J. Membrane Biol. 106, 203–210 (1988)

The Journal of
Membrane Biology

Growth Factors Modulate Junctional Cell-to-Cell Communication

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Summary. The epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) inhibit gap junctional communication in the mammalian cell lines NRK and BalbC 3T3: cell-to-cell transfer of a 400-dalton tracer molecule is reduced and junctional conductance is reduced. The inhibition of cell-to-cell transfer is reversible and dose dependent; half-maximal effects are obtained at 10^{-9} and 10^{-11} M concentrations of EGF and PDGF, respectively. The response of junctional conductance is detectable within 2 min of EGF application and reaches a maximum within 10 min. It is among the earliest cellular responses to this growth factor and may be significant in the regulation of growth. The response is lacking in EGF receptor-deficient NIH 3T3 cells. The transforming factor β (TGF_β) enhances junctional communication in BalbC 3T3: cell-to-cell transfer is increased over a period of 8 hr. But in NRK cells, where it upregulates EGF receptors, TGF_β reduces junctional communication synergistically with EGF.

Key Words gap junction · growth factors · EGF · PDGF · TGF_β · cell-to-cell communication · junctional communication

Introduction

The 16–20 Å-wide cell-to-cell membrane channels of gap junctions are the conduits of a ubiquitous mode of intercellular communication in organs and tissues (Loewenstein, 1987). This communication is under the control of the cellular *src* gene (Azarnia et al., 1988*a*), whose product is a 60-kD membrane-bound protein tyrosine kinase (Hunter & Cooper, 1985). This protein downregulates the communication; overexpression of the protein or enhancement of its specific enzyme activity leads to reduction of junctional permeability (Azarnia & Loewenstein, 1987; Azarnia et al., 1988*a*).

In the present work we examine the action on junctional permeability of two other membrane-bound protein tyrosine kinases: the receptor of the epidermal growth factor, EGF (Carpenter & Cohen, 1979), and the receptor of the platelet-derived growth factor, PDGF (Balk, 1971; Heldin & Westermark, 1984). These receptors are expressed in a variety of cell types. As their tyrosine kinase activities are turned on by the binding of their ligands, one can readily manipulate the enzyme activity by external application of the growth factors to cells in culture. We show that such application leads to fast reduction of junctional permeability in NRK and BalbC 3T3 cells.

Furthermore, we explore the action of the transforming factor β, TGF_β (De Larco & Todaro, 1978; Tucker et al., 1984; Sporn et al., 1986; Massagué, 1987), which upregulates the EGF receptors in NRK cells (Assoian et al., 1984). We show that the effect of TGF_β on junctional permeability is synergistic with that of EGF in these cells.

Materials and Methods

Cell Culture

NRK ("Normal Rat Kidney," 49F), BalbC 3T3 (A-31) and NIH 3T3 cells were cultured in Dulbecco Modification of Eagle Minimal Essential Medium (DMEM) plus 10% calf serum in the case of NRK and BalbC 3T3 and 5% calf serum in the case of NIH 3T3. RL cells (epithelioid rat liver cells, ATCC CRL 1439, Clone 9) were cultured in Basal Medium Eagle (BME) plus 5% fetal bovine serum. During culture, cells were at 37°C in an atmosphere of 5% CO_2, and during the tests of junctional transfer or the electrical measurements, they were exposed to room air and temperature for periods not exceeding 30 min.

Electrical Measurements and Permeability Probings

For measurement of electrical coupling, microelectrodes connected to balanced bridge circuits were inserted into two contiguous cells of near-confluent cultures (Fig. 1, inset *a*). Current pulses ($i = 5 \times 10^{-10}$ A, 100–150 msec duration, 1/20 sec) were passed between cell interior and exterior (grounded) in each cell and the resulting membrane voltages, namely the input voltages (V_1^1, V_2^2) and transfer voltages (V_2^1, V_1^2) were recorded (superscripts denote the cells into which i was injected and subscripts, the cells in which V was recorded). Currents and voltages were displayed on an oscilloscope and steady-state and zero-current

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values were sampled during each test sequence by a computer (Fig. 1, inset *b*). Bridge balance was verified after retraction of the microelectrodes from the cells. In successful experiments the microelectrodes could be kept continuously inside the cells for up to 30 min.

Measurements of membrane resistance (V/i) were taken in sparse cultures by pulsing current ($i = 2.5 \times 10^{-11}$ A) between the interior and exterior of single cells (without neighbors in contact) and measuring the resulting membrane voltages (V) over periods of 10–20 min.

For determination of junctional fluorescent tracer transfer, Lucifer Yellow CH (Molecular Probes; 10% aqueous solution) or glutamic acid covalently labeled with lissamine rhodamine B (LRB-Glu) (Simpson, Rose & Loewenstein, 1977) were microinjected into cells of near-confluent cultures; we used Lucifer Yellow for NRK, BalbC 3T3 and NIH 3T3 cells, and LRB-Glu for the more junction-permeant RL cells. The microinjection was done with the aid of a micropipette connected to a pneumatic system delivering sharp solenoid-controlled pressure pulses. The cells in the regions tested were of similar size. The microinjection (usually lasting 1–3 sec) and the cell-to-cell diffusion of the tracer were monitored by means of a television system coupled to a phase contrast/fluorescence microscope, video-taped and played back for analysis (Yada, Rose & Loewenstein, 1985). The total number of cells to which the tracer was transferred within 1 min of the injection served as index of junctional permeability (*transfer index*). At this time interval, the transfer index was not saturated, thus offering a sensitive indicator for changes in junctional permeability, as the tracers were chosen for their closeness to the threshold of junction permeation in the respective cells (Flagg-Newton, Dahl & Loewenstein, 1981). In one set of experiments on BalbC 3T3 cells, specially identified in the legend of Fig. 6, the interval was 40 sec throughout the entire set. (For details of the techniques of electrical measurement and fluorescent tracer transfer, *see* Socolar and Loewenstein, 1979).

Growth Factor Application

Aqueous stock solutions of EGF, PDGF and TGF_β (Collaborative Research, Inc.) were diluted in an aliquot of medium withdrawn from the culture dish.

For the experiments in which junctional communication was measured electrically, the growth factor solution was delivered by a coarse-tipped micropipette (P) to the region of the two test cells (Fig. 1, inset *a*). The micropipette was connected to a pressure reservoir, supplying a constant flow throughout the 20–35 min period of electrical measurements. The doses given in the Results are the growth-factor concentrations in the micropipette. For controls, the cells were superfused the same way, but growth factors were not added to the micropipette medium.

For the experiments in which junctional permeability was probed with fluorescent tracers, the growth factor stock solution was mixed with 1 ml medium withdrawn from the culture and then added to the culture dish medium and stirred. The doses given are the concentrations of the growth factors in the dish medium (2 ml). For controls, medium was withdrawn from the culture and re-applied the same way.

Results

Actions of EGF and PDGF

Changes in Junctional Conductance

To demonstrate the action of EGF on junctional communication and to study the time course of this action, we measured junctional communication electrically. In nearly confluent cultures, the electrical coupling between adjacent cells was measured in both directions across the cell junction. Currents of constant intensity ($i = 5 \times 10^{-10}$ A) were pulsed into contiguous cells (*1, 2*) and the resulting input voltages (V_1^1, V_2^2), transfer voltages (V_2^1, V_1^2) and transfer resistances (V_2^1/i, V_1^2/i) were determined (Fig. 1, top inset). This allowed us to monitor the entire time course of the growth-factor action, and the simultaneous measurement in both cells provided a test of symmetry, insuring against artifactual uncoupling through injury or trivial uncoupling through changes in permeability in nonjunctional cell membrane.

Figure 1 illustrates the result of an application of EGF (80 nM) to NRK cells. Within 2 min of the growth factor application, the junctional transfer resistances fell symmetrically in both cells, while the input voltages rose. The transfer resistance reached steady levels within 10 min, which were maintained for as long as we were able to record from inside the cells—up to 30 min. This result was typical of five experiments. When, in controls, only medium was applied, the transfer resistances (open symbols in Fig. 1) and input voltages (*data not shown*) did not change (10 experiments).

The crucial point in these results is that the fall in junctional transfer resistance was associated with a rise in input voltage. This unambiguously showed that the junctional conductance, that is, the junctional ion permeability, was reduced.

The permeability of nonjunctional cell membrane was not significantly affected. We measured the membrane conductance in single NRK cells without neighbors in contact (sparse cultures); there were no detectable changes upon EGF treatment (five experiments) (Fig. 2).

Changes in Junctional Permeance to 400–600 Dalton Tracers

With the action of the growth factor on junctional permeability established, we proceeded to examine the threshold conditions in various cell types. To this end we tested junctional permeability 20 min after growth factor application, when the junctional response was maximal in the preceding results.

In these experiments we probed the junctions with fluorescent tracer molecules: the dye Lucifer Yellow (443 daltons) and the lissamine rhodamine B-labeled glutamic acid (LRB-Glu; 688 daltons). We probed near the threshold of permeation where the method is most sensitive to changes in junctional permeability (Simpson et al., 1977). RL cells exhibit a higher basal level of junctional permeability than NRK, BalbC 3T3 and NIH 3T3 cells (Flagg-

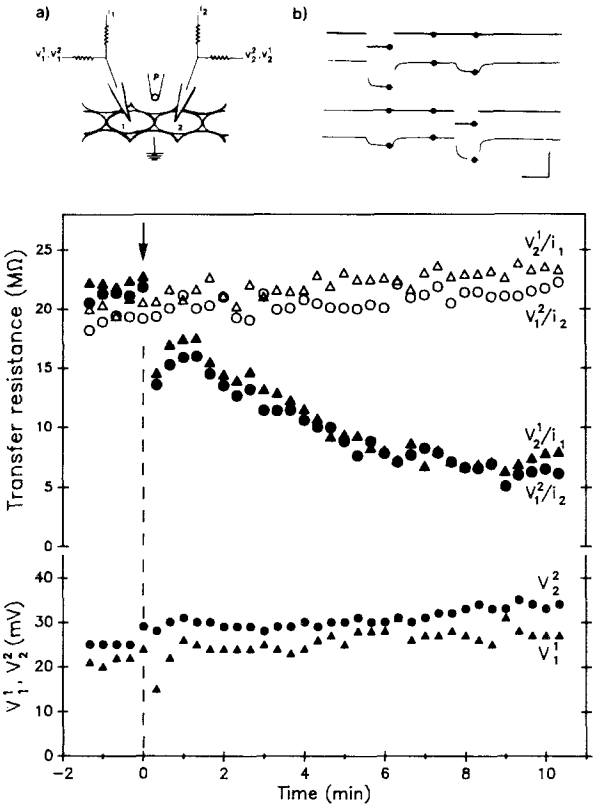

Fig. 1. EGF reduces junctional conductance. EGF (80 nM) was applied (arrow) while the electrical coupling between two contiguous NRK cells was measured. The main figure shows the computer plots of the steady-state values of transfer resistance (V_2^1/i_1, ▲; V_1^2/i_2, ●) and input voltage (V_1^1, ▲; V_2^2, ●), as current was pulsed alternately into the two cells. The transfer resistances measured from either side are nearly identical, and the fall in transfer resistance in response to EGF is concomitant with a rise in input voltage, demonstrating a fall in junctional conductance. The open-symbol data (△, ○) are from a control in which medium without EGF was applied (arrow). *Insets:* (*a*) The arrangement: a micropipette (*P*) serves to superfuse the test cells with growth factor-containing medium. Microelectrodes connected to balanced bridge circuits were inserted into cells *1* and *2* to pulse current ($i = 5 \times 10^{-10}$ A) between cell inside and outside (grounded) and to record the resulting input voltages (V_1^1, V_2^2) and transfer voltages (V_2^1, V_1^2) (superscripts denote the cells into which i was injected and subscripts, the cells in which V was recorded). (*b*) Sample of an oscilloscope record of the currents i_1, i_2 (1st and 3rd trace, respectively) and voltages V_1^1, V_2^2 (2nd and 4th trace) from both cells. The computer sampled the steady-state current and voltages at the times marked by dots. Calibration: vertical, 20 mV and 1 nA; horizontal, 100 msec

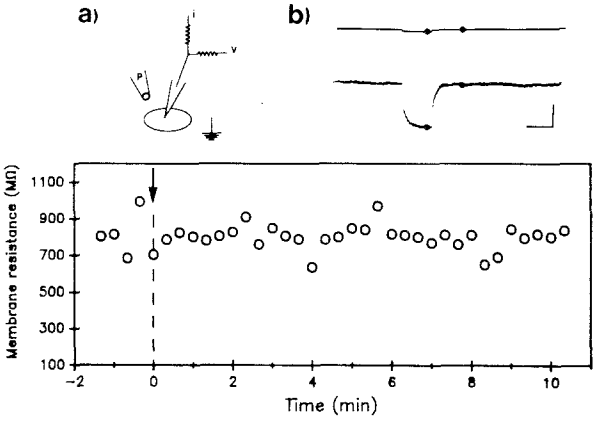

Fig. 2. EGF does not sensibly affect nonjunctional membrane conductance. Computer plot of steady-state membrane resistance (V/i) in an NRK cell. EGF (80 nM) was applied at arrow. *Inset:* (*a*) Current ($i = 2.5 \times 10^{-11}$ A) was pulsed between interior and exterior of a single cell without neighbors (sparse culture) and the resulting membrane voltage (V) was measured. The current passing/voltage recording microelectrode was connected to a balanced bridge circuit, and EGF-containing medium was delivered to the cell by micropipette (*P*). (*b*) An oscilloscope record of i (top trace) and V (bottom trace); the steady-state data were sampled by the computer at the times marked by dots. Calibration: vertical, 20 mV, 1 nA; horizontal, 100 msec

Newton, Simpson & Loewenstein, 1979). Therefore, we used the larger and more polar LRB-Glu for the probings of RL cells and Lucifer Yellow for the other cells. The cell-to-cell diffusion of the fluorescent tracers was video-recorded for analysis. This allowed us to scan a population of cells and to obtain information about responses of permeability of a relatively large number of cell junctions by microinjecting several cells (10–15 per culture dish) in rapid succession. This has obvious advantages over the electrical method, which gives information only about one junction at a time (and which, besides, requires inserting two microelectrodes instead of one). Used in combination with measurements of nonjunctional membrane permeability and close to the threshold for tracer permeation, the fluorescent tracer method already has proven its worth in the discoveries and analyses of the actions of a number of regulators of cell-to-cell communication (e.g., Flagg-Newton et al., 1981; Radu, Dahl & Loewenstein 1982; Wiener & Loewenstein, 1983; Yada et al., 1985; Azarnia et al., 1988*a*).

Our probings with fluorescent tracers corroborated the results obtained with the electrical measurements in NRK cells and extended their validity to BalbC 3T3 cells: both types of cells showed reduced junctional permeability in response to EGF, as indexed by the number of cell neighbors to which the fluorescent tracer diffused from the injected test cells (Fig. 3).

Furthermore, these experiments showed that the junctional response was dose-dependent and reversible: half-maximal responses were obtained at 10^{-9} M concentrations of EGF (Fig. 4*A*); the control level of junctional permeability was restored within 2 hr of washout of the growth factor (Fig. 5).

NIH 3T3 cells, in which EGF-receptors are below detection level (Livneh et al., 1986), served as negative controls. These cells exhibited no change in junctional permeability in response to doses of EGF (1.6×10^{-8} M) which produced maximal re-

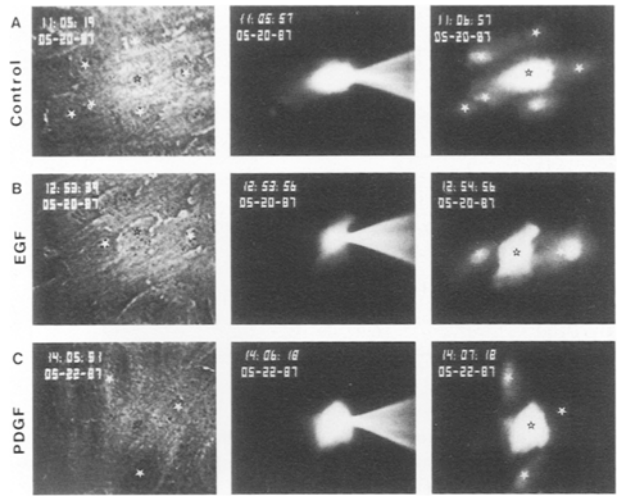

Fig. 3. EGF and PDGF reduce junctional transfer of a 443-dalton fluorescent tracer. Samples of junctional probings with Lucifer Yellow in NRK cells. Video-pictures of the cells in phase contrast (left), in fluorescence mode at the time of the microinjection (center), and 1 min after the microinjection (right), showing the spread of the fluorescent tracer from the injected cell (marked by black asterisk) to neighboring cells (white asterisk) 1 min after the microinjection. Each videoframe displays the time during the actual experiment on a digital clock in hours: minutes: seconds (and the date). (*A*) Control. (*B*) Treatment with EGF (16 nM). (*C*) Treatment with PDGF (0.6 nM). The probings of junctional transfer were done 20 min after the application of the growth factor-containing solution or of the control medium to the cell cultures

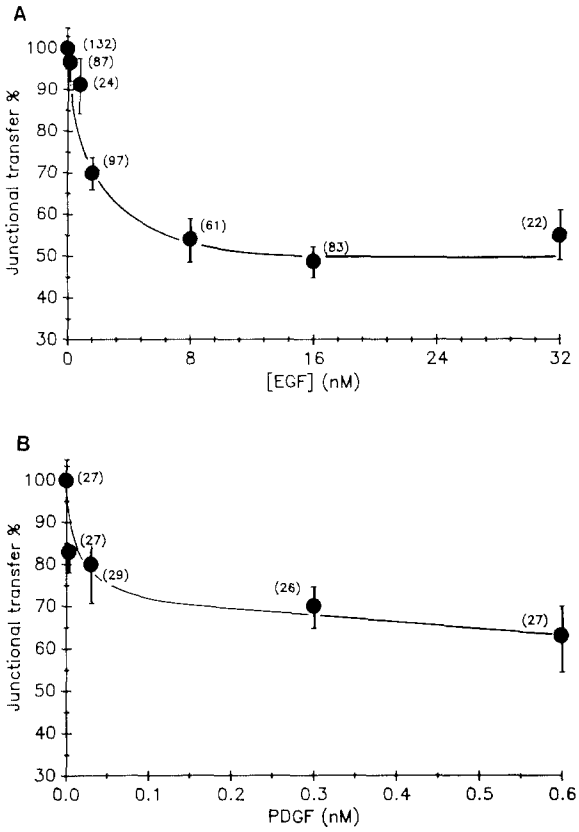

Fig. 4. Dose-response curves for EGF and PDGF. The junctional transfer, as indexed by the number of cells to which fluorescent tracer (Lucifer Yellow) is transferred 1 min after its microinjection, at various growth-factor concentrations. Values are means ± SE, normalized with respect to the corresponding untreated controls. In parentheses, the number of trials, i.e., the number of individually injected cells. BalbC 3T3 treated (*A*) with EGF and (*B*) with PDGF. Junctional transfer was tested 20 min after growth-factor application (or control medium application). The absolute mean values of the controls were 8.6 cells for *B* and ranged 6.8–7.3 cells for *A*

sponses in NRK and BalbC 3T3 cells. Table 1 summarizes the results (and their statistical significance levels; legend) of a set of parallel experiments and their respective controls in which NRK, BalbC 3T3 and NIH 3T3 cells were treated with such a dose of EGF. (This table lists only the experiments carried out with that particular dose. The data of six sets of such experiments with EGF at different concentrations are plotted in Fig. 4.)

PDGF produced a reduction of junctional permeability in NRK and BalbC 3T3 cells, which was comparable to that produced by EGF (Fig. 3 and Table 1), but the sensitivity was higher; half-maximal responses were obtained at 10^{-11} M concentrations of PDGF (Fig. 4*B*).

RL cells showed a slight, but statistically significant, reduction of junctional transfer in response to PDGF (6×10^{-10} M). They did not respond to EGF at concentrations (1.6×10^{-8} M) that produced maximal effects in NRK and BalbC 3T3 cells. We don't know whether RL cells are EGF-receptor deficient.

Actions of TGF_β

The effects of TGF_β (1×10^{-11} M) were tested 8 hr after its application to NRK and BalbC 3T3 cells. Both types of cells possess receptors for this growth factor (Massagué & Like, 1985). The junctional responses were opposite in these cells: whereas in NRK cells the junctional permeability was reduced, in BalbC 3T3 cells it was enhanced (Fig. 6) and both effects were substantial (Table 2).

In a variant of these experiments the TGF_β treatment was carried out conjointly with EGF treatment. Here the cells were pre-treated for 8 hr with TGF_β (1×10^{-11} M) and junctional transfer was tested 20 min after application of EGF (1.6×10^{-8} M). This protocol was guided by the finding of Roberts and Sporn and colleagues that the EGF receptors are maximally upregulated 8 hr after TGF_β treatment in NRK cells (Assoian et al., 1984).

The effect of the conjoint treatment in NRK cells was a reduction in junctional transfer greater than that produced by either TGF_β alone or EGF alone (Table 2).

Table 1. Effects of EGF and PDGF on junctional transfer

	NRK	BalbC 3T3	NIH 3T3	RL
Control	100 ± 3 (26)	100 ± 12 (29)	100 ± 14 (33)	100 ± 4 (30)
EGF (16 nM)	48 ± 6 (30)	48 ± 8 (33)	102 ± 13 (30)	95 ± 3 (27)
Control	100 ± 2 (19)	100 ± 10 (30)	100 ± 12 (34)	100 ± 3 (30)
PDGF (0.6 nM)	76 ± 6 (30)	63 ± 7 (27)	105 ± 17 (29)	89 ± 2 (27)

Mean transfer index values normalized with respect to untreated controls ± SE; in parentheses, the number of individual injections. The tracers were Lucifer Yellow (443 mol wt) for NRK, BalbC 3T3 and NIH 3T3, and LRB-Glu (688 mol wt) for RL. The statistical significance levels (P) of the differences between the mean values of the growth factor-treated condition and controls were: NRK, $\leqq$ 0.0004; BalbC 3T3, $\leqq$ 0.001; NIH 3T3 and RL, not significant except for RL treated with PDGF, 0.003 (standard t test). The absolute mean values of transfer index of the controls were 7.4 (NRK), 8.5 (BalbC 3T3), 3.1 (NIH 3T3), 5.6 (RL). The controls for each treatment were carried out on sister cultures in strictly parallel experimental conditions.

Table 2. Differential effects of TGF_β on junctional transfer in NRK and BALBC 3T3 cells

	NRK			BalbC 3T3			NIH 3T3	RL
		P_1*	P_2*		P_1*	P_2*		
Control	100 ± 3 (26)			100 ± 12 (29)			100 ± 14 (33)	100 ± 4 (30)
TGF_β (10 pM)	63 ± 5 (26)	0.00001		153 ± 11 (34)	0.001		106 ± 16 (31)	108 ± 4 (28)
TGF_β (10 pM) + EGF (16 nM)	37 ± 8 (26)	0.00001	0.002	61 ± 8 (33)	0.004	0.00001	90 ± 18 (31)	95 ± 4 (27)

Mean transfer index values normalized with respect to untreated controls ± SE; in parentheses, the number of individual injection trials. The tracers were Lucifer Yellow for NRK, BalbC 3T3, NIH 3T3, and LRB-Glu for RL.

* The statistical significance levels (t test) of the difference between the mean values of the growth factor-treated condition and control (P_1) and between the TGF_β-treated and TGF_β + EGF-treated (P_2) conditions. In NIH 3T3 and RL cells the corresponding differences are not statistically significant. The experiments summarized in this table were carried out, for each cell type, in parallel with those of EGF listed in Table 1 (the control data are the same). The difference between the data of the EGF-treated and TGF_β + EGF-treated conditions are not statistically significant for any cell type.

In BalbC 3T3 cells, where TGF_β (alone) enhanced junctional transfer, the combined treatment produced reduction of the transfer (Fig. 6, Table 2). Evidently, the inhibitory action on junctional permeability of EGF here dominated over the enhancement action of TGF_β.

Discussion

Our results show that the growth factors EGF and PDGF cause reduction in junctional permeability. This brought to light an interaction between the hormonal form and the junctional form of intercellular communication, an interaction where the former modulates the latter. Such a modulatory action already was known for cyclic AMP-releasing hormones (Loewenstein, 1985), but in that case the junctional response generally is in the opposite direction; junctional permeability increases by that hormone action in many cell types (Loewenstein, 1987).

Both the EGF receptor and PDGF receptor possess endogenous protein tyrosine kinase activity (Heldin & Westermark, 1984; Hunter & Cooper, 1985) and that activity presumably is responsible for the modulation of junctional permeability. The effects on junctional permeability are in the same direction as those of the membrane-bound protein tyrosine kinases of the normal cellular *src* protein (Azarnia et al., 1988*a*) and the pathogenic viral *src* protein (Atkinson et al., 1981; Azarnia & Loewenstein, 1984*a*). The EGF effect on junctional permeability sets in within 2 min, fast enough to suggest an action on the open state of the cell-to-cell channels, the unit conduits of junctional communication (Loewenstein, 1981). Two modes of action on these channels by the receptor kinases may be pondered at this time: a channel closure due to direct phosphorylation of the channel protein or a channel closure mediated by calcium ion. The first mechanism, a phosphorylation on channel-protein tyrosine residues, is conceivable because one of the gap-junction proteins contains consensus sequences for this type of phosphorylation (Paul, 1986; Kumar & Gilula, 1986). The second mechanism, an action by way of Ca^{2+}, may be envisioned because both EGF

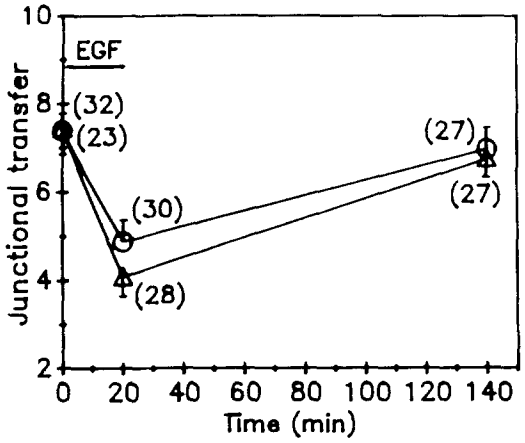

Fig. 5. Reversibility of the EGF effect. The cells were treated with 8 nM (○) or 16 nM (△) EGF. The growth factor was applied to the cultures at time zero and washed out at 20 min. Junctional transfer was tested just before EGF application, at 20 and at 140 min (on near-confluent cultures). Junctional transfer is not normalized here

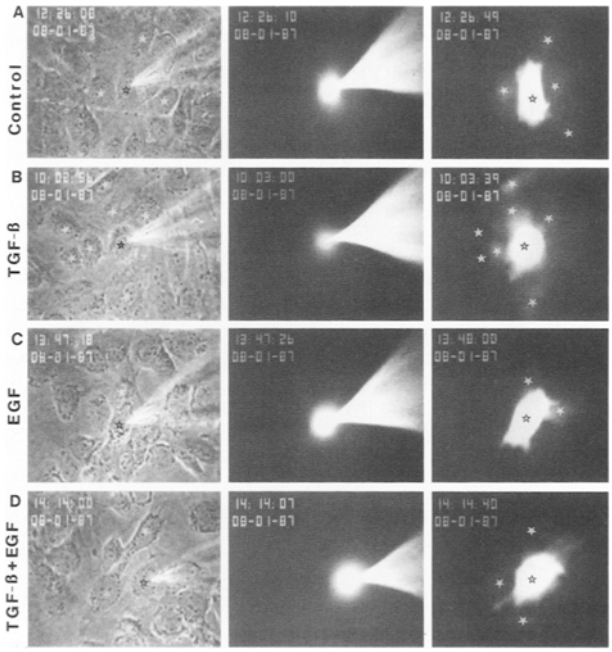

Fig. 6. Long-term effects of TGF_β and TGF_β + EGF on junctional transfer in BalbC 3T3 cells. Videoframes of the cell-to-cell spread of Lucifer Yellow in (*A*) a control culture and (*B*) a culture treated with TGF_β (10 pM) for 8 hr, (*C*) a culture treated for 20 min with EGF (16 nM), and (*D*) a culture treated for 8 hr with TGF_β (10 pM) and then for 20 min with EGF (16 nM). From left to right: in phase contrast mode; at the time of microinjection of the tracer; and 40 sec after microinjection. The microinjected cell is marked by black asterisk, the neighbors seen as fluorescent in the microscope, by white asterisks. In the photographic reproduction some of the weakly fluorescent cells (visible in the original) do not show up clearly; note the stronger fluorescence of the first-order neighbors after TGF_β treatment (*B*) compared with that in the control (*A*)

and PDGF raise cytosolic Ca^{2+} concentration (Moolenaar, Tertoolen & deLaat, 1984; Hesketh et al., 1985) and cell-to-cell channels close upon sufficient elevation of that concentration in the junctional locale (Rose & Loewenstein, 1975; *see also* Unwin & Ennis, 1984; Loewenstein, 1987). In connection with the Ca^{2+}-mediated mechanism, we note that the inhibitor of intracellular Ca^{2+} mobilization TMB-8 [8-N,N-(diethylamine) octyl-3, 4, 5-trimethoxybenzoate] prevents the reduction of junctional permeability by the *v-src* protein (Yada et al., 1985).

The changes in junctional permeability are among the earliest cellular responses to EGF and PDGF known. They precede the morphological (cytoskeletal) changes by many hours. This echoes the situation involving *v-src* protein (Atkinson et al., 1981; Rose, Yada & Loewenstein, 1986), where the junctional permeability response could be genetically segregated from morphological changes (Azarnia & Loewenstein, 1984*b*).

The well-known growth-stimulating action of EGF and PDGF manifested itself as an increase in saturation density in NRK and BalbC 3T3 cells. For example, at 16 nM EGF, a concentration which maximally reduced junctional permeability in NRK cells (Fig. 4*A*), the saturation density of these cells about doubled (Fig. 7). Such an inverse relationship between growth and junctional communication also prevails in two other conditions of physiological interest: when junctional permeability is varied by the cellular *src* protein level (Azarnia et al., 1988*b*) and when it is varied by retinoic acid (a possible morphogen) or other retinoids (Mehta, Bertram & Loewenstein, 1988). All this leads us to suspect that the junctional communication response is an important link in the regulation of cellular growth.

We have no clues as yet on the mechanism of the enhancement of junctional communication by TGF_β in Balbc 3T3 cells. This point must await elucidation of the molecular biology of the TGF_β receptor. However, the reduction of junctional permeability found after prolonged TGF_β treatment (8 hr) of NRK cells may be interpreted in the light of the upregulation of EGF receptors reported for these cells by Assoian et al. (1984). Thus, the reduction of junctional permeability may reflect an amplification of the responsiveness to EGF, namely to EGF contained in the serum. We were unable to test the effect of TGF_β in serum-free medium because the NRK cells then lost their contact relationships within 30–60 min and eventually detached. But the results obtained with EGF receptor-defi-

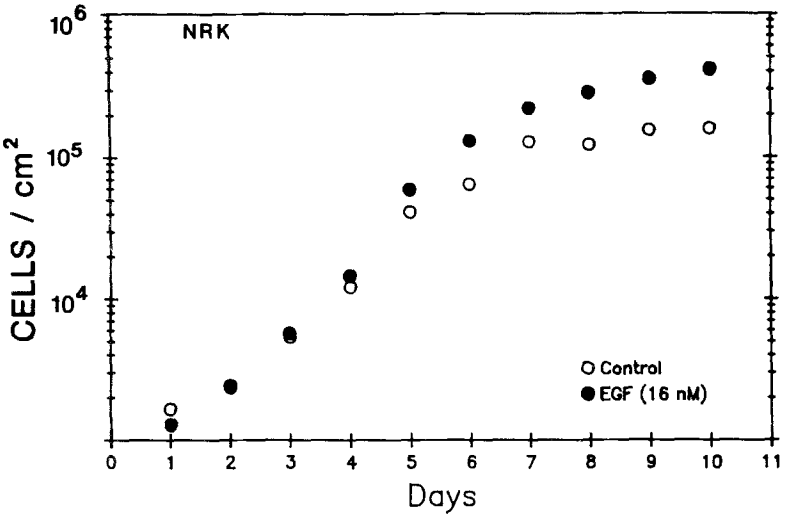

Fig. 7. Effect of EGF on growth of NRK cells. 40 dishes (35 mm) were seeded each with 20,000 cells on day 0. EGF, 16 nM, was added to the medium of 20 dishes from day 0 onward (filled symbols). Medium was changed every other day. Each day, the cell number/dish was determined (Coulter Counter) in two dishes each for control and EGF-treated cultures. Plotted are the means of the cell counts in two dishes (the highest count difference within each set was 5%)

cient NIH 3T3 cells are consistent with this notion; in these cells there was no significant reduction of junctional transfer in response to TGF_{β}, and this also was so in the EGF-unresponsive RL cells (Table 2).

We thank Maria de Luz Aylwin for cell culture. The work was supported by research grant CA14464 from the National Cancer Institute, National Institutes of Health.

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Received 30 September 1988