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Epidermal Growth Factor Receptors on PC12 Cells: Alteration of Binding Properties by Lectins

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The PC12 cell line displays cell surface receptors for both nerve growth factor (NGF) and epidermal growth factor (EGF). It has been previously shown that the lectin wheat germ agglutinin (WGA) alters the properties of NGF receptors on these cells. We now report that preincubations with either WGA or concanavalin A (Con A) decrease the binding of ^{125}I-EGF to PC12 cells by greater than 50%. The inhibition of binding occurred at 37°C and 4°C and could be blocked or reversed by the addition of sugars which bind specifically to WGA or Con A. Scatchard analysis revealed that these lectins decreased binding primarily by lowering the affinity of the receptor and to a lesser extent by decreasing receptor number. Succinylation of Con A (sCon A) produced a derivative that was less effective than the native lectin in decreasing EGF binding; however, addition of an antibody against Con A restored the ability of sCon A to decrease binding. Similar to results obtained with ^{125}I-NGF binding, WGA but not Con A was found to increase, by severalfold, the proportion of ^{125}I-EGF binding that is resistant to solubilization by Triton X-100 detergent. A potential association of the EGF receptor with cytoskeletal elements is discussed which could account for such results.

Key words: lectins, EGF receptors, Triton X-100, cytoskeletons, receptor clustering, PC12 cells

The PC12 cell line, derived by Greene and Tischler [1] from a rat pheochromocytoma, has been extensively studied as a model system for neuronal differentiation. These cells respond to the hormone nerve growth factor (NGF) by ceasing cell division, extending extensive neurites, and becoming electrically excitable. More recently, it has been shown that epidermal growth factor (EGF) also produces a number of biochemical and morphological effects on PC12 cells. EGF shares in common with NGF the ability to increase cellular adhesion and glucose uptake as well as to induce the synthesis of ornithine decarboxylase [2]. However, in marked contrast to NGF, EGF enhances proliferation of PC12 cells [2]. It has been suggested

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that both NGF and EGF may play important but different roles in the development of the nervous system [3].

The biological responses elicited by these hormones are mediated by specific cell surface receptors. Although the receptors for NGF [4] and EGF [2] on PC12 cells have been characterized in terms of numbers and affinities, the precise mechanism through which these receptors generate intracellular signals which trigger the number of complex biochemical events which occur has yet to be resolved. We have approached this problem by investigating the effects of agents that could potentially perturb membrane receptors. We have previously reported that the plant lectin wheat germ agglutinin converted one subtype of NGF receptor with rapidly dissociating kinetics into a type with slowly dissociating kinetics [5]. This conversion was accompanied by the association of the NGF receptor with cytoskeletal elements.

In this report, we have examined the effects of lectins on ^{125}I-EGF binding to PC12 cells. We have found both similarities and differences in the manner in which lectins influence EGF and NGF binding in this cell type.

MATERIALS AND METHODS

Materials

Lectins were purchased from Vector or EY Laboratories. Affinity-purified antibody against concanavalin A was purchased from Vector labs. Succinylated Con A (sCon A) was prepared by the method of Gunther et al [6]. EGF was prepared by the method of Savage and Cohen [7] and radiolabeled with ^{125}I to a specific activity of 55–90 cpm/pg using chloramine T as described by Aharanov et al [8].

Cell Preparation

PC12 cells (obtained from D. Schubert) were cultured in 150 mm^2 Falcon dishes in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 5% horse serum (Grand Island Biological Co) and were passaged every week. Cells were washed gently on the plate twice with Dulbecco's phosphate-buffered saline containing 1 mg/ml of glucose and bovine serum albumin (referred to as binding buffer) and were removed by trituration. Cell clumps were partially dispersed by gently passing the cells through a nylon mesh loaded Swinex filter. Cells were counted in a hemocytometer and diluted between 0.75 and 1.5 $\times$ 10^6 cells/ml unless otherwise indicated. Experiments were performed immediately after cell preparation.

^{125}I-EGF Binding

^{125}I-EGF was added to cells at a concentration of 50 ng/ml unless otherwise indicated. This concentration fully saturates EGF receptors on PC12 cells [2]. To determine nonspecific binding, some samples received ^{125}I-EGF along with a 500-fold excess (25 μg/ml) of unlabeled hormone. After the indicated time of incubation, three 100-μl samples of cells containing both bound and free ^{125}I-EGF were layered over 200 μl of 150 mM sucrose (in binding buffer) in 400-μl microfuge tubes. Samples were centrifuged at 10,000g for 30 sec in a microfuge, and tubes were frozen in a dry ice/ethanol bath and cut just above the cell pellet. The bottoms and tops of the tubes (containing bound and free ^{125}I-EGF respectively) were counted in a Beckman gamma counter. Total ^{125}I-EGF binding had nonspecific binding subtracted to yield specific binding, which is reported for all experiments. Nonspecific generally accounted for 10–20% of the total binding.

Triton X-100–insoluble binding was conducted in the following manner. Three 100-μl aliquots of medium containing ^{125}I-EGF and PC12 cells were layered over 200 μl of 0.5% Triton X-100 in 0.3 M sucrose, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4. This buffer has been used by Ben-Ze'ev et al [9] to prepare Triton X-100–insoluble cytoskeletal structures. Samples were spun at 10,000g for 30 sec in a microfuge and frozen and cut as described above. Radioactivity which pelleted to the bottom of the tube was considered Triton X-100–insoluble. Nonspecific binding samples were also centrifuged through the Triton X-100–containing buffer and were subtracted from total Triton X-100–insoluble counts to yield specific Triton X-100–insoluble binding. Approximately 75% of the nonspecific binding was Triton X-100–insoluble.

RESULTS

Carpenter and Cohen [10] have previously reported that WGA, Con A, and ricin I block binding of ^{125}I-EGF to cultured human fibroblasts. More recently, Hock and Hollenberg [11] documented that human placental membranes treated with WGA, Con A, or phytohemagglutinin exhibited decreased levels of EGF binding. These investigators subsequently used lectin affinity chromatography to purify the EGF receptor [12]. A number of lectins with different sugar specificities were tested for their effects on ^{125}I-EGF binding to PC12 cells (Table I). Only preincubations with WGA or Con A significantly reduced ^{125}I-EGF binding (both by approximately 75%). WGA was also found to decrease the amount of ^{125}I-NGF binding by 50% in PC12 cells, whereas Con A only produced a slight effect (5).

Both Con A and WGA produced a maximal inhibition of EGF binding at 20 μg/ml (Fig. 1). This inhibition could be prevented by adding the competing sugar for the lectin in the incubation medium. N-acetyl-D-glucosamine (100 mM) blocked the decrease in binding seen even at 100 μg/ml WGA, and α-methyl-mannoside (100 mM) prevented similar concentrations of Con A from inhibiting binding. Neither of the sugars alone significantly affected binding. Thus, the effects of the lectins appear to be specifically related to their sugar binding capabilities.

Figure 2 shows a time course of ^{125}I-EGF binding at 37°C and 4°C and the effects of two types of treatments with WGA. At 37°C, ^{125}I-EGF binding to untreated PC12 cells reached a maximum after 15 min. Thereafter, binding declined so that by

TABLE I. The Effect of Various Lectins on ^{125}I-EGF Binding

Lectin	^{125}I-EGF binding (cpm)	Percent binding of control
None	1,886 ± 294	100.0%
Ricin communis agglutinin I	1,678 ± 225	88.9%
Soybean agglutinin	1,714 ± 129	90.9%
Dolichos biflorus agglutinin	1,795 ± 166	95.2%
Limulus polyphemus agglutinin	1,813 ± 108	96.1%
Peanut agglutinin	1,822 ± 44	96.6%
Ulex europeus agglutinin I	1,737 ± 88	92.1%
Concanavalin A	424 ± 21	22.5%
Wheat-germ agglutinin	531 ± 130	28.2%

PC12 cells (0.75×10^6/ml) were incubated with the indicated lectin (50 μg/ml) for 30 min at 37°C. ^{125}I-EGF (63 cpm/pg) was added and binding was determined 30 min later. Values represent the mean and standard deviation of triplicate determinations.

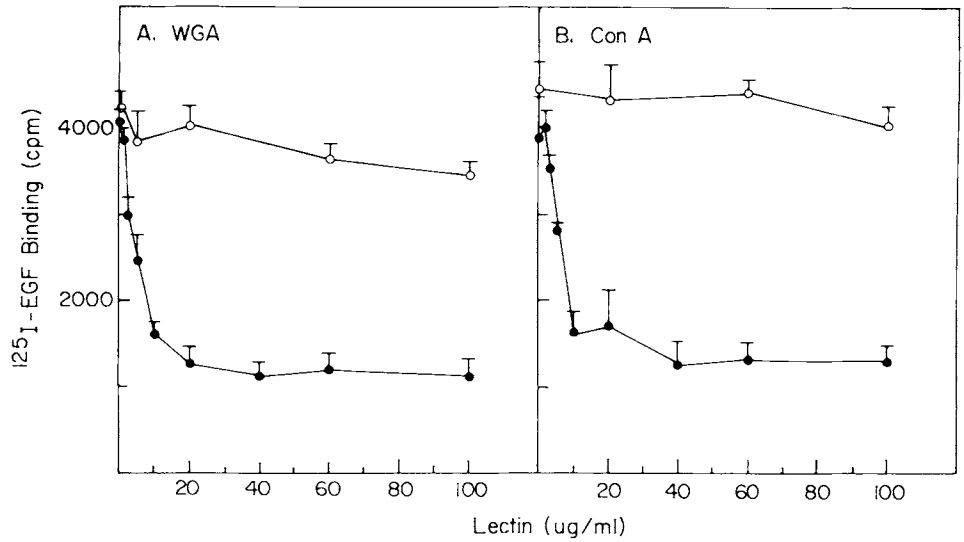

Fig. 1. Dose response of the lectin-induced inhibition of ^{125}I-EGF binding in the presence and absence of competing sugars. PC12 cells (1.2 × 10^6/ml) were incubated for 30 min at 37°C with WGA (panel A) or Con A (panel B) at the indicated concentrations and with (○) or without (●) 100 mM of the specific competing sugar (N-acetyl-D-glucosamine for WGA-treated cells and α-methyl-mannoside for Con A–treated cells). ^{125}I-EGF (85 cpm/pg) was added and binding was determined 30 min later. Values represent the mean and standard deviation of triplicate determinations.

90 min it had dropped to 40% of the maximal level. This loss or down regulation of receptor binding has been observed for EGF [13] as well as other ligands, and there is evidence that suggests this phenomenon is due to the internalization of the ligand-receptor complex [14,15]. On the other hand, binding of ^{125}I-EGF at 4°C was lower than at 37°C but was maintained at a steady state over 95 min as endocytosis is blocked at this temperature (Fig. 2B). Preincubating PC12 cells at 37°C or 4°C with WGA resulted in a substantial reduction in ^{125}I-EGF binding (33% and 20% of the control at 37°C and 4°C respectively). Inhibition of binding was generally greater when binding was assayed at lower temperatures. Conversely, when WGA was introduced 35 min after ^{125}I-EGF had been added to the cells, no subsequent alteration in binding was seen at either temperature (Figs. 2A, B). Similar findings were observed with Con A (data not shown). These results indicate that lectins cannot displace ^{125}I-EGF once it is bound to its receptor. Furthermore, WGA added 35 min after ^{125}I-EGF at 37°C did not alter the time course of down-regulation. Thus, this lectin appears not to interfere with the steady-state regulation of EGF receptors.

The inhibition of EGF binding by lectins was further investigated by Scatchard analysis. These experiments were conducted at 4°C in order to minimize internalization which can complicate equilibrium binding data (16). Figure 3 shows an example of a Scatchard plot with or without lectin pretreatment. This analysis demonstrates that Con A and WGA decrease binding primarily by causing a 4- to 5-fold lowering of receptor affinity. The apparent change in receptor affinity could be due to a conformational change in the receptor or, alternatively, could be produced by a

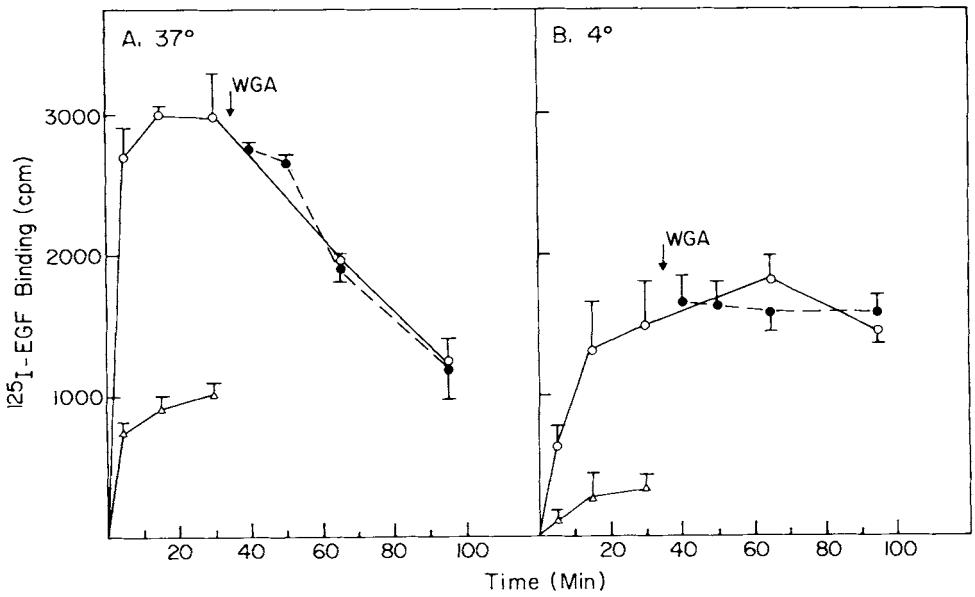

Fig. 2. Effects of temperature and time of lectin addition on the inhibition of ^{125}I-EGF binding by WGA. Experiments were either conducted entirely at 37°C (panel A) or 4°C (panel B). PC12 cells (1 × 10^6/ml) were incubated in the presence (△) or absence (○) of WGA (50 μg/ml) for 60 min. ^{125}I-EGF (81 cpm/pg) was then added, and binding was determined at the indicated times. At 35 min, half of the control sample received 50 μg/ml WGA (●) and binding continued to be assayed. Values represent the mean and standard deviations of triplicate determinations.

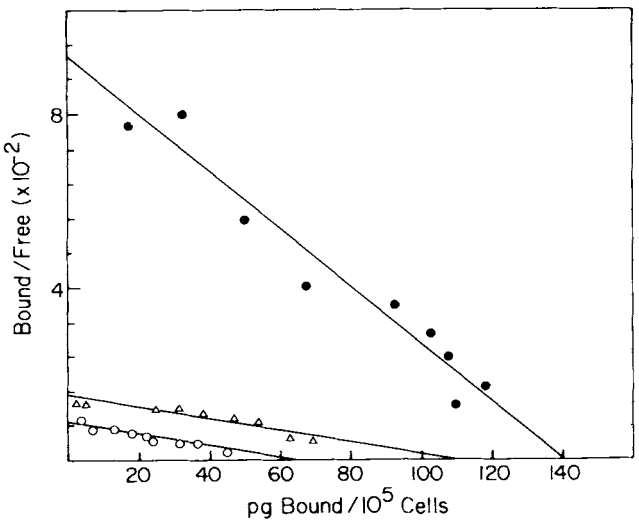

Fig. 3. Scatchard analysis of ^{125}I-EGF binding from WGA treated and untreated cells. PC12 cells (4 × 10^6/ml) were incubated with WGA (50 μg/ml) (○), Con A (50 μg/ml) (△), or without lectin treatment (●) for 30 min at 4°C. ^{125}I-EGF (71 cpm/pg) was added at concentration varying from 1 ng/ml to 200 ng/ml with nonspecific binding determined for each concentration. After 1 hr at 4°C, binding was assayed, and the results were transformed into a Scatchard plot. Lines through the data points are the best fit provided by linear regression.

competition of lectins and EGF for binding sites on the receptor. There is not sufficient information from our data to allow one to distinguish between these two possibilities. The lectins also affected the apparent number of EGF receptors. WGA consistently had a greater effect on receptor number than Con A, which had only a slight effect. An average of three experiments yielded the following values for the affinity and receptor number of lectin-treated and untreated cells: Control cells, $K_d = 3.28 \pm 1.85 \times 10^{-9}$ M, sites/cell = 55,200 ± 1,680; Con A–treated cells, $K_d = 1.37 \pm 0.21 \times 10^{-8}$ M, sites/cell = 46,000 ± 28,000; and WGA-treated cells, $K_d = 1.54 \pm 0.60 \times 10^{-8}$, sites/cell = 33,100 ± 10,000.

The effects of lectins on EGF receptors are reversible. Figure 4 shows that the binding properties of lectin-treated receptors can be restored to normal by removing the lectins from the cell surface with specific sugars. In this experiment performed at 4°C, Con A and WGA inhibited ^{125}I-EGF binding by 76% and 91% respectively. Unbound lectins were then removed by centrifuging cells and adding medium containing either 100 mM α-methyl-mannoside or N-acetyl-D-glucosamine to Con A- or WGA-treated cells respectively. By 30 min, ^{125}I-EGF binding to lectin-treated cells increased severalfold and reached values close to the control cells. Binding to WGA-treated cells increased 10-fold within 5 min after sugar addition, whereas the recovery of binding to Con A-treated cells showed a slower time course. This result could be

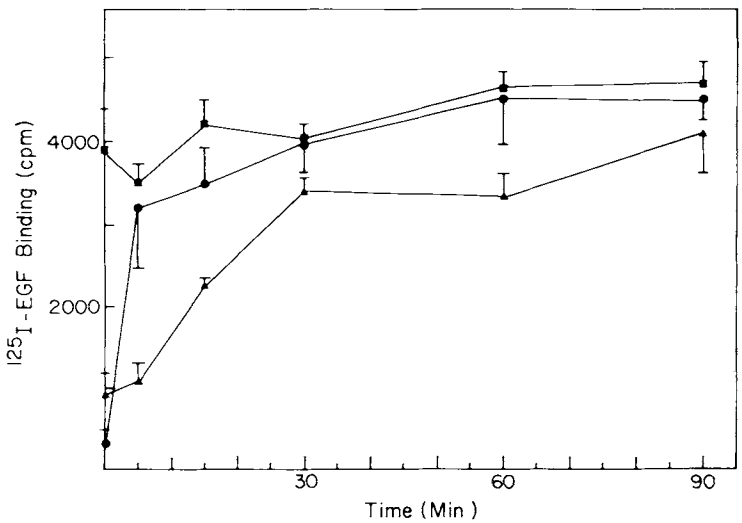

Fig. 4. Reversal of the lectin-induced inhibition of ^{125}I-EGF binding by competing sugars. PC12 cells (1×10^6/ml) were incubated with Con A (50 μg/ml) (▲), WGA (50 μg/ml) (●), or without lectins (■) for 30 min at 37°C. Cells were cooled on ice for 5 min, and ^{125}I-EGF (90 cpm/pg) was added for 45 min at 4°C. Binding was determined at this time (time "O" binding measurement). Cells were then centrifuged at 500g for 5 min at 4°C in order to remove the medium and were resuspended either in binding buffer (control cells), binding buffer containing 100 mM N-acetyl-D-glucosamine (WGA-treated cells), or binding buffer containing 100 mM α-methyl-mannoside (Con A–treated cells). ^{125}I-EGF (50 ng/ml) was present in all of the above resuspension buffers, and binding was determined as a function of time after the addition of the competing sugars. Values represent the mean and standard deviations of triplicate determinations.

due to a slower dissociation rate of Con A from the EGF receptor. While the effects of WGA were fully reversible, recovery of Con A–inhibited binding was 70–90% of the control in different experiments.

The ability of Con A to decrease ^{125}I-EGF binding was diminished by chemically derivatizing the lectin with succinic anhydride. Succinylation of Con A (sCon A) converts this normally tetravalent lectin into a divalent form [6]. Although both forms have identical carbohydrate specificities, sCon A has markedly reduced cross-linking abilities manifested by its weak ability to agglutinate sheep erythrocytes. Table II (averaged from four experiments) shows that sCon A inhibited EGF binding by only 15% compared with 53% for Con A. The impaired ability of sCon A to inhibit binding could be due to a reduced capacity to cross-link receptors or simply due to a lower affinity constant, as the tetravalent lectin has the potential to form a higher affinity complex than divalent sCon A by virtue of its ability to form multivalent attachments more readily.

It has been previously shown that the agglutinating capabilities of sCon A could be enhanced by adding an antibody to Con A [6]. As demonstrated in Table II, we have found that an antibody against Con A also enhanced the ability of sCon A to inhibit ^{125}I-EGF binding from 15% to 43%. This finding further supports the notion that multivalent lectin attachments are in some way important for affecting the binding properties of EGF receptors.

We have previously shown that WGA increases the proportion of ^{125}I-NGF binding that is resistant to Triton X-100 solubilization [5]. It was proposed [5,17] that this Triton X-100–resistant binding may reflect an association of the NGF receptor with underlying cytoskeletal elements. Triton X-100 is a detergent that has been used to solubilize membranes in order to prepare cytoskeletal structures [9]. Extraction of PC12 cells with 0.5% Triton X-100 leaves only a nucleus and an array of cytoskeletal fibers (unpublished observations). We have investigated the Triton X-100 solubility of ^{125}I-EGF binding by centrifuging a cell suspension containing bound ^{125}I-EGF through a 0.3 M sucrose solution containing 0.5% Triton X-100 as described by Schechter and Bothwell [17]. This procedure effectively solubilizes PC12 cells, and,

TABLE II. Antibodies to Con A Enhance the Inhibitory Effect of sCon A on ^{125}I-EGF Binding

	^{125}I-EGF binding Treatment with Con A antibody		
Lectin treatment	−	+	$P < 0.05$
	Percent of control ± SD		
None	100.0	89.8 ± 6.6	No
Con A	46.8 ± 1.5	47.7 ± 5.1	No
sCon A	85.1 ± 6.4	57.3 ± 8.2	Yes

Con A or sCon A was added to PC12 cells for 30 min at 37°C. Cells were then centrifuged at 500g for 5 min in order to remove medium containing unbound lectins and were then resuspended in binding buffer containing 100 μg/ml of an antibody against Con A. After a 30-min incubation at 37°C, ^{125}I-EGF was added and binding was assayed 30 min later. Results were normalized in reference to a cell sample that received neither lectin nor Con A antibodies (100.0% binding). Data presented here represent the mean and standard deviations from four such experiments. Student's paired t-test was used to assess the statistical significance of the Con A antibody treatment.

since it is very rapid, problems such as proteolytic degradation or nonspecific binding of ^{125}I-EGF to cytoskeletal structures are minimized.

Figure 5 shows the effects of lectins on the Triton X-100 solubility of ^{125}I-EGF binding. In these experiments, ^{125}I-EGF was first bound to cells, and the indicated lectin was added subsequently. As previously indicated in Figure 2, lectin addition after ^{125}I-EGF did not significantly decrease binding. In the absence of any lectin treatment, only 10% of the ^{125}I-EGF binding was not solubilized by the detergent. However, after the addition of WGA, 60% of the binding became insoluble in Triton X-100. Con A, which is equally effective as WGA in inhibiting EGF binding, produced only a small increase in the resistance of binding to Triton X-100 solubilization. Lectins, such as soybean agglutinin (SBA), that did not inhibit binding also did not overtly affect the Triton X-100 solubility properties of the EGF receptor.

DISCUSSION

Lectins have proved to be useful tools to probe the structure and function of hormone receptors. These carbohydrate-binding proteins can interact with cell surface glycoproteins and have been shown to influence the binding properties of a variety of hormone receptors [10,18,19]. In some instances, lectins can mimic the action of the hormone itself. For example, binding of lectins to the insulin receptor has been shown to induce a number of insulin-like responses [19], and recent evidence suggests that binding of lectins and insulin may generate identical second messengers [20]. There-

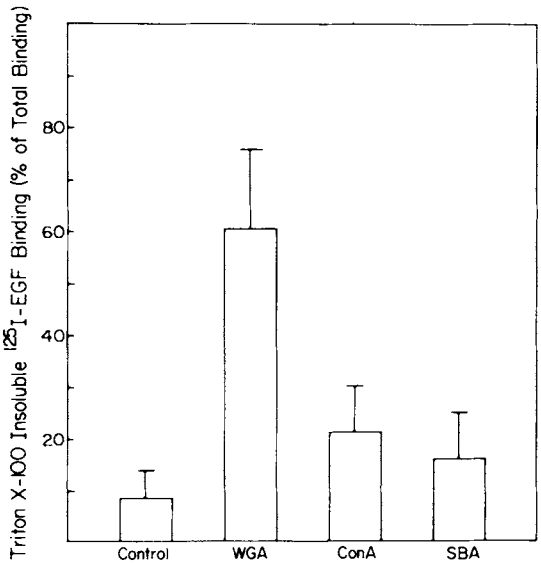

Fig. 5. The effects of lectins on the Triton X-100 solubility of ^{125}I-EGF binding. PC12 cells were incubated with ^{125}I-EGF for 20 min at 37°C. The indicated lectin (50 μg/ml) was then added for an additional 10 min prior to a determination of specific total and Triton X-100–insoluble binding. Control cells received no lectin treatment. The percent Triton X-100–insoluble binding of the total binding was averaged from four independent experiments and the mean and standard deviations are presented in this figure.

fore, lectin perturbations of cell surface receptors may lead to important insights into the mechanism of hormone action.

We have shown in this and a previous report [5] that certain lectins alter the binding properties of EGF and NGF receptors on PC12 cells. The lectin specificities for the two receptors are slightly different. Both WGA and Con A decrease ^{125}I-EGF binding with equal effectiveness, whereas only WGA substantially reduces the binding of ^{125}I-NGF. However, there are a number of similarities in the lectin-induced inhibition of binding to NGF and EGF receptors. For both receptors, maximal inhibition of binding occurs at lectin concentrations of approximately 25 μg/ml. The decrease in binding can be prevented by simultaneously adding specific competing sugars with the lectins to the medium and can be reversed by adding these same sugars to compete for lectins that are bound to the cell surface. The decrease in ^{125}I-EGF and ^{125}I-NGF binding by WGA are due to a change in receptor affinity and number (Fig. 3 and unpublished results). On the other hand, Con A decreases ^{125}I-EGF binding almost exclusively by lowering receptor affinity. Lectin-induced clustering of proteins may be important for this effect since sCon A is much less effective than Con A in decreasing binding. Reestablishing clustering activity of sCon A with a bivalent antibody to Con A restores the potency of sCon A to close to that of the native lectin. The decrease in receptor numbers by WGA is apparently not due to internalization since full binding can be rapidly recovered by removing the lectins from the cell surface by adding N-acetyl-D-glucosamine. Furthermore, lectin inhibition of EGF and NGF binding occurs at 4°C, a temperature that excludes the participation of endocytosis.

The mechanism whereby lectins decrease the binding of EGF and NGF to their receptors on PC12 cells is unclear. The two simplest explanations are that lectins either induce a receptor conformational change or sterically block the EGF binding site in the receptor so that the ligand no longer has free access. It is also possible that the lectin disrupts an interaction between the receptor and a protein which modulates receptor affinity. Evidence for the existence of such modulating proteins has been obtained for a few hormone receptors [21,22]. However, once the hormone-receptor complex is established, our results show that lectins cannot readily displace the bound lectin.

The lectin WGA also increases by severalfold the resistance of EGF receptors to Triton X-100 solubilization. Similar results were obtained for the Triton X-100 solubility of NGF receptors where there was an excellent correlation between the extent of Triton X-100–insoluble binding and the amount of NGF bound to slowly dissociating receptors [5]. From these results we speculate that WGA can induce an attachment of NGF and EGF receptors to cytoskeletal structures. It is unclear whether these receptors are linked to the cytoskeleton directly or whether the lectin cross-bridges the receptor to another membrane protein which is connected to the cytoskeletal matrix. Interestingly, Con A, which is as effective as WGA in decreasing EGF binding, does not dramatically change the Triton X-100 solubility of EGF binding. Since Con A does not change Triton X-100–insoluble EGF binding, this result indicates that the cytoskeleton does not play a role in the inhibition of EGF binding produced by WGA or Con A.

An alternative explanation is that WGA induces aggregation of receptors which are rendered insoluble in detergent. Linsley and Fox [23] have observed that half of the EGF-binding activity from A-431 human epidermal carcinoma cell membranes

was not solubilized by Triton X-100 extraction. They concluded that EGF receptors display a tendency to aggregate in nonionic detergent solutions. However, our results are more consistent with an association with cytoskeletal structures, since Triton X-100–insoluble EGF binding in PC12 cells can be pelleted at very low centrifugal forces and since Con A, which interacts with EGF receptors and has the potential to aggregate molecules, produces only a small change in the Triton X-100 solubility of EGF binding.

EGF produces a number of rapid morphological changes in cells, changes which likely involve cytoskeletal elements. Addition of EGF to A431 cells results in rapid but transient appearance of surface ruffles and extension of filopodia [24], and actin and α-actinin have been found to reorganize in this same cell line after EGF treatment [25]. These studies suggest that EGF induces the interaction of cytoskeletal proteins with certain membrane structures. EGF has also been reported to increase the number of long microvilli in PC12 cells, indicating its influence on the cytoskeletal organization of this cell type as well [26].

An interaction of the EGF-receptor complex directly with the cytoskeleton or through an actin-binding protein linked to the plasma membrane such as vinculin [27] could initiate events such as membrane ruffling or even the internalization of the hormone-receptor complex. Lectins such as WGA, which can modulate associations between membrane glycoproteins and the cytoskeleton [28,29], may be useful in investigating the role of the cytoskeleton in the mechanism of action and internalization of hormone receptors such as the EGF receptor.

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