone A31 mouse cells as described (5).

Cells. Bovine vascular smooth cells were grown from primary explants as described (6). The cells (passages 2 through 6) were plated at a density of 2×10^5 cells per 75-cm^2 flask (Corning) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, penicillin (100 units), and streptomycin (100 μg). The cells were used for binding assays 5–7 days later (without a change of medium). Prior to the assay, the cells were suspended in 0.05% EDTA (10 min) and were washed three times in binding buffer (phosphate-buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$).

Binding Assay. ^{125}I-PDGF (10,000–15,000 cpm unless otherwise specified) was incubated with cell suspensions (2×10^5 cells per ml) in binding buffer (total volume, 600 μl). Some experiments were done in the presence of 0.1% albumin, which did not affect the results. In most experiments, incubations

Abbreviations: PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF.

were terminated by rapid filtration with Millipore EAWP filters, followed by one slow wash (10 ml for 5 min) and two rapid washes (10 ml each) of the filters with wash buffer (phosphate-buffered saline with 5% fetal calf serum) at 4°C. The amount of ^{125}I-PDGF bound to the filters in the absence of protein was <10% of the total binding. In other experiments, binding was assayed by rapid centrifugation of the incubation mixture in a Fisher model 59 microcentrifuge followed by three washes of the pellets with cold wash buffer prior to radioassay of the pellets. Nonspecific binding to cells, estimated by the amount of binding in the presence of 10 nM unlabeled PDGF, was usually between 20% and 40% of the total binding.

Reduction and Alkylation of PDGF. Pure PDGF (10 μg in 100 μl) was treated with 20 mM dithiothreitol/0.5 M ammonium bicarbonate for 2 hr at room temperature in the dark, followed by alkylation with 40 mM iodoacetamide for 15 min to prevent recombination of disulfide bonds. The material was either used directly at a 1:3,000 dilution or was first dialyzed to remove the reagents.

RESULTS

After radioiodination, PDGF retained its ability to stimulate DNA synthesis, and it bound specifically to vascular smooth muscle cells over the same concentration range over which it stimulated cell proliferation (Fig. 1). The density of binding sites calculated from Scatchard analysis is about 50,000 sites per cell (Fig. 1, *Inset*). Half-maximal occupancy of the binding sites occurred at 0.14 nM, which agrees closely with the concentration of ^{125}I-PDGF (0.1–0.2 nM) that gave a half-maximal proliferative response (Fig. 1). The same amount of binding was observed whether the assays were performed by filtration or by centrifugation of suspended smooth muscle cells. In other experiments, we attempted to measure specific binding to cells adherent to tissue culture plates using the method of Heldin *et al.* (2), but the large amount of PDGF binding to the plates precluded the routine use of this technique, especially for kinetic studies. For this reason, most of our assays were performed on suspended cells, and bound and free PDGF were separated by centrifugation or filtration.

Unlabeled PDGF had a high affinity in competing for the binding sites (Fig. 2), which correlates well with its mitogenic effect. Reduction and alkylation of PDGF, treatment which is known to irreversibly cleave the M_r 35,000 molecule into at least two lower molecular fragments (M_r of ≈14,000 and ≈18,000), caused a total loss of mitogenic activity and a concomitant loss in ability to compete for the receptor sites at a concentration of 0.1 nM (Fig. 2). This finding indicates that the integrity of disulfide bonds is necessary for PDGF to fully interact with its receptors. Epidermal growth factor, nerve growth factor, and fibroblast growth factor at 1 nM did not compete for the binding sites (not shown). EDTA (1 mM) and histone B (10 nM) did not affect ^{125}I-PDGF binding.

Kinetic studies indicated that the binding of PDGF reached a steady-state level within 15 min at 37°C and achieved a somewhat lower level of binding at 4°C (Fig. 3). During prolonged incubation at 37°C, the binding to smooth muscle cells remained constant for 6 hr (Fig. 3 *Inset*). After a steady-state level of binding had been attained, the addition of an excess of unlabeled PDGF caused only a slight diminution of ^{125}I-PDGF binding, indicating that the binding had become largely nondissociable (Fig. 3). The reversibility of binding was tested also in the cold and after a brief incubation at 37°C (Fig. 4). After incubation at 4°C for 1 hr, the binding was only slowly reversible. However, warming the cells to 37°C for 1 min (after the 1-hr incubation at 4°C) allowed the bound PDGF to be rapidly and totally dissociated from the cells. Similar experiments (Fig. 5) demonstrated that during a more prolonged warming period, the binding gradually became nondissociable; thus, the binding

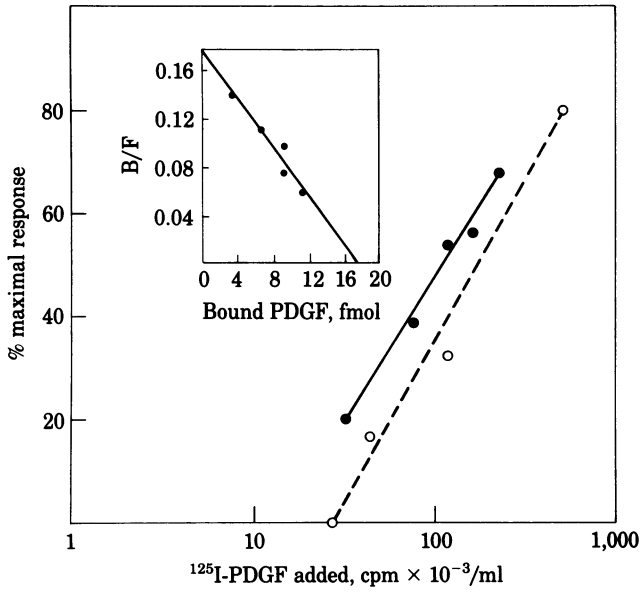

FIG. 1. Comparison of specific binding and mitogenic activity of ^{125}I-PDGF. ^{125}I-PDGF at the indicated concentrations was incubated with vascular smooth muscle cells and specific binding (●) was determined after 3 hr of incubation at 22°C. Mitogenic activity (○) was measured on the same samples by [^{3}H]thymidine uptake. Each value represents the mean of duplicate or triplicate determinations. Maximal binding (100%) refers to the level of specific binding defined by the intercept of the Scatchard plot (*Inset*) with the abscissa. Maximal mitogenic activity refers to [^{3}H]thymidine uptake stimulated by 10% fetal calf serum (10-fold above background). Half-maximal responses occurred at ≈100,000 cpm/ml, which corresponds to a concentration of 0.1 nM ^{125}I-PDGF.

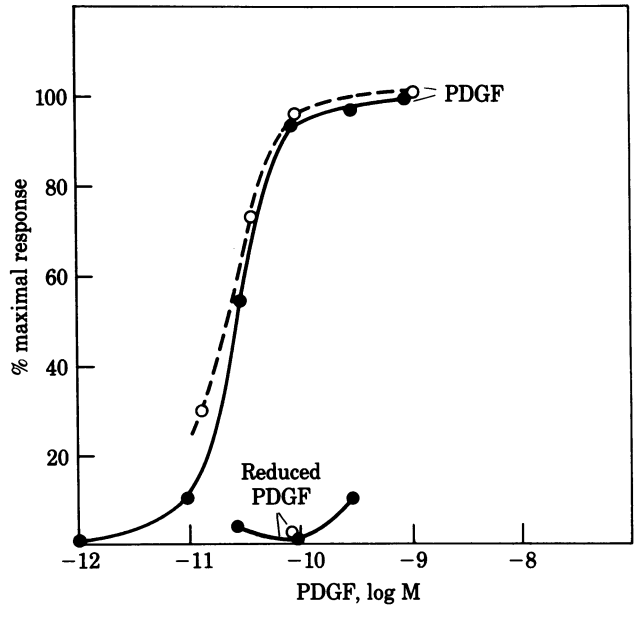

FIG. 2. Comparison of mitogenic activity with the ability of PDGF and its reduction products to compete for ^{125}I-PDGF binding sites. Pure PDGF was tested for its ability to stimulate DNA synthesis ([^{3}H]thymidine uptake) (○) or to inhibit specific binding of ^{125}I-PDGF (●). Reduced PDGF was prepared as described in *Methods*. The maximal mitogenic response was the response to 10% fetal calf serum. Maximal inhibition of binding refers to the total inhibition of specific binding, which was determined after 2 hr of incubation at 37°C.

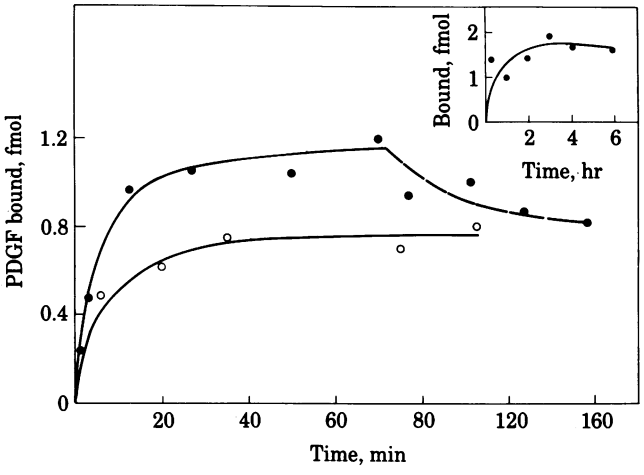

FIG. 3. Kinetics of specific binding of ^{125}I-PDGF to vascular smooth muscle cells. Smooth muscle cells were incubated with ^{125}I-PDGF at either 4°C (○) or 37°C (●) for the indicated time intervals, and specific binding was measured. At 72 min of incubation, an excess (10 nM) of unlabeled PDGF was added to the 37°C incubation. The inset shows that the binding remained constant at 37°C for up to 6 hr.

was dissociable after 2 min of warming but was not dissociable after 40 min of warming.

In order to follow the time course of formation of the nondissociable state, ^{125}I-PDGF was allowed to bind to cell surface receptors at 4°C for 1 hr, and the cells were washed extensively to remove the unbound ligand. The cells were then warmed rapidly to 37°C to follow the fate of the bound ^{125}I-PDGF. The nondissociable state was rapidly formed at the expense of dissociable binding with a half-time ranging from 4 to 10 min in three determinations (Fig. 6). Because in this experiment there was no ^{125}I-PDGF in the incubation medium available for additional binding during the warming period, the nondissociable state was formed directly from bound ^{125}I-PDGF, which initially (after 1 min of warming) had been dissociable. When this

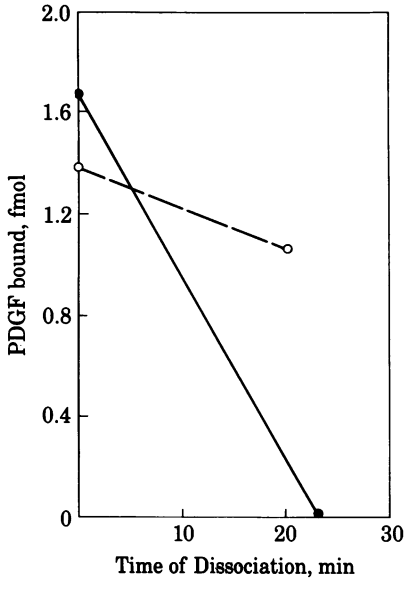

FIG. 4. Reversibility of specific PDGF binding at 4°C and after brief warming. ^{125}I-PDGF was bound to smooth muscle cells by incubation for 1 hr at 4°C. Some samples were then warmed to 37°C (●) for 1 min and other samples were maintained at 4°C (○). At zero time, an excess (10 nM) of PDGF was added to each sample and specific binding was measured. Each point represents the mean of six determinations.

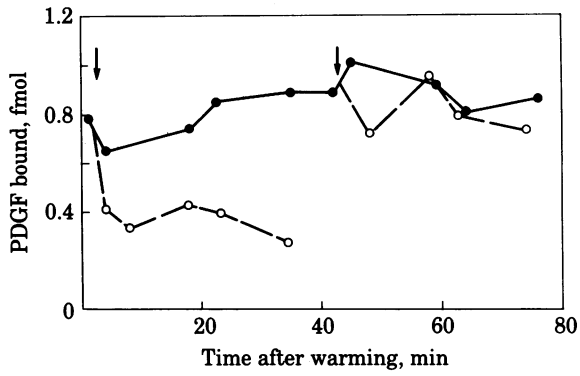

FIG. 5. Reversibility of specific PDGF binding after short and long periods of warming. ^{125}I-PDGF was incubated with smooth muscle cells for 1 hr at 4°C. Then at zero time, the incubations were warmed to 37°C, and specific binding was measured (●). After 2 min and after 42 min of warming (see arrows), an excess of unlabeled PDGF was added to some samples to test the reversibility of binding (○). This experiment is representative of two similar experiments.

experiment was performed in the continuous presence of ^{125}I-PDGF, there was a higher level of nondissociable binding attained, and there was persistence of a small amount (15–30% of the total) of dissociable binding even after 40 min of warming (not shown).

In summary, the kinetic data indicate that PDGF binding is slowly reversible at 4°C but can be reversed upon brief warming to 37°C (Fig. 5). Continued application of heat (37°C) after the cold incubation (Figs. 5 and 6) or initial incubation at 37°C for prolonged periods (Fig. 3) results in the formation of a nondissociable state, which arises directly from the dissociable state of binding (Fig. 6).

DISCUSSION

These data demonstrate that biologically active ^{125}I-PDGF binds to high-affinity sites on vascular smooth muscle cells and

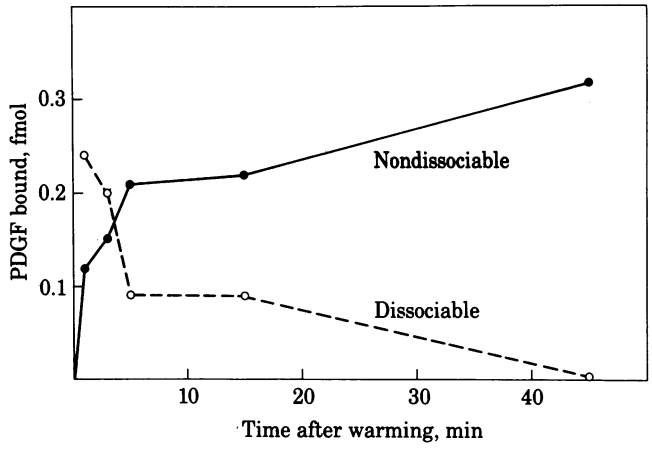

FIG. 6. Time dependence of the formation of the nondissociable state of specific PDGF binding. ^{125}I-PDGF was incubated with smooth muscle cells for 45 min at 4°C, and the cells were washed three times in cold binding buffer to remove the unbound ligand. At zero time, the incubations were warmed; at the indicated times, the amount of binding that could be dissociated by addition of excess (10 nM) unlabeled PDGF for an additional 20 min was determined (○). The portion of binding that remained 20 min after the addition of excess PDGF was considered to be nondissociable (●). The experiment shown is representative of two experiments, each done in duplicate determinations, which varied from the mean by <10%. The absolute level of binding is low because the ^{125}I-PDGF dissociated during the first 2 min of warming.

that the characteristics of the sites are those expected of the PDGF receptors through which PDGF acts as a mitogen. Over a large range of concentrations, the correlation of mitogenic activity and binding was excellent for both ^{125}I-PDGF and native PDGF. Reduction of PDGF caused a loss of its ability to compete for the binding sites and a concomitant loss of mitogenic activity. The same amount of specific binding was observed whether the assay was performed by filtration through Millipore filters or by centrifugation of the incubation mixture. Human osteosarcoma cells, which do not respond to PDGF, had no detectable PDGF-specific binding sites (not shown). For these reasons, it seems likely that the sites we have labeled by these methods are, in fact, PDGF receptors.

Our kinetic studies demonstrate that PDGF binding at 4°C is slowly reversible (Fig. 4) and can be totally reversed after a brief (1 min) incubation at 37°C (Figs. 4 and 5). However, the same bound ^{125}I-PDGF that was reversible after 1 min of warming becomes irreversible after more prolonged incubation at 37°C, with a half-time of 4–10 min for formation of the irreversible state. By contrast, the nondissociable state of binding is not formed at 4°C, even after prolonged incubation. A plausible interpretation of these data is that the binding becomes nondissociable because the ligand and the receptors are internalized into the cell at 37°C but not at 4°C. This seems especially likely because other polypeptides such as epidermal growth factor (7), insulin (8), low density lipoproteins (9), asialoglycoproteins (10), and diphtheria toxin (11) are internalized through specific binding sites over approximately the same time course we observed for formation of the nondissociable state.

An alternative explanation for the kinetic data is that the nondissociable state of binding represents the expression at 37°C of a high-affinity state of the receptor, which still might be located on the cell surface. Recent studies have demonstrated a temperature and time-dependent formation of high-affinity receptor sites for nerve growth factor, (12), epidermal growth factor (13), growth hormone (14), and insulin (15). In each case, the formation of the slowly dissociable state appears not to depend on ligand internalization. It has been suggested that the high-affinity state of binding plays an important role in the regulation of cell proliferation (13).

The demonstration of nondissociable cellular binding is especially relevant to the understanding of the action of PDGF. Previous studies in which quiescent cells were exposed to PDGF, washed, and treated subsequently with plasma devoid of PDGF, led to the suggestion that a brief (2 hr) exposure of cells to PDGF confers competence to enter the cell cycle, which persists after the PDGF in the medium is removed (16). Our data suggest that the persistance of cellular PDGF, which cannot be easily removed by simple washing procedures, may contribute to this phenomenon.

Heldin *et al.* (2) have reported binding experiments with ^{125}I-PDGF, which bound to 3T3 cells with a K_d of $\approx 1 \times 10^{-9}$ M. This K_d is approximately 10-fold higher than the concentration that we find to cause half-maximal stimulation of cell growth in 3T3 cells and in smooth muscle cells. Our K_d from binding experiments is in excellent agreement with the half-maximal mitogenic concentration for both 3T3 and vascular smooth muscle cells. The reasons for the discrepancy between our data and those of Heldin are unclear because neither the biological activity of the ^{125}I-PDGF nor kinetic studies were reported in Heldin's study (2), precluding a direct comparison with our data. It is noteworthy that Heldin *et al.* performed binding experiments on cells adherent to culture plates, a technique that we have found difficult to interpret because of the tendency of ^{125}I-PDGF to adhere to the plates. It is possible that adherent cells have receptors of different affinity than cells in suspension have, thus accounting for the discrepancies in our data. Finally, it should be noted that although the concentration (0.1 nM) at which there is 50% of maximal binding (Fig. 1) can be used to compare binding data and biological activity, this concentration may not be the absolute value of the equilibrium dissociation constant of interaction of ^{125}I-PDGF with its receptor because the kinetic data indicate that the system is not truly at equilibrium.

The study of PDGF binding and internalization is especially important in view of the large amount of information about the regulation of action of epidermal growth and nerve growth factor which has been gained from such studies. Although these hormones share many similarities in their actions, there are important differences in chemical structure, physical properties, kinetics of the growth responses, regulation by other hormones, and tissue specificity. Hence, future studies of PDGF receptors may lend support to the theories of the action of other growth hormones and also may reveal important differences between these hormones in receptor regulation and function.

Note Added in Proof. Since submission of this manuscript, Huang *et al.* (17) and Bowen-Pope and Ross (18) have described binding sites for PDGF on Swiss mouse 3T3 cells.

We are grateful to K. Burke for help in assaying mitogenic activity, to P. Moran for help in performing HPLC, to Dr. M. Moskowitz for help in culturing smooth muscle cells, and to G. Easterly for preparing the manuscript. This work was carried out during the tenure of an American Heart Association Clinician Scientist Award and with funds contributed by the Massachusetts affiliate. This work was also supported by National Institutes of Health Grant CA30101, the Council for Tobacco Research USA, Inc., and the Milton Fund of Harvard Medical School.

1. Ross, R. & Glomset, J. (1976) *N. Engl. J. Med.* **295**, 369–377.
2. Heldin, C. H., Westermark, B. & Wasteson, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3664–3668.
3. Antoniades, H. N. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7314–7317.
4. Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857.
5. Abelson, H., Antoniades, H. N. & Scher, C. D. (1979) *Biochim. Biophys. Acta* **561**, 269–275.
6. Coughlin, S. R., Moskowitz, M. A., Antoniades, H. N. & Levine, L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7134–7138.
7. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–169.
8. Goldfine, I. D., Smith, G. J., Wong, K. Y. & Jones, A. L. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1368–1372.
9. Anderson, R. G. W., Brown, M. S. & Goldstein, J. L. (1977) *Cell* **10**, 351–364.
10. Tolleshaug, H., Berg, T., Nilsson, M. & Noruny, K. R. (1977) *Biochim. Biophys. Acta* **499**, 74–84.
11. Dorland, R. B., Middlebrook, J. L. & Leppla, S. H. (1979) *J. Biol. Chem.* **254**, 11337–11342.
12. Landreth, G. E. & Shooter, E. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4751–4755.
13. King, A. C. & Cuatrecasas, P. (1982) *J. Biol. Chem.* **257**, 3053–3060.
14. Donner, D. B., Martin, D. W. & Sonenberg, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 672–676.
15. Corin, R. E. & Donner, D. B. (1982) *J. Biol. Chem.* **257**, 104–110.
16. Pledger, W. J., Stiles, C. D., Antoniades, H. N. & Scher, C. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2839–2843.
17. Huang, J., Huang, S., Kennedy, B. & Deuell, T. F. (1982) *J. Biol. Chem.* **257**, 8130–8136.
18. Bowen-Pope, D. & Ross, R. (1982) *J. Biol. Chem.* **257**, 5161–5171.