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Alteration of Insulin Binding and Cytoskeletal Organization in Cultured Fibroblasts by Tertiary Amine Local Anesthetics

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Tertiary amine local anesthetics cause a time- and dose-dependent, reversible increase in insulin binding sites in cultured chick embryo fibroblasts. Incubation of fibroblasts with 0.2 mM dibucaine for 3 h at 37°C results in a twofold to threefold increase in insulin binding, with an increase in average number of binding sites ($K_a = 3.0 \times 10^7 M^{-1}$) from 9×10^3 to 29×10^3 per cell. Trypsin or ethyleneglycoltetraacetic acid (EGTA) alone increases insulin binding twofold to threefold, but fails to further increase ^{125}I-insulin binding in cells pretreated with dibucaine. Transformation of chick embryo fibroblasts with Rous sarcoma virus causes a threefold to fivefold increase in insulin binding, which is not further increased by incubation with dibucaine. As demonstrated by transmission electron microscopy, dibucaine and trypsin also induce changes in the cytoskeleton of chick embryo fibroblasts, characterized by disorganization and disappearance of microfilament and microtubule bundles. These alterations are accompanied by gross morphologic changes, including rounding of cells and appearance of numerous ruffles and blebs on the cell surface. These observations are consistent with the hypothesis that expression of surface receptors in cultured chick embryo fibroblasts is related to the organization and disorganization of cytoskeletal structures.

Key words: insulin receptors, ^{125}I-insulin binding, microtubules and microfilaments, cultured fibroblasts, local anesthetics

The changes in distribution of cell surface receptors after binding of ligands, a two-step process involving clustering of receptors into patches and accumulation of these patches at one pole of the cell, demonstrate the importance of fluidity of plasma membranes and the movement of membrane components in receptor processing [1, 2]. In turn, the movement of membrane receptors (and cytoplasm) in cells has been shown to be a function of the organizational pattern of microfilaments and microtubules [3].

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In previous studies of the growth-promoting action of insulin, we have demonstrated that cultured chick embryo fibroblasts (CEF) have specific insulin receptors, the occupancy of which is closely correlated with the magnitude of the pleiotypic responses to insulin [4, 5]. Brief incubation with trypsin or transformation of CEF by Rous sarcoma virus (RSV) results in a severalfold increase in the number of these receptors [6]. Similarly, treatment of untransformed CEF with cytochalasin B causes an increase in the number of insulin receptors as well as changes in cell shape and surface morphology. Cytochalasin B fails to increase these receptors in transformed CEF [7], which already have a round cell shape, numerous ruffles, and disorganized cytoskeletal structure.

Recently it has been reported that tertiary amine local anesthetics are able to produce structural and organizational changes in both microtubules and microfilaments, and alter ligand-induced distribution of concanavalin A receptors [8, 9]. In this study, we have examined the effects of local anesthetics in cultured CEF with regard to cytoskeletal integrity and insulin binding. Our observations demonstrate that tertiary amine local anesthetics, like other agents that alter cell shape and cytoskeletal organization, have major effects on the expression of insulin receptors in cultured CEF.

MATERIALS AND METHODS

Fertilized eggs were supplied by Spafes, Inc. (Norwich, Connecticut). Modified Eagle's medium (Temin) and fetal bovine serum were purchased from Grand Island Biological Company (Grand Island, New York). Bovine serum albumin (fraction V) and and cytochrome C were from Sigma Chemical Co. (St. Louis), trypsin (190 units/mg) was from Worthington Biochemical Corp. (Freehold, New Jersey), Rous sarcoma virus (Schmidt Ruppin subgroup A) from American Type Culture Collection (Rockville, Maryland), and ^{125}I-insulin (specific activity ~ 100 μCi/μg) from New England Nuclear (Boston). Dibucaine-HCl was obtained from Ciba Pharmaceuticals (Worchester, Massachusetts), and mepivacaine-HCl, procaine-HCl, and tetracaine-HCl from Sterling Winthrop Laboratories, Worcester, Massachusetts). Porcine insulin was a gift from Dr. R.E. Chance, Eli Lilly Company (Indianapolis).

Cell Culture

Chick embryo fibroblasts, prepared from 12-day-old embryos according to a previously published procedure [10] were cultured in modified Eagle's medium (Temin) containing 4% fetal bovine serum at 37°C with 5% CO_2. Primary cultures were dissociated with 0.25% trypsin, and 2.5×10^5 cells were subcultured in 35-mm Falcon tissue culture dishes in the same medium. Cells were fed on day 2 and used for experiments 24 h later.

Infection of CEF With Rous Sarcoma Virus

Primary cultures of CEF were dissociated with trypsin, and 1×10^6 cells were incubated in 100-mm Falcon tissue culture dishes with modified Eagle's medium (Temin) without serum for 6 h. CEF were infected with Rous sarcoma virus, Schmidt Ruppin subgroup A, as described previously [11]. After 4 days, confluent cultures of infected CEF were dissociated with trypsin, and 2.5×10^5 cells were plated in 35-mm dishes. Cells were fed on day 2 and used for experiments 24 h later.

Measurement of ^{125}I-Insulin Binding

The specific binding of ^{125}I-insulin to confluent cultures of untransformed and transformed CEF was measured with intact cells attached to culture dishes as described else-

where [4]. All binding assays were performed in triplicate, with the results expressed as femtomoles of ^{125}I-insulin bound per milligram cell protein. Specific binding was obtained by subtracting the amount of radioactivity bound to cells in the presence of 16.6 μM unlabeled insulin from that bound in the absence of unlabeled insulin. The protein content of cultures was determined by the method of Lowry, with a bovine serum albumin standard.

Transmission Electron Microscopy

For visualization of cytoskeletal organization, cultured fibroblasts were treated with Triton X-100 by the method of Small and Celis [12]. Approximately 2.5×10^5 CEF were seeded and grown in 35-mm culture dishes containing four gold electron microscope grids which had been precoated with carbon, incubated at 110°C overnight, and sterilized with ultraviolet radiation. On day 3, grids were removed, rinsed twice with phosphate-buffered saline, pH 7.4 (PBS), and immersed in 0.1% Triton X-100 in Pipes buffer. Cells were fixed with 2.5% glutaraldehyde for 30 min at room temperature. Samples were negatively stained and prepared for microscopic examination essentially as described by Small and Celis [12]. Cytoskeletal structures were examined and photographed with a Hitachi HV-125E electron microscope at 75 kV.

For visualization of microtubules and microfilaments in thin sections of CEF, cells grown in 35-mm culture dishes were fixed with 2.5% glutaraldehyde, postfixed with osmium tetraoxide and tannic acid, stained with uranyl acetate, dehydrated, and infiltrated in Spurr's low-viscosity resin according to previously described procedure [7]. Sections of 500 Å were cut perpendicularly to the dish, poststained with uranyl acetate and lead citrate, and examined under a Hitachi HV-125E electron microscope at 50 kV.

RESULTS

Effect of Anesthetic Agents on ^{125}I-Insulin Binding

Treatment of confluent cultures of untransformed CEF with tertiary amine local anesthetics of varying hydrophobicity caused a twofold to threefold increase in the specific binding of ^{125}I-insulin (Fig. 1). The concentration required to produce maximum binding differed from each anesthetic (Table I), dibucaine being the most and procaine the least potent. The concentration required to produce the maximum effect on ^{125}I-insulin binding in CEF was similar to that required for redistribution of concanavalin A receptors in 3T3 cells [8, 9], and was related to the hydrophobicity of the anesthetic (reviewed in Seeman [13]).

Specific binding increased with increasing concentration of anesthetic. A 2.7-fold increase was observed with 0.2 mM dibucaine (Fig. 2a), a concentration which did not affect attachment of cells to plates. Although treatment of CEF with 0.4 mM dibucaine produced a threefold increase in binding, it also resulted in a 60–65% decrease in the amount of protein per plate, due to detachment and loss of cells. The increase in binding was time-dependent, reaching a maximum 3 h after incubation of cultures at 37°C with 0.2 mM dibucaine (Fig. 2b).

The increase in ^{125}I-insulin binding induced by dibucaine was due to an increase in the total number of binding sites, determined by Scatchard analysis, from 9×10^3 to 29×10^3 per cell (Table I). The affinity constant for binding ranged from 3.0 to $3.6 \times 10^7 M^{-1}$, and was indistinguishable from the constant for low-affinity binding sites of untreated CEF (Table II). Treatment of untransformed CEF with either trypsin or cytochalasin

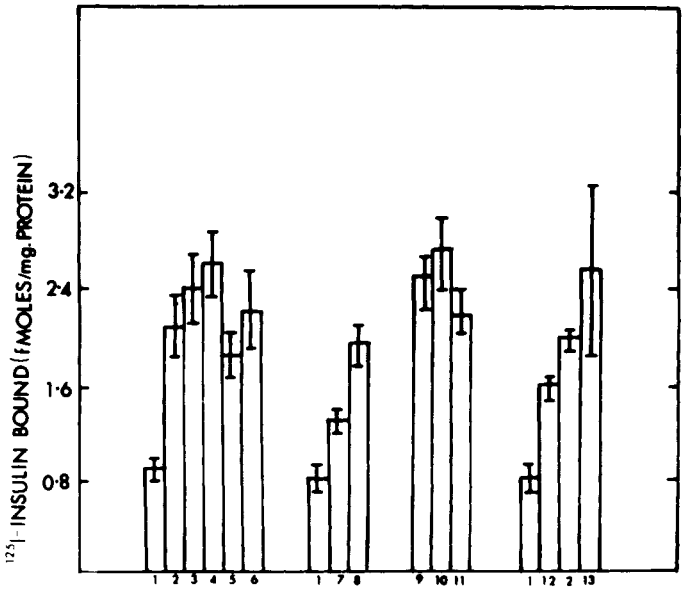


Fig. 1. Effect of tertiary amine local anesthetics, trypsin, and EGTA on ^{125}I-insulin binding in cultured chick embryo fibroblasts. Confluent cultures of untransformed CEF were incubated with modified Eagle's medium (Temin) containing 4% fetal bovine serum (1,12), 0.2 mM dibucaine (2,13), 0.5 mM tetracaine (3), 5 mM lidocaine (4), 5 mM mepivacaine (5), 10 mM procaine (6) for 3 h at 37°C; and containing 4 and 10 mM EGTA (7, 8) for 40 min at 37°C. Similarly, cultures of RSV-transformed CEF were incubated with growth medium (9), 0.2 mM dibucaine (10), and 5 mM procaine (11) for 3 h at 37°C. Cultures were washed twice with PBS and used for ^{125}I-insulin binding, except groups 12 and 13, which were treated with 10 μg/ml trypsin for 7 min at room temperature prior to insulin binding as described previously [4]. Data are given as the mean ± SEM of triplicate determinations.

B [7] also caused an increase in the number of low-affinity binding sites; a similar increase in specific binding was observed when confluent cultures of untransformed CEF were treated with EGTA, 5–10 nM, for 40 min at 37°C (Fig. 1).

The effect of removing dibucaine from the medium on binding of ^{125}I-insulin by CEF was investigated. Fibroblasts were incubated with 0.2 mM dibucaine for 3 h at 37°C, washed twice with modified Eagle's medium (Temin) containing 4% fetal bovine serum, and incubated up to 6 h in the same medium. A decrease in ^{125}I-insulin binding was observed 2 h after removal of dibucaine. Binding fell to control levels within 6 h (Fig. 2c), with a $T_{1/2}$ of approximately 2.5 h.

Virus-transformed CEF have been shown to have a threefold to fivefold increase in the number of low-affinity binding sites. Treatment of transformed cells with either 0.2 mM dibucaine or 10 mM procaine for 3 h at 37°C did not lead to a further increase in binding (Fig. 1).

Effect of Trypsin on ^{125}I-Insulin Binding

Treatment of confluent cultures of untransformed CEF with trypsin, 10 μg/ml, causes a twofold to threefold increase in specific binding of ^{125}I-insulin [4]. This is due to an increase in the number of low-affinity binding sites from 9×10^3 to 29×10^3 per cell [5]. The effect of sequential treatment of CEF with local anesthetics and trypsin was

TABLE I. Effect of Tertiary Amine Local Anesthetics on ^{125}I-Insulin Binding in Cultured Chick Embryo Fibroblasts

Local anesthetics	Concentration (mM)	^{125}I-Insulin bound (fmoles/mg protein ± SEM)
None	–	0.9 ± 0.08
Dibucaine	0.2	2.0 ± 0.05
Tetracaine	0.25	1.7 ± 0.10
Tetracaine	0.50	2.4 ± 0.20
Lidocaine	2.5	1.5 ± 0.08
Lidocaine	5.0	2.7 ± 0.27
Mepivacaine	5.0	2.3 ± 0.10
Mepivacaine	10.0	2.2 ± 0.30
Procaine	5.0	1.8 ± 0.10
Procaine	10.0	2.0 ± 0.50

Experimental conditions are described in legend to Figure 1.

TABLE II. Affinities and Number of Insulin Binding Sites in Chick Embryo Fibroblasts Treated With Dibucaine and Cytochalasin B

Treatment	Affinity constant K_1 ($\times 10^8 M^{-1}$)	Affinity constant K_2 ($\times 10^7 M^{-1}$)	Binding sites ($\times 10^3$)
Untreated	2–6	0.8–3	8.5–9
Dibucaine, 0.2 mM, 3 h at 37°C	–	3.0–3.6	20–29
Cytochalasin B, 10 μg/ml, 24 h at 37°C	–	0.8–3.6	18–22

The affinity constants and number of ^{125}I-insulin binding sites per cell were determined by Scatchard analysis for confluent cultures of untreated CEF [4], of CEF treated with CB [7], and of CEF treated with dibucaine.

studied. Since local anesthetics and trypsin each disrupt the organization of cytoskeletal structure, the effect of sequential treatment was investigated. Although treatment of CEF with trypsin or dibucaine alone caused a (2–2.5)-fold increase in ^{125}I-insulin binding (Fig. 1), no further increase was observed when cultures were treated with dibucaine followed by trypsin. Similarly, no increase in binding was observed when CEF were sequentially treated with other local anesthetics and trypsin.

Effect of Dibucaine and Trypsin on CEF Morphology and Cytoskeletal Structure

In previous studies we have demonstrated that treatment of confluent cultures of CEF with cytochalasin B caused an increase in the number of low-affinity insulin binding sites. The increase in receptors was accompanied by changes in cell shape from flat and

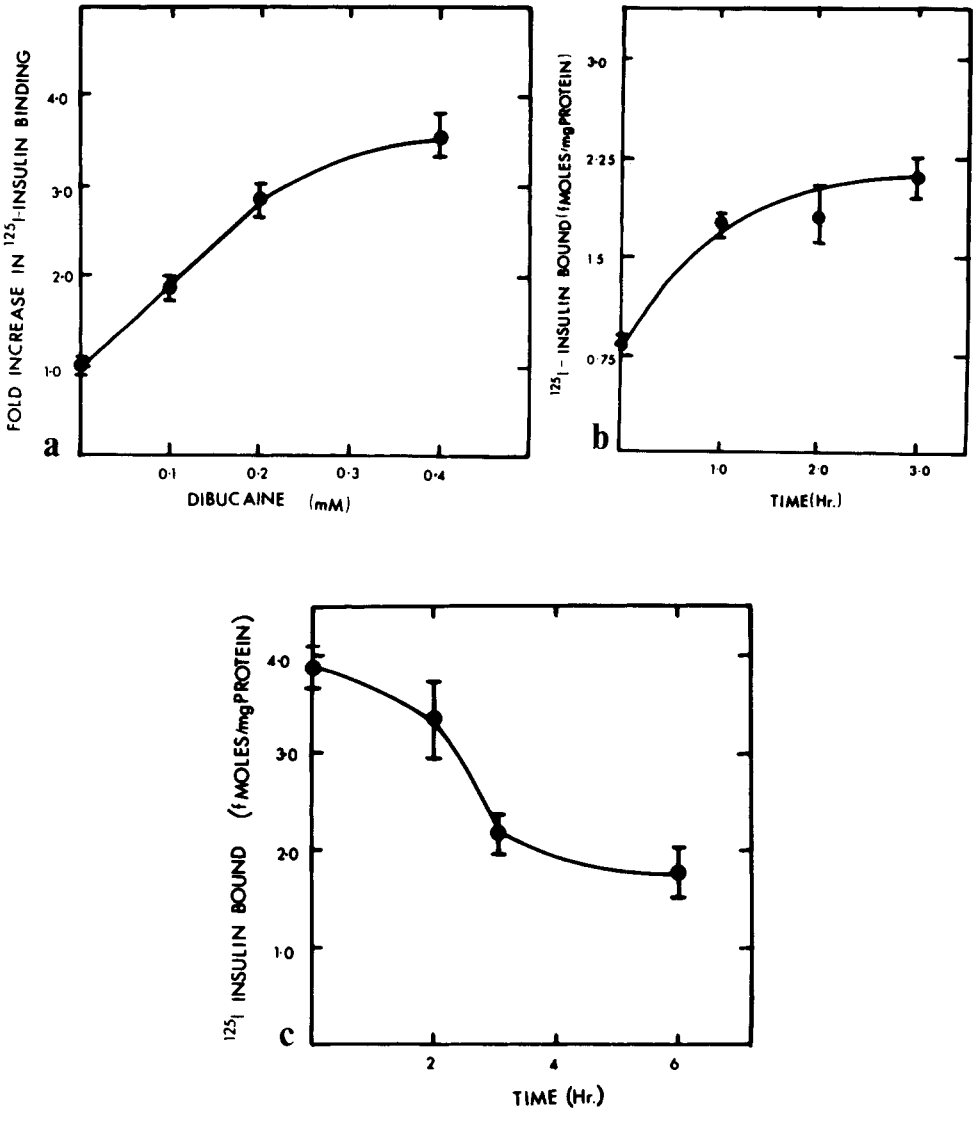


Fig. 2. Effect of dibucaine on ^{125}I-insulin binding to chick embryo fibroblasts. a: Concentration dependence. Confluent cultures of CEF were incubated with several concentrations of dibucaine for 3 h at 37°C. Specific binding is given as mean of triplicates ± SEM. b: Time dependence. Confluent cultures of CEF were incubated with 0.2 mM dibucaine in PBS for indicated time periods and specific binding of ^{125}I-insulin was determined as described in text as a mean of triplicates ± SEM. c: Effect of dibucaine removal on ^{125}I-insulin binding. Confluent cultures of CEF were incubated with 0.2 mM dibucaine for 3 h at 37°C, washed twice, and incubated in modified Eagle's medium (Temin) containing 4% fetal bovine serum. At indicated time periods, specific binding of ^{125}I-insulin was determined; untreated cultures (not shown) bound 1.8 fmoles ^{125}I-insulin/mg protein.

fusiform to round, by development of numerous ruffles and blebs on the cell surface, and by disruption and disorganization of cytoskeletal structures, including microfilament bundles [7]. Since local anesthetics cause an increase in specific binding of ^{125}I-insulin, we have investigated the effects of dibucaine on cell shape and cytoskeletal organization.

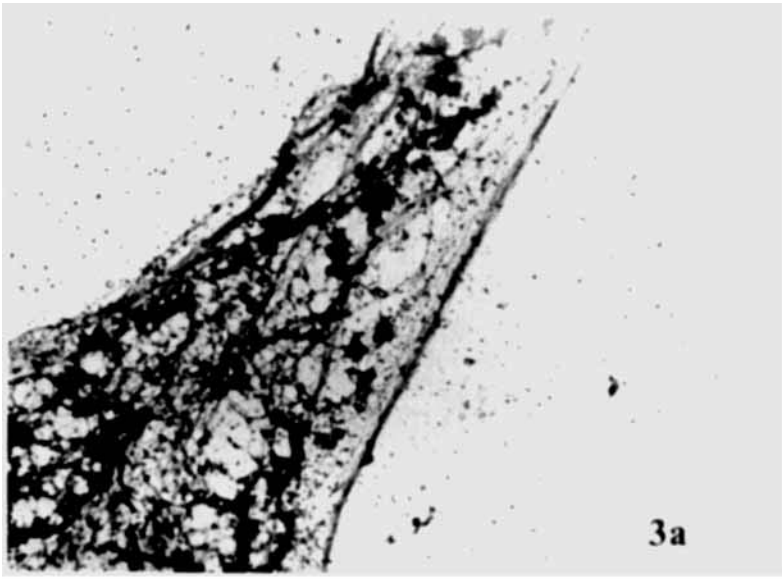
Examination of Triton X-100-treated intact CEF by transmission electron microscopy after negative staining shows a well-organized cytoskeleton with filaments running parallel to the plasma membrane (Fig. 3) and particularly prominent near the periphery of the cell. The cytoskeleton, as visualized by this method, consists of actin- and tubulin-containing fibers of various sizes in a three-dimensional array [12, 14]. Although in untreated CEF these appear as a highly organized network of banded and unbanded filaments (Fig. 3a), there is a prominent loss of cytoskeletal organization after treatment with 0.2 mM dibucaine for 3 h at 37°C (Fig. 3c). Observation of the cytoskeleton of untreated cells at high magnification reveals multiple filamentous structures joined together in bundle-like arrays (Fig. 3b). In dibucaine-treated cells, the remaining filament bundles show a significant decrease in thickness (Fig. 3d).

Electron microscope examination of thin sections of untreated CEF demonstrated a fusiform cell shape and relatively smooth cell surface (Fig. 4a). At high magnification, bundles of microfilaments predominently are seen close to the plasma membrane and running parallel with it (Fig. 4b). Microfilaments of different sizes (Fig. 4c), as well as microtubules (Fig. 4d), are also seen throughout the cytoplasm.

Treatment of CEF with trypsin, 10 μg/ml (Fig. 5a), or with 0.2 mM dibucaine (Fig. 4e) results in significant modification in cell shape and surface morphology, including rounding of fibroblasts and development of large and small ruffles and blebs. These changes are accompanied by disappearance of membrane-associated microfilament bundles and a significant reduction of microtubules and microfilaments in the cytoplasm (Fig. 4f and 5b).

DISCUSSION

The present investigation supports our earlier finding [7] that polymerization and depolymerization of cytoplasmic microfilaments and microtubules are associated with changes in the expression of insulin binding sites on the cell surface. Other investigators have shown that in 3T3 and BHK-21 cells, tertiary amine local anesthetics cause an alteration in the organization of microtubules and microfilaments and affect the ligand-induced distribution of concanavalin A receptors [8, 9]. In this study, treatment of confluent cultures of CEF with these agents caused a time- and dose-dependent increase in low-affinity insulin binding sites, the occupancy of which stimulates pleiotypic responses in the target cell [4]. An increase in these sites was accompanied by changes in the cell shape from fusiform to round, with development of numerous ruffles and blebs on the cell surface and disappearance of microfilament bundles closely associated with the plasma membrane. Brief treatment of cells with trypsin also caused a similar change in cell shape, surface morphology, and insulin receptor number, but incubation with both dibucaine and trypsin did not have an effect greater than either agent alone. In addition, RSV-transformed CEF, which have a greater number of insulin binding sites, a rounded cell shape, irregular cell surface morphology, and disorganized cytoskeletal structures, do not increase number of receptors in response to local anesthetics.



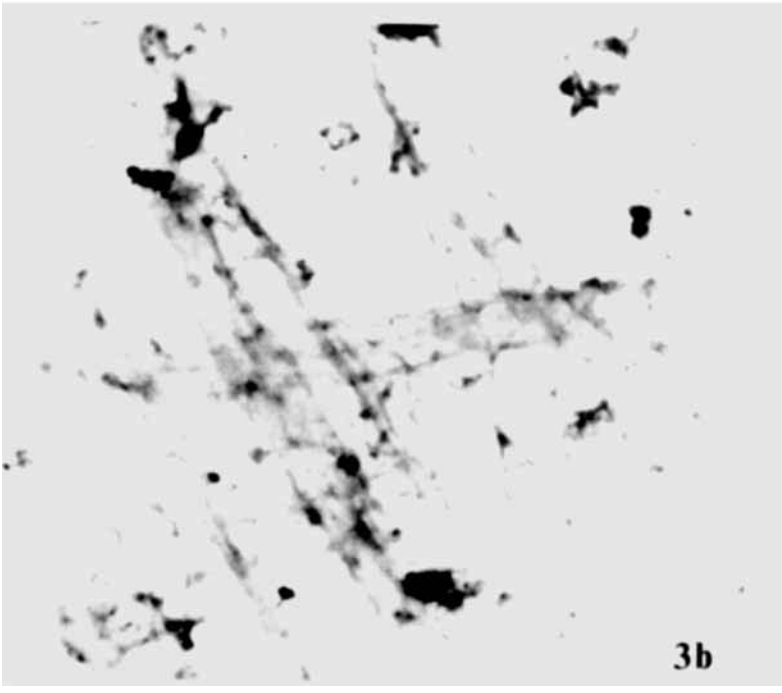
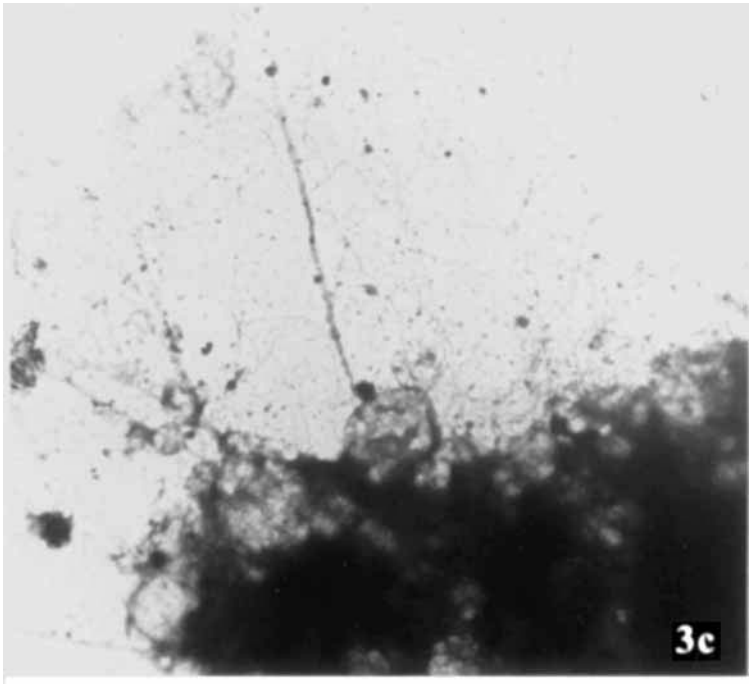


Fig. 3. Distribution of cytoskeletal structures in untreated and dibucaine-treated chick embryo fibroblasts. Confluent cultures were incubated with 0.2 mM dibucaine for 3 h at 37°C, treated with Triton X-100, and processed for electron microscope examination as described in Methods. a: Untreated CEF, region near cell periphery (× 5,500). b: Filaments of untreated CEF (× 47,200). c: Dibucaine-treated CEF, nucleus stains dark (× 5,500). d: Filaments of dibucaine-treated CEF (× 47,200).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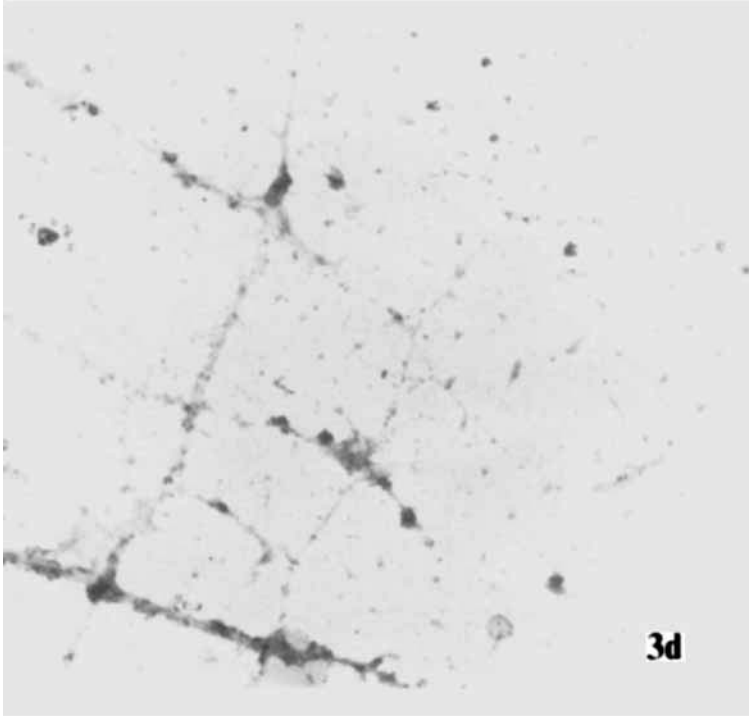
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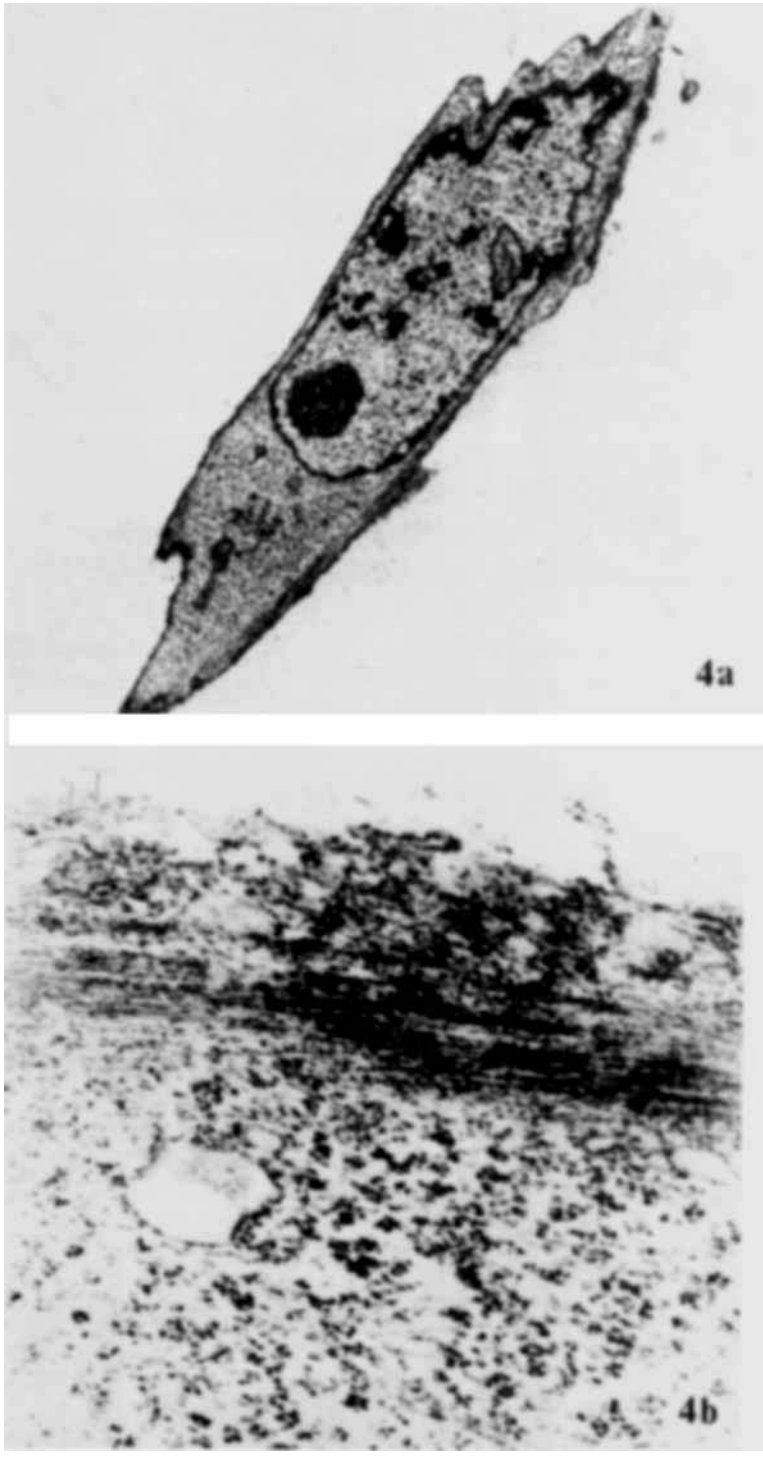


Fig. 4. Electron photomicrographs of thin sections of untreated and dibucaine-treated chick embryo fibroblasts. a: Untreated CEF demonstrating fusiform shape and relatively smooth cell surface (× 5,500). b: Untreated CEF with prominent microfilament bundles in close association with plasma membrane (× 47,200).

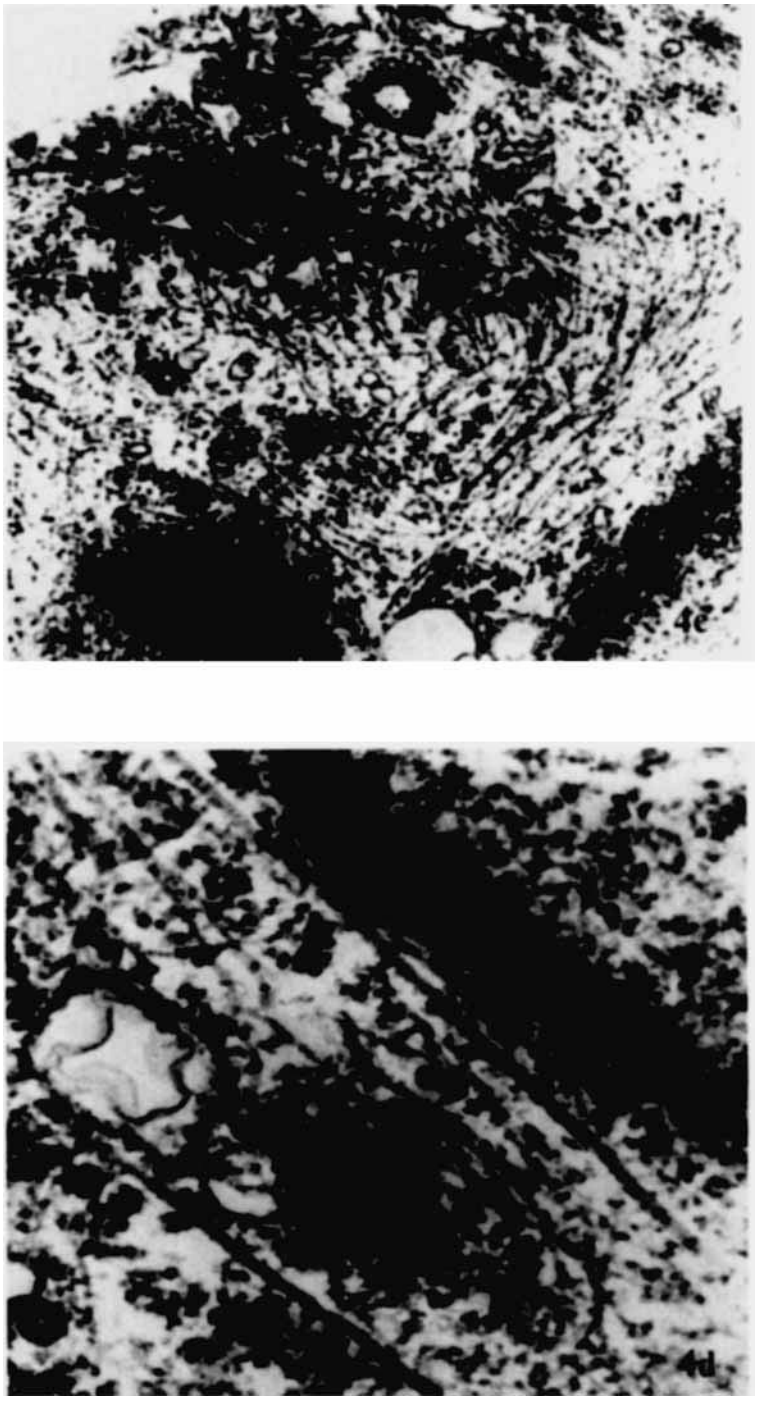
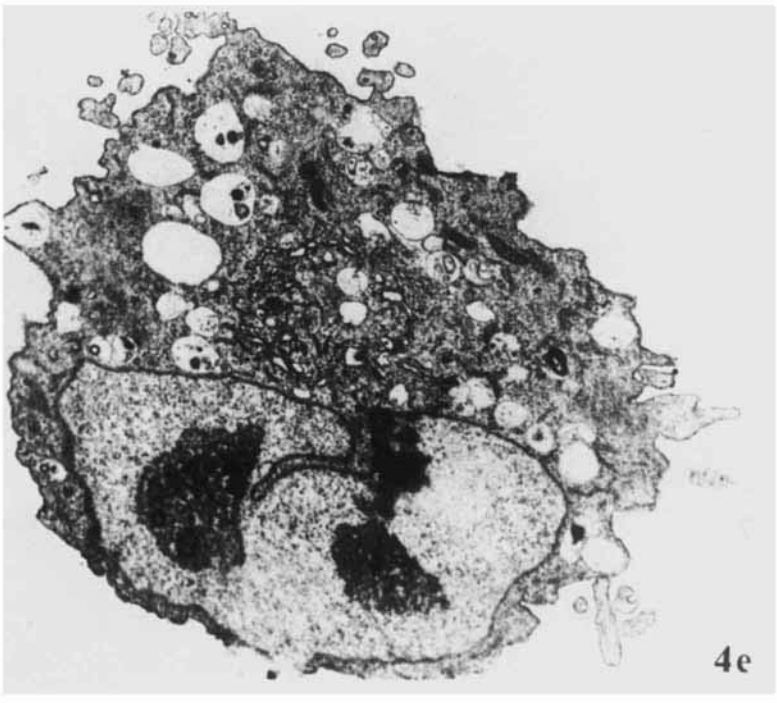


Fig. 4. Electron photomicrographs of thin sections of untreated and dibucaine-treated chick embryo fibroblasts continued. c: Untreated CEF with microfilaments present in the cytoplasm and away from the plasma membrane (× 47,200). d: Untreated CEF showing prominent microtubules (× 64,000).



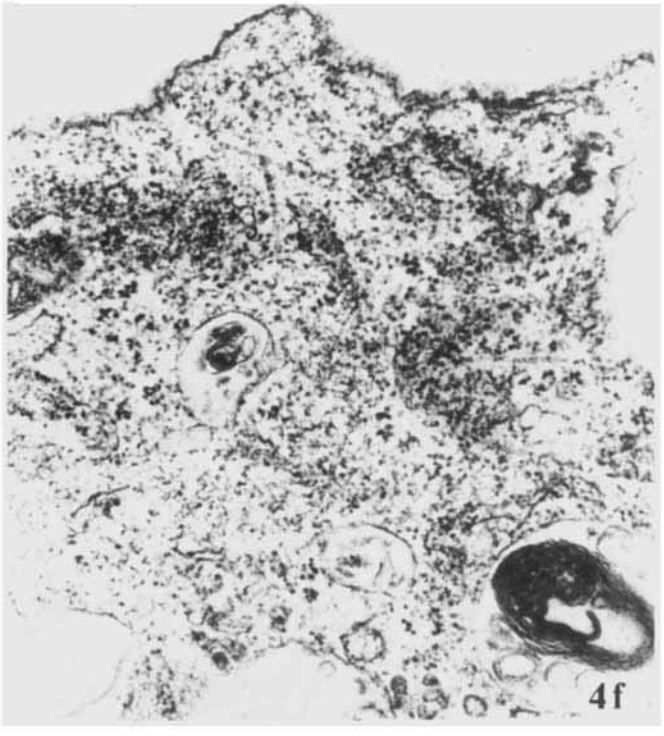


Fig. 4. Electron photomicrographs of thin sections of untreated and dibucaine-treated chick embryo fibroblasts continued. e: Dibucaine-treated CEF demonstrating round cell shape with numerous microvilli and blebs on the cell surface (× 5,500). f: Dibucaine-treated CEF representing the absence of microfilament and microtubules (× 47,200).

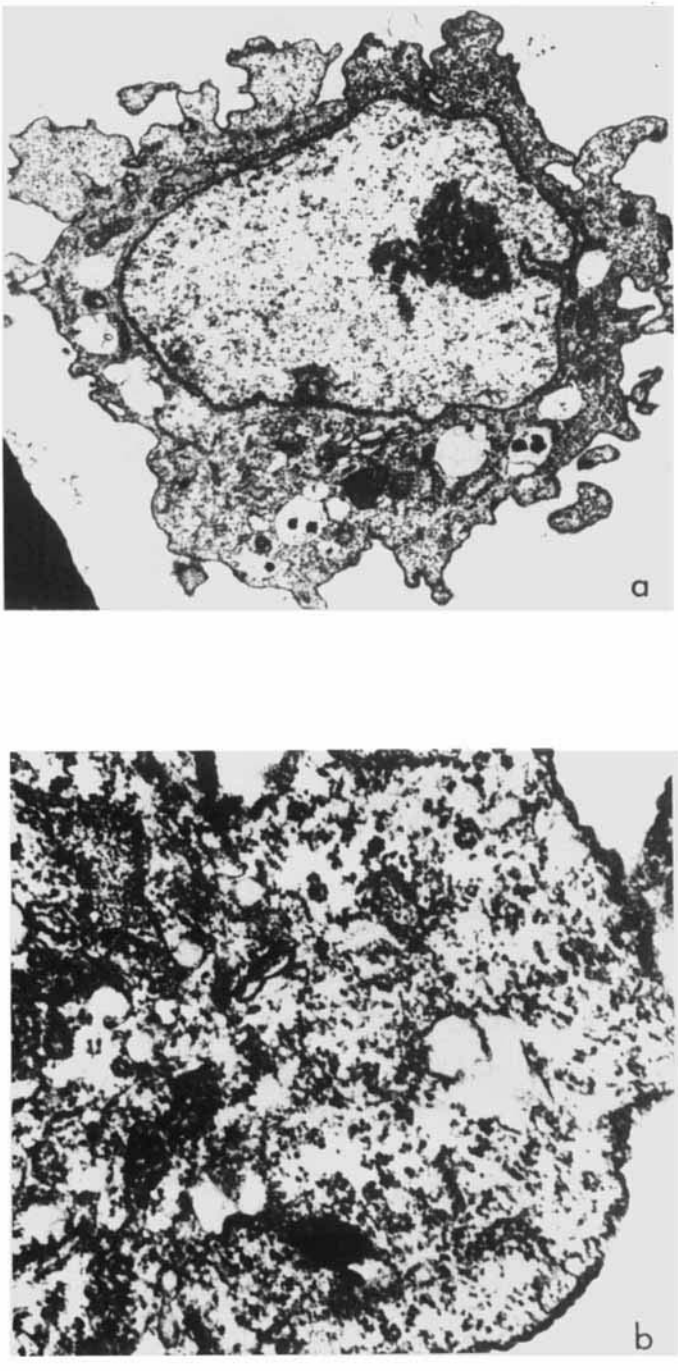


Fig. 5. Electron photomicrographs of thin section of trypsin-treated chick embryo fibroblasts. a: Trypsin-treated CEF demonstrating round cell shape with numerous microvilli and blebs on the cell surface (× 5,500). b: Trypsin-treated CEF showing the absence of microfilament bundles (× 47,200).

The finding that tertiary amine local anesthetics, trypsin, EGTA, and viral transformation produce similar effects on cytoskeletal organization and insulin binding sites suggests that the effect on insulin binding sites is mediated by a common cellular mechanism dependent on cytoskeletal integrity. The mechanism by which changes in the cytoskeleton might bring about changes in insulin binding sites is speculative at this time. It is possible that disorganization of these structures by local anesthetic inhibits the rate of endocytosis of cell surface receptors, resulting in an increase in their number. It is also possible that receptors "hidden" in the membrane are exposed as a result of disorganization.

Insulin receptor macromolecules on the cell surface may be directly linked to the cytoskeleton by a structure or structures which can be modified by chemical agents, transformation, or rapid growth. This type of organized relationship between cell surface macromolecules and cytoskeleton has been proposed by Ash et al [15]. Schlessinger and co-workers have demonstrated that movement of concanavalin A receptors in the plane of plasma membrane involved microtubules and microfilaments [16, 17]. It is reasonable to suggest that this relationship exists where receptor exposure and movement can be related to the organization of cytoskeletal structures, although the molecular evidence for such a linkage and the mechanism by which it may bring about changes in the expression of receptors has not been established.

It is possible that such a mechanism may involve a cellular "messenger." A likely candidate for this is calcium, since it is involved in many aspects of membrane function, as well as in the organization and disorganization of microtubules and microfilaments [8, 18, 19]. The effects of local anesthetics and cytochalasin B [7] on mitogen receptors, cell shape, and surface morphology are indistinguishable from those produced by EGTA. Thus, displacement of membrane-bound calcium by EGTA may, by raising cytoplasmic levels, cause depolymerization of the cytoskeleton. This is supported by the report of Poste et al [9] that elevation of intracellular calcium by calcium ionophore A 23187 has an effect on concanavalin A receptor mobility similar to that of colchicine or vinblastine.

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