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Diacylglycerol Downregulates Junctional Membrane Permeability. TMB-8 Blocks this Effect

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Summary. We tested the question whether junctional cell-to-cell communication is regulated by the diacylglycerol branch of the phosphoinositide transmembrane signal pathway. Cultured epithelial rat liver cells were treated with the synthetic diacylglycerol 1-oleoyl-2-acetyl glycerol, while their junctional permeability was probed with the microinjected 443-dalton fluorescent tracer Lucifer Yellow. The treatment reduced junctional permeability (without affecting Lucifer permeability of nonjunctional cell membrane). The effect was dose dependent, with a threshold of about 25 μg diacylglycerol/ml in sparse cultures and about 50 μg/ml in confluent cultures. The reduction of junctional permeability began within 3 min of diacylglycerol application, peaked within 20 min, and reversed spontaneously within 90 min. The phorbol ester TPA mimicked the diacylglycerol effect, but the (spontaneous) reversal was slower. We propose that cell-to-cell communication is under dual physiological control: an upregulatory one, as exerted by the cyclic AMP signal route (Loewenstein, W.R., 1985, *Biochem. Soc. Symp. London*, **50:** 43–58), and a downregulatory one, by the diacylglycerol signal route.

TMB-8 (54–70 μM)—a blocker of intracellular Ca^{2+} mobilization—impeded the diacylglycerol action on junctional permeability. It prevented the effect of low diacylglycerol doses completely and it markedly reduced the effect of high doses. (It also counteracted the effect of TPA.) Ca^{2+} thus emerges as a possible candidate for a role in the junctional downregulation by the diacylglycerol signal route. We tentatively advance two models. In one, leaning closely on the Calcium Hypothesis of cell-to-cell channel regulation (Loewenstein, W.R., 1966, *Ann. N.Y. Acad. Sci.* **137:**441–472), Ca^{2+} mediates the action of the route on the channel. In the other, Ca^{2+} acts farther removed from the channel, on protein kinase C.

Calmidazolium (5–10 μM)—an inhibitor of calmodulin-activated proteins—did not prevent the diacylglycerol-induced reduction of junctional permeability. Nor did sodium orthovanadate (25 or 50 μM)—an inhibitor of tyrosyl phosphatase—prevent the reversal of diacylglycerol-induced (or TPA-induced) reduction of junctional permeability.

Key Words junctional permeability · gap junction · diacylglycerol · phosphatidylinositol · calcium ion · TMB-8 · phorbol esters · TPA

Introduction

The protein products of the viral and cellular *src* genes, $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, affect cell-to-cell communication; they downregulate junctional permeability in vertebrate cells (Atkinson, Menko, Johnson, Sheppard & Sheridan, 1981; Azarnia & Loewenstein, 1984*a*; Loewenstein, 1985; R. Azarnia & W.R. Loewenstein, *unpublished results*), and this action is independent of their action on cytoskeleton (Azarnia & Loewenstein, 1984*b*). The mechanism of this effect is not known, but we suspect that it is tied in somehow with the peculiar phosphorylation these proteins promote in the host cell: they phosphorylate proteins at tyrosine residues (Collett, Purchio, & Erikson, 1980; Hunter, 1980; Bolen, Thiele, Israel, Yonemoto, Lipsich & Brugge, 1984). Junctional permeability depends on protein kinase A (Wiener & Loewenstein, 1983; Loewenstein, 1985), a cyclic AMP-activated enzyme which phosphorylates at serine or threonine residues (Krebs, 1972; Rosen & Krebs, 1981).

The $pp60^{v\text{-}src}$ also promotes phosphorylation of membrane lipids, namely phosphoinositide precursors of diacylglycerol and inositol triphosphate (and the diacylglycerol itself) (Sugimoto, Whitman, Cantley & Erikson, 1984; Macara, Marinetti & Balduzzi, 1984) probably by enhancing the activities of cellular lipid kinases (MacDonald, Kuenzel, Glomset & Krebs, 1985; Sugimoto & Erikson, 1985). It can thus perturb the signal route that uses diacylglycerol and inositol triphosphate as intracellular messengers of hormonal information (Michell, 1982; Nishizuka, 1984, 1985; Berridge & Irvine, 1984; Sekar & Hokin, 1985). At high enough concentration, it causes overproduction of the messenger precursors (*cf.* Macara, 1985). The messengers

themselves may then become overabundant, nursing a continuous intracellular signal flow rather than an intermittent one as in the normal cell. Somehow as a result of this excessive signal flow, a 42K protein becomes phosphorylated at tyrosine (Cooper & Hunter, 1981; Cooper, Bowen-Pope, Raines, Ross & Hunter, 1982), a phosphorylation suspected to be at the root of the oncogenic cell transformation. The diacylglycerol signal branch by way of protein kinase C (rather than the inositol triphosphate branch) is a likely candidate to mediate this *src* effect (Berridge, 1984) because this 42K-protein phosphorylation can be mimicked by administration of exogenous diacylglycerol to the cells (Gilmore & Martin, 1983).

Could the diacylglycerol signal route also mediate the *src* effect on junctional permeability? As a first step in exploring this question, we inquired here whether diacylglycerol affects junctional permeability. We treated epithelial rat liver cell cultures with the synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol, and probed their junctions with the 443-dalton fluorescent tracer Lucifer Yellow. The junctions of these cells are normally highly permeable in culture (Flagg-Newton, Simpson & Loewenstein, 1979) and this diacylglycerol readily penetrates cell membranes from the outside and activates protein kinase C, bypassing the cell surface receptors of the phosphoinositide signal route (Kaibuchi, Takai, Sawamura, Hoshijima, Fugikura & Nishizuka, 1983). We show that this lipid causes reduction of junctional permeability.

Furthermore, we explored whether a rise in the cytosolic Ca^{2+} concentration might play a part in this diacylglycerol effect. Ca^{2+} is a regulator of junctional permeability; it causes closure of the cell-to-cell channels, the unit-conduits of junctional communication (Loewenstein, Kanno & Socolar, 1978; Unwin & Ennis, 1983). Junctional permeability falls when the cytosolic Ca^{2+} concentration in the junctional locale rises through excessive Ca^{2+} influx into cells (Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1975) or through excessive Ca^{2+} release from intracellular stores (Rose & Loewenstein, 1976). Thus, we examined whether 8-N,N-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), a blocker of intracellular Ca^{2+} mobilization (Chiou & Malagodi, 1975; Mix, Dinerstein, Villereal, 1984), affects the diacylglycerol-induced reduction of junctional permeability. We find that TMB-8 can prevent that reduction.

Materials and Methods

Cell Culture

Clone 12-B was isolated by P. Mehta from rat liver cells (ATCC CRL 1439, Clone 9, passage 30). This clone was selected for its flat and clearly epithelioid morphology and for its sharp inhibition of growth at confluence. These traits were quite stable over 35 passages; the present experiments were performed on passages 18–25.

The cells were cultured in Eagle's Basal Medium (GIBCO), supplemented with 10% fetal bovine serum (HyClone Labs) and 40 μg/ml Gentamycin (Elkins-Sinn) on 35-mm plastic dishes (Falcon) at 37°C in a 95% air/5% CO_2 atmosphere. The medium (2 ml) was changed every other day, including the day before experiments. Confluent cultures had a density of about 1×10^6 cells/dish.

Culture dishes from the same passage, maintained under strictly parallel conditions, including cell density, are referred to in the Results as *sister* cultures and designated with the same small letter (e.g., $a_1 \ldots a_4$).

Treatments

The diacylglycerol, 1-oleoyl-2-acetyl-rac-glycerol (Sigma) or the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (Sigma) were added to 0.5 ml medium withdrawn from a culture dish; the mixture was sonicated for 5–6 min at room temperature, added to the 1.5 ml remaining in the culture dish, and mixed by shaking. Stock solutions of diacylglycerol (12.5 and 25 mg/ml DMSO) and TPA (4 and 20 μg/ml DMSO) were kept under an atmosphere of nitrogen and stored frozen. Calmidazolium (R-24571, Boehringer) was applied directly from a 50 mM stock solution (DMSO), and TMB-8 (8-N,N-(diethylamino)octyl-3,4,5-trimethoxybenzoate, Calbiochem) and sodium orthovanadate (Sigma) were applied from stocks in Hanks' balanced salt solution or distilled water.

Determination of Junctional Cell-to-Cell Transfer

For observation and microinjection of the cells, we used a television camera (DAGE MTI 65 SIT) coupled to a Leitz Diavert microscope equipped with phase contrast and fluorescence optics (100 W mercury arc lamp). The cells and the microinjection pipette were viewed on a TV monitor. In the phase contrast mode, the micropipette (IB 100F, WP-Instruments) containing Lucifer Yellow CH (Molecular Probes, Inc.; 10% aqueous solution) was brought into contact with a cell. The illumination was then switched to the fluorescence mode and the cell was impaled. Lucifer was injected by means of a sharp, solenoid-controlled gas pressure pulse. 10 sec after the injection, timed by a clock displayed on the TV monitor, we scored the number of fluorescent first-order neighbors of the injected cell and the total number of its first-order neighbors (usually 6 to 8 in confluent cultures). *The incidence of permeable interfaces*, the parameter used throughout the present study, is the ratio *fluorescent first-order neighbors : total first-order neighbors*, at 10 sec, for each injection trial. The experiments were videotaped and played back for evaluation.

The cultures were at room temperature and room air during the testing of cell-to-cell transfer. We usually limited the tests to a period of 40 min, in order to avoid effects of the lower temperature and lack of CO_2 on junctional permeability. This left a safety margin; in untreated cultures, the incidence of permeable interfaces was not affected by these conditions over periods of up to 70 min.

Measurement of Fluorescence Loss

Cells without neighbors (very sparse cultures) were injected with Lucifer. The cell was videotaped in the fluorescence mode dur-

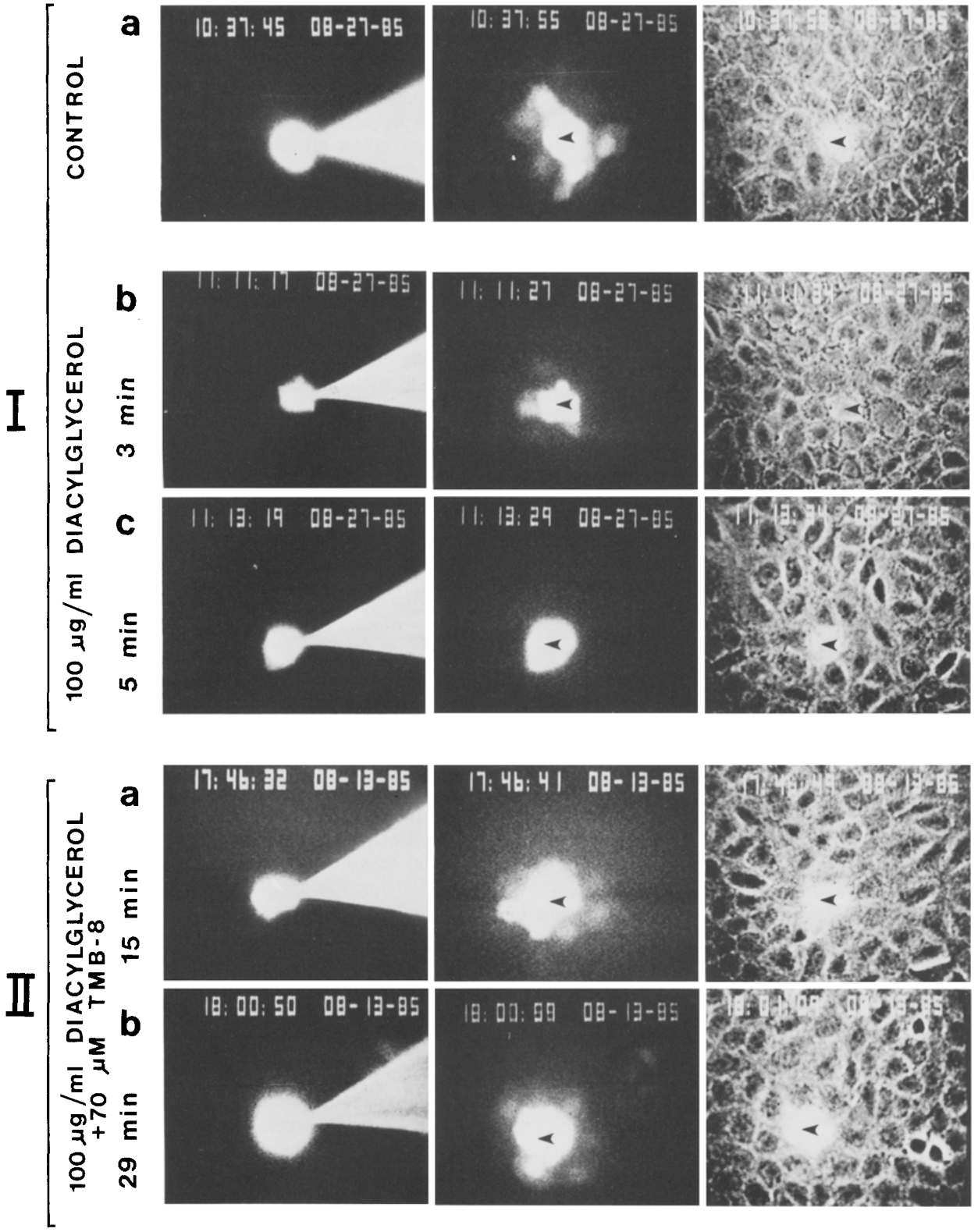

Fig. 1. *I*, Effect of diacylglycerol on junctional transfer. Video pictures of three sample microinjections of Lucifer Yellow in the same culture (*a*) before, (*b*) 3 min, and (*c*) 5 min after treatment with 100 µg/ml diacylglycerol. Confluent cultures. The left (vertical) panel shows the micropipette and the cell during the injection. The middle panel shows the cells 10 sec thereafter (the micropipette was retracted right after the injection). Right panel, cells in phase contrast. Injected cells—each a different cell—are marked with an arrowhead. Each frame displays the time of a digital clock in hours: minutes: seconds.

II, Diacylglycerol plus TMB-8. 70 µM TMB-8 was applied 15 min before treatment with 100 µg/ml diacylglycerol. Two sample injections of Lucifer (*a*) 15 min, (*b*) 29 min after diacylglycerol application.

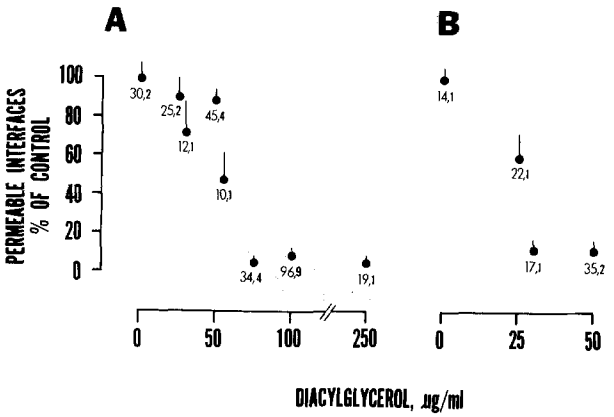

Fig. 2. Effect of exogenous diacylglycerol on junctional transfer of Lucifer Yellow in rat liver cells. Ordinates: mean incidence (%) of permeable interfaces (±SE), that is, the proportion of first-order neighbors of the injected cell exhibiting fluorescence 10 sec after injection. Abscissa: diacylglycerol concentration, µg/ml. (*A*) Confluent cultures. Cell density about 10^6 cells/dish. (*B*) Sparse cultures. Cell density $<10^5$ cells/dish. Expanded abscissa scale. The data are from the period 10 to 40 min after diacylglycerol application. Below each point, the number of injection trials followed by the number of dishes tested. Here and in Fig. 11, the incidence is normalized with respect to a control, namely 0.4% DMSO for *A* and 0.3% DMSO for *B*, the highest concentration used in each series. The absolute mean incidence of the control was 72 ± 6% SE and 85 ± 5% SE for *A* and *B*, respectively. (For the datum 250 µg/ml diacylglycerol in *A*, the DMSO concentration was 1%, the only experiment with a concentration exceeding 0.4%. The control here had an incidence of 64 ± 8% (n = 24))

ing the injection, during the following 15 to 40 sec, and thereafter for periods of 10 sec, spaced 50 sec apart—a total recording period of up to 10 min. This protocol minimized photodamage to the cell. Intracellular fluorescence was measured by video-analysis (Colorado Videoanalyzer, model 321) of a spot in the cell's video image. The signal amplitude from this spot was displayed against time on a chart recorder (e.g., Fig. 4), and this was also done with the signal (and noise level) from a spot in a dark region containing no fluorescent cells, to evaluate the video gain (the gain control of the TV camera was automatic). We chose conditions where the signals from the test spot were not saturated and the gains were comparable in the various trials.

Results

Diacylglycerol Reduces Junctional Permeability

Treatment with the synthetic diacylglycerol 1-oleoyl-2-acetyl glycerol caused reduction of cell-to-cell transfer: the proportion of interfaces of the injected cell transferring Lucifer Yellow (the incidence of permeable interfaces) decreased. Figure 1,*I* illustrates an example in which, 5 min after a 100-µg/ml diacylglycerol application, all transfer was blocked at all interfaces (*c*).

The incidence of permeable interfaces fell in a dose-dependent manner in both confluent (growth-arrested) and sparse (growing) cultures. The threshold of the effect lay at about 25 µg/ml in sparse cultures and at 50 µg/ml in confluent ones; at concentrations of 75 µg/ml and above, the incidence of permeable interfaces was close to zero (Fig. 2). The effect developed within 3 min of application of the agent and was complete generally within 20 min (Fig. 3).

The treatment caused no change in the *non*junctional membrane permeability to the fluorescent tracer. We determined the rates of Lucifer loss from single cells by video analysis of the cellular fluorescence. Treated or untreated, the cells exhibited negligible rates of loss over periods of several minutes (Fig. 4*A*), more than ten times the length of the 10-sec period for the scoring of permeable interfaces.

Thus, the reduction of cell-to-cell transfer reflects a reduction of junctional permeability.

TMB-8 Impedes the Reduction of Junctional Permeability

TMB-8 counteracted the effect of exogenous diacylglycerol. Figure 5 illustrates an experiment in which one member of a pair of cultures treated with 75 µg/ml diacylglycerol was, in addition, treated with 54 µM TMB-8. TMB-8 was applied 7 min before diacylglycerol, and the culture was kept in TMB-8/diacylglycerol medium for the duration of the test. In the TMB-8-treated condition the usual junctional response to diacylglycerol did not develop. The average incidence of permeable interfaces was less than in the untreated controls (*compare* Fig. 5*B* with 5*C*), but most of the cells exhibited an incidence of 60% throughout the 43-min period of observation. By contrast, the incidence in the culture treated only with diacylglycerol fell to zero within 10 min of the application of that lipid (Fig. 5*A*).

TMB-8 (54–70 µM) was effective over a broad range of exogenous diacylglycerol concentration (Fig. 6). It completely prevented the effect of 30 µg/ml diacylglycerol in the more sensitive sparse cultures, and it counteracted even up to 100 µg/ml diacylglycerol in the confluent cultures (Table 1; and Fig. 1,*II*).

We used TMB-8 at 54 and 70 µM, except for two experiments at 100 µM. The last concentration may have been toxic; the incidence of permeable interfaces was lower than at 70 µM (Table 1; *compare* $3a_3$ and $3a_4$ with $3a_2$ and 4).

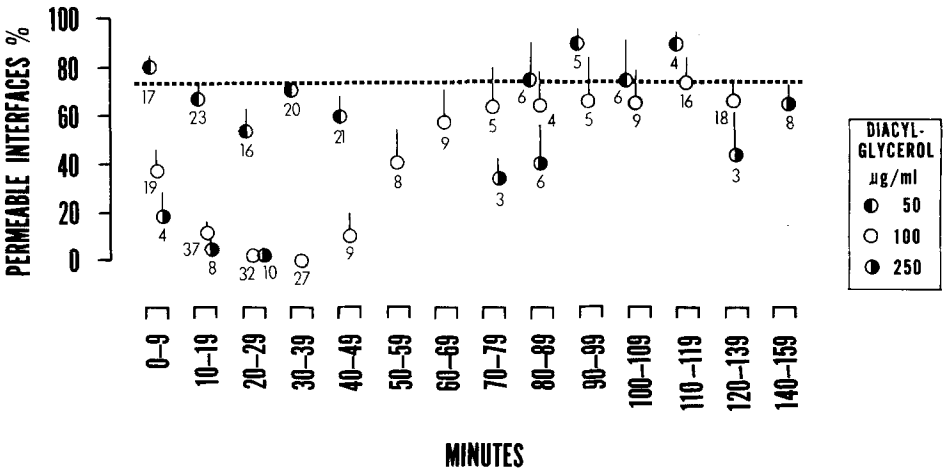

Fig. 3. Time course of the change in junctional permeability. Confluent cultures. The data points (means ± SE) for each concentration were pooled from different dishes for the test periods bracketed. Below each point, the number of injection trials. To avoid graphical overlap, the data from the three concentrations within a time bracket were slightly offset horizontally from one another. (The maximum test period outside the incubator for any given dish was generally 40 min, in a few cases up to 60 min; the dishes were then re-incubated for at least 30 min before the next test period.) Dashed line, mean incidence (72%) of permeable interfaces of the control (0.4% DMSO)

Calmidazolium

The Ca-binding protein calmodulin mediates the action of Ca^{2+} in many cellular processes (Cheung, 1980; Means, Tash & Chafouleas, 1982). Several pieces of evidence suggest that it might have such a mediator role also in the Ca^{2+}-induced closure of the cell-to-cell channel: calmodulin was found associated with the gap-junction protein (Hertzberg & Gilula, 1982; Welsh et al., 1982); addition of Ca-conjugated calmodulin reduced the permeability engendered by gap-junction protein in lipid vesicles (Girsch & Peracchia, 1985); the calmodulin inhibitor calmidazolium prevented CO_2-evoked depression of electrical coupling (Peracchia, 1984); and bilateral internal perfusion of a cell junction rendered the cell-to-cell channel unresponsive to Ca^{2+}, presumably through washout (Johnston & Ramon, 1981).

We examined, therefore, whether calmidazolium, an inhibitor of calmodulin-activated proteins (Van Belle, 1981), influenced the diacylglycerol action on junction. In these experiments the cultures were pretreated with 5 or 10 μM calmidazolium for 1.5 and 1 hr, respectively, and were continued at that concentration during the diacylglycerol (75 μg/ml) treatment.

Calmidazolium did not prevent the fall of junctional permeability (Fig. 7).

Recovery of Junctional Permeability

The cells recovered spontaneously from the depression of junctional permeability; the incidence of permeable interfaces came back to about pretreatment level, without any medium exchange. This was so even with treatments in which the incidence had fallen to zero in *all* cells tested (Figs. 8 and 3). Recovery was noticeable within 40–50 min of the exposure to diacylglycerol and was about complete within 90 min.

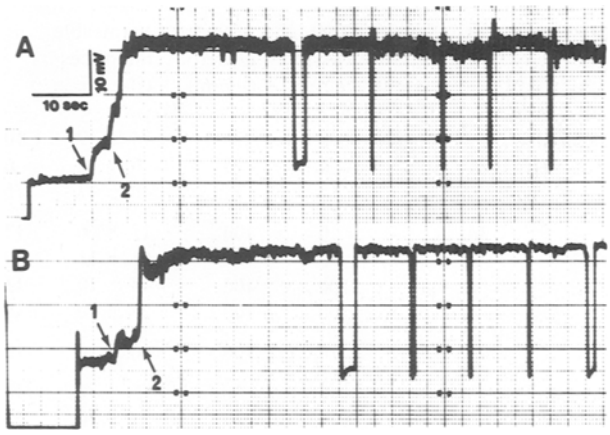

Fig. 4. Fluorescence loss from single cells (very sparse cultures). (*A*) Treated with 100 μg/ml diacylglycerol for 30 min, and (*B*) with 10 ng/ml TPA for 50 min. Chart records of the video signal from a point in the cell image (*see* Methods). Injection of Lucifer at arrow *1*. Removal of the brightly fluorescent micropipette from the cell and image field produces an abrupt rise in the signal (arrow *2*) due to an increase in the (automatic) video gain. The downstrokes to baseline correspond to 50-sec interruptions of video recording and illumination. The last records shown were taken about 5.5 min after injection

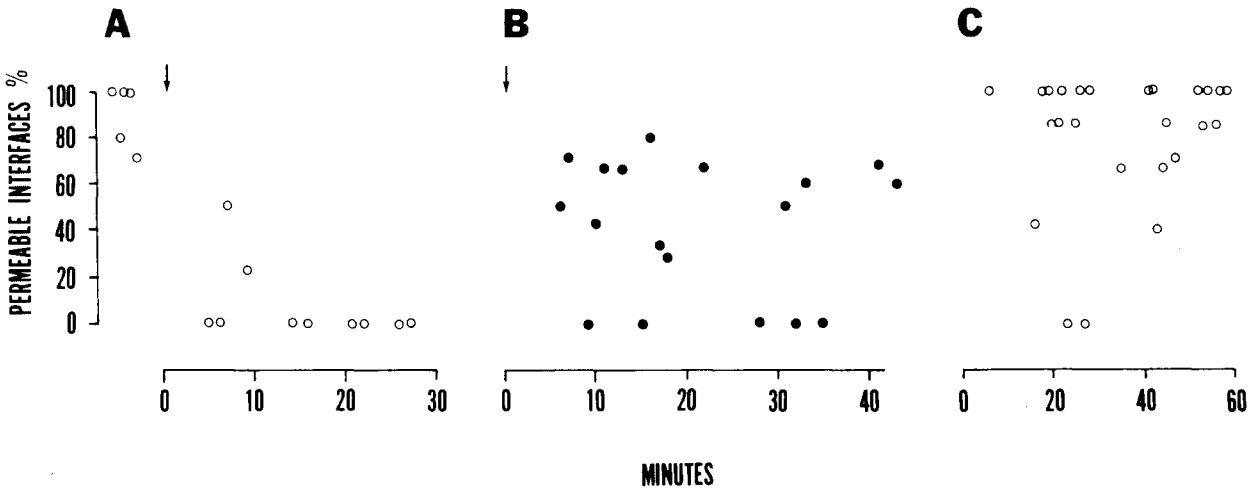

Fig. 5. TMB-8 counteracts the effect of diacylglycerol. (*A*, *B*) Diacylglycerol, 75 μg/ml, is applied to two sister dishes at arrows (time zero). In *B*, TMB-8, 54 μM, was applied 7 min earlier. The data points before time zero in *A* were collected 10 to 5 min before diacylglycerol application. (*C*) For comparison, data from a control dish to which only the solvent (0.4% DMSO) was applied at time zero. Each data point in this figure pertains to an individual injection trial. Confluent cultures

Table 1. TMB-8/diacylglycerol treatment

Culture	Experiment No.	TMB-8 (μM)	Diacyl-glycerol (μg/ml)	Permeable interfaces (%)[a]	*n*
Confluent	1	70	0	74 ± 9	10
	$2a_1$	0	75	0	6
	a_2	54	75	40 ± 7	15
	$3a_1$	0	100	17 ± 6	12
	a_2	70	100	61 ± 11	10
	a_3	100	100	44 ± 10	11
	a_4	100	100	27 ± 10	10
	4	70	100	60 ± 10	10
Sparse	5[b]*a*	0	30	9 ± 6	15
	b	54	30	71 ± 10	30
	$6a_1$	0	50	2 ± 1	16
	a_2	54	50	9 ± 4	17
	a_3	54	50	24 ± 7	15

[a] Mean ± SE; *n*, the number of injection trials. In each experiment, data from sister dishes were collected in the same time periods after application of diacylglycerol, generally 10–40 min.
[b] *a* and *b* are not sister dishes; cells were seeded (from the same stock cultures) on the same day but at much lower density in *b*; however, culture *b* was allowed to grow 16 hr longer before the experiment. In the other experiments, a_1 . . . a_4 denote sister cultures.

Is this recovery due to the cells becoming resistant to the exogenous diacylglycerol, to the diacylglycerol being unstable in the medium, or to the diacylglycerol being metabolized by the cells?

The first two possibilities were ruled out by the following experiments:

(*i*) Fresh diacylglycerol, 100 μg/ml, was applied to a culture (a_1) after it had recovered from the fall of junctional permeability produced in a preceding round of 100 μg/ml diacylglycerol treatment. Junctional permeability fell again in the second round (Fig. 9*A*).

(*ii*) The medium from a culture (b_1) was removed and incubated with the diacylglycerol (100 μg/ml), but without cells, at 37°C, 5% CO_2, for 2 hr. This medium was then applied to a sister culture (b_2). It produced a fall in junctional permeability comparable to that produced by fresh diacylglycerol medium (Fig. 9*B*).

The last possibility—metabolization—seems likely because of the following result:

(*iii*) The medium from a culture (a_2), which had been treated with diacylglycerol, 100 μg/ml, and which had recovered from the ensuing depression of junctional permeability, was applied to an untreated sister culture (a_3). This had little, if any, effect on junctional permeability in a_3 (Fig. 9*C*).

In sum, exhaustion of the exogenous diacylglycerol in the medium by its metabolization in the cells appears to be the cause of the recovery of junctional permeability. Indeed, there is evidence that this synthetic diacylglycerol, like the endogenous one, is rapidly phosphorylated in cells and converted to its phosphatidate (Kaibuchi et al., 1983; Sugimoto et al., 1984.)

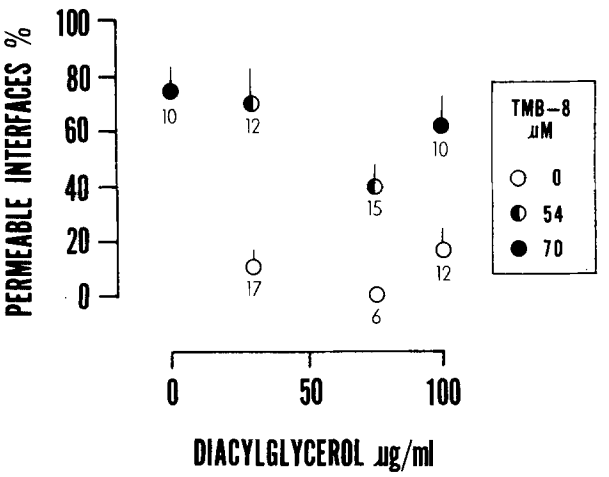

Fig. 6. TMB-8 is effective over a range of diacylglycerol concentration. The data (mean incidence of permeable interfaces ± SE) for each diacylglycerol concentration are from parallel dishes: one treated with diacylglycerol (○), the other with diacylglycerol plus TMB-8, 54 μM (◐) or 70 μM (●). Below each data point is the number of injection trials for equal periods for each pair of dishes. TMB-8 was applied 7 to 18 min earlier than diacylglycerol. The first point gives the mean incidence value for 70 μM TMB-8 alone for a test period of 55 min. The data for 30 μg/ml diacylglycerol are from sparse, the others from confluent, cultures. (Not included here are the data for 50 μg/ml diacylglycerol from sparse cultures where TMB-8 was little effective; *see* Table 1 and Discussion)

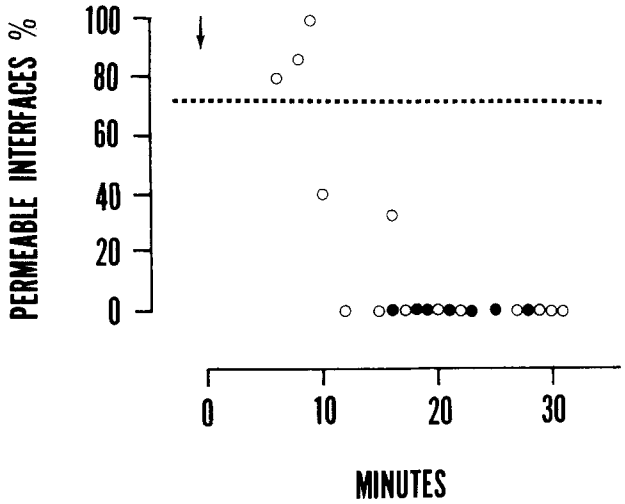

Fig. 7. Calmidazolium does not prevent reduction of junctional permeability by diacylglycerol (75 μg/ml, applied at arrow). (○) 5 μM calmidazolium was applied 90 min earlier than diacylglycerol; (●), 10 μM calmidazolium was applied to another culture 60 min earlier. For clarity of presentation, four zero % data points (two for each calmidazolium concentration) were omitted between time 15 and 23 min. Confluent cultures

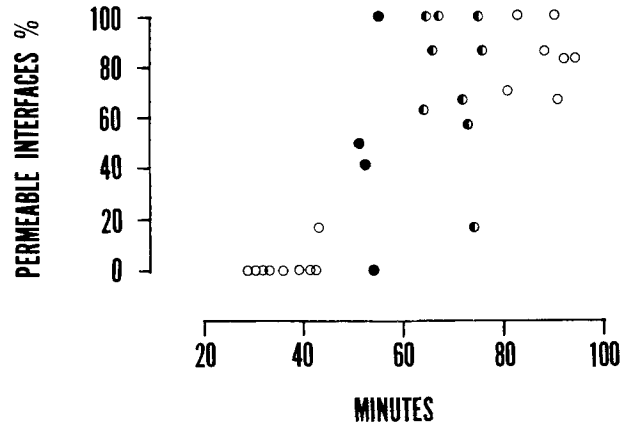

Fig. 8. Recovery from diacylglycerol-induced depression of junctional permeability. At time zero (not shown) 100 μg/ml diacylglycerol was applied to three confluent sister cultures ○, ◐, ●. The dishes were then kept in the incubator, except for the test periods (about 20 min) plotted

Action of a Protein Kinase C-Activating Phorbol Ester

The phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), also denominated phorbol-12-myristate-13-acetate, activates the diacylglycerol signal branch at protein kinase C. It has a molecular site similar to diacylglycerol, the endogenous activator of this enzyme (Nishizuka, 1984). Like the exogenous diacylglycerol, it can readily penetrate the cell membrane because of its lipid moiety. But it is not metabolized as fast and, presumably because of this, it has a longer lasting action on a number of cellular activities ruled by the diacylglycerol signal pathway (Nishizuka, 1984). We used TPA here to compare its effect with that of exogenous diacylglycerol, including the recovery of junctional permeability. TPA has been found to reduce metabolic cooperation (Yotti, Chang & Trosko, 1979; Murray & Fitzgerald, 1979), electrical coupling and cell-to-cell dye transfer (Enomoto et al., 1981; Yamasaki et al., 1985) in mammalian cells.

TPA caused the incidence of permeable interfaces to fall (Figs. 10*a* and 11), without sensibly affecting nonjunctional membrane permeability (Fig. 4*B*). TPA, thus, reduced junctional permeability.

The threshold concentration for the effect was <4 ng/ml, less than one-thousandth the threshold for exogenous diacylglycerol. At low doses, the time courses of the fall of junctional permeability produced by the two agents were not very different, but the time courses of the recovery were. With TPA the recovery was slower and less complete: in the diacylglycerol treatments the incidence of per-

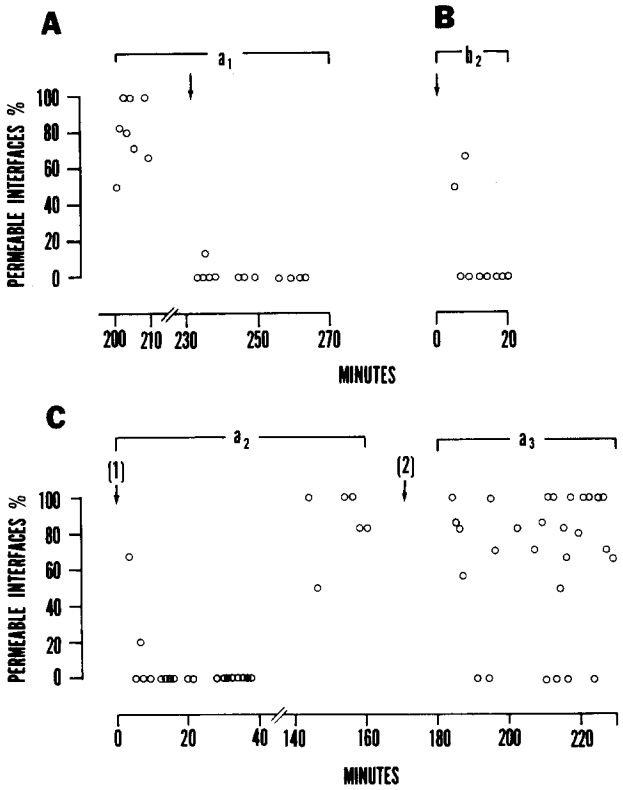

Fig. 9. Is the recovery of junctional permeability due to exhaustion of exogenous diacylglycerol by metabolization? (*A*) Culture a_1 had been treated with 100 μg/ml diacylglycerol at time zero causing the incidence of permeable interfaces to fall to 0% (not shown). Recovery ensued and is shown from time 200 min onward. At arrow, fresh 100 μg/ml diacylglycerol was applied. (*B*) Medium was removed from an untreated culture (b_1, not shown) and 100 μg/ml diacylglycerol added. After a 2-hr incubation (without cells), this medium was applied (arrow) to sister culture b_2. (*C*) At arrow *1*, diacylglycerol, 100 μg/ml, was applied to culture a_2. Recovery from depression of junctional permeability was tested during the period 140–160 min after the diacylglycerol application. At arrow *2*, the medium from culture a_2 was transferred to culture a_3. (a_1, a_2, and a_3 were sister cultures.) Confluent cultures

meable interfaces came back to about control level within 90 min in confluent cells; in the TPA treatments most of the incidence values were then still at about 50% of the controls (Fig. 12*A*, *compare* with Fig. 3).

In sparse cultures the effect of TPA was even more prolonged. There the incidence was about 10% even after 2 hr of treatment, and the values were widely scattered (Fig. 12*B*).

TMB-8

TMB-8 (54 μM) prevented the depression of junctional permeability produced by 10 ng/ml TPA (Fig. 13). The antagonistic effect of TMB-8 is thus comparable to that in the diacylglycerol-treated cells. TMB-8 (70 μM) was effective even against TPA concentrations more than 10 times threshold (40 ng/ml) (Table 2; Fig. 10*b*).

The effect of TMB-8 seemed to wear off with time. The incidence of permeable interfaces tended to fall after about 1 hr in TMB-8/TPA medium (Table 2, Expts. $2a_2$ and $3a_2$).

Vanadate

Vanadate inhibits phosphatases (Lopez, Stevens & Lindquist, 1976), particularly those de-phosphorylating at tyrosine (Leis & Kaplan, 1982; Swarup, Cohen & Garber, 1982). Since activity in the diacylglycerol messenger pathway is often associated with phosphorylation of tyrosine, it seemed of interest to see whether vanadate would impede the recovery of junctional permeability from diacylglycerol- or TPA-induced depression. We added 25 or 50 μM sodium orthovanadate to the medium containing diacylglycerol or TPA. The vanadate enters cells by the transport route of phosphate (Cantley, Resh & Guidotti, 1978), and at those concentrations it is not toxic to various types of rat and mouse cells (Klarlund, 1985).

In one experiment the cultures were treated with vanadate 25 min after diacylglycerol (100 μg/ml) application. The incidence of permeable interfaces, determined 4–4.5 hr later, was 69%. In another experiment, we treated a culture with vanadate plus 10 ng/ml TPA for about 12 hr. Here, too, junctional permeability recovered to a level comparable to that of the sister culture treated with TPA alone (Table 3) .

These experiments were too few to tell whether vanadate retards the recovery, but they showed that it does not prevent it.

Morphological Changes

Diacylglycerol and, more so, TPA caused changes in cell morphology: the cells became more refractile and got thicker in their central portions, as seen in phase contrast; but they did not lose contact (Figs. 1 and 10). These changes occurred at their earliest 5–10 min after the fall in junctional permeability (Figs. 1,*Ic* and 10*a*) and they persisted long after the junctional permeability had recovered (Fig. 10*c*).

The morphological and permeability changes were so out of phase with each other that they are unlikely to be linked. This notion is corroborated by the finding that the two changes can be dissociated by TMB-8: while TMB-8 blocked the junctional-permeability change, it did not block the morphological one (Fig. 10*c*).

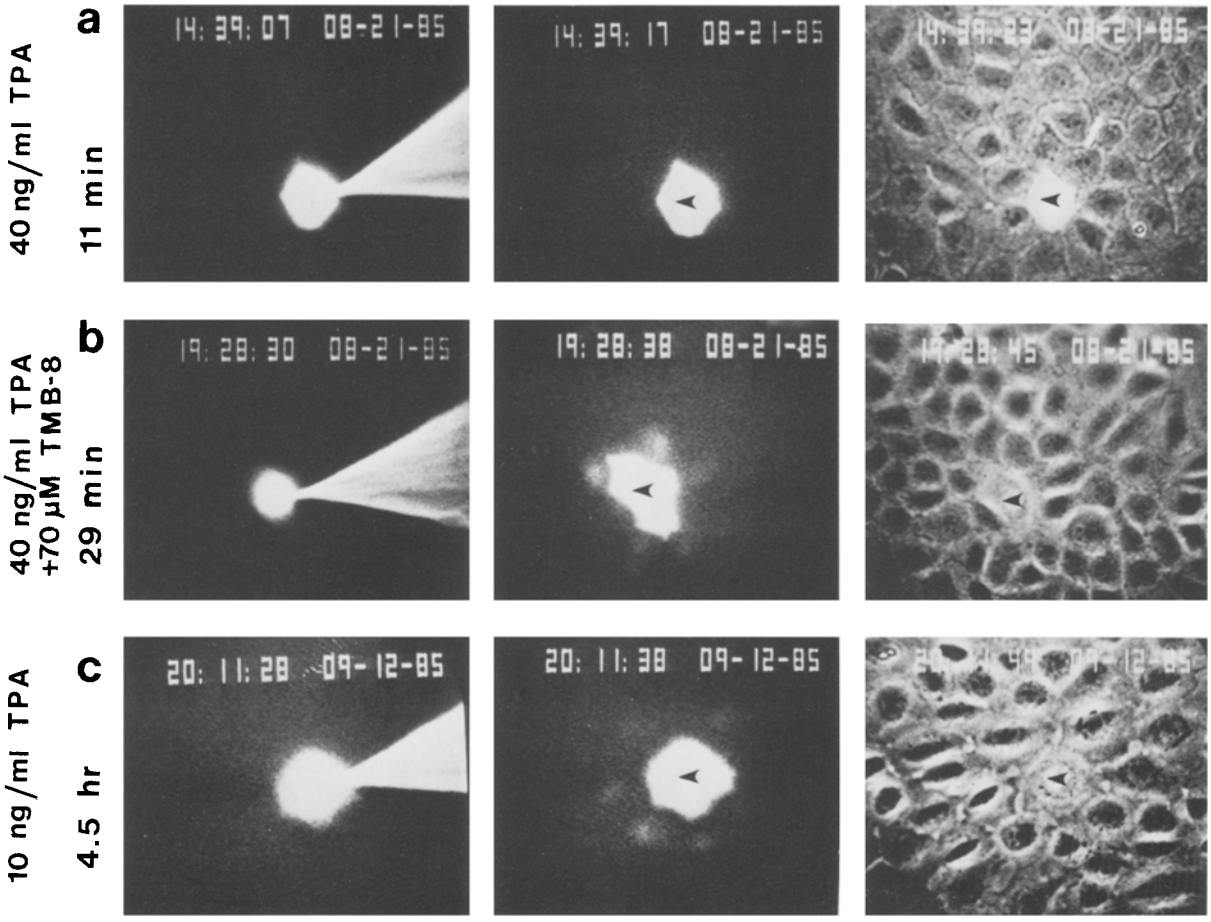

Fig. 10. Effect of TPA on junctional transfer and cell morphology. Video pictures of sample Lucifer microinjections (*a*) 11 min after application of 40 ng/ml TPA, (*b*) 29 min after application of 40 ng/ml TPA plus 70 μM TMB-8 (sister culture of *a*); (*c*) recovery of junctional transfer (but not of morphology) at 4.5 hr after application of 10 ng/ml TPA. *Left panel*, microinjection; *middle panel*, 10 sec after injection; *right panel*, phase contrast view

Table 2. TMB-8/TPA treatment

Culture	Experiment No.	TMB-8 (μM)	TPA (ng/ml)	Test period[a] (min)	Permeable interfaces (%)[b]	*n*
Confluent	$1a_1$	0	10	20 to 50	5 ± 3	13
	a_2	54	10	20 to 50	72 ± 7	20
	$2a_1$	0	40	10 to 37	12 ± 4	23
	a_2	70	40	10 to 36	70 ± 7	11
				43 to 66	30 ± 13	8
Sparse	$3a_1$	0	10	20 to 30	47 ± 14	8
				60 to 70	11 ± 7	8
				303 to 315	57 ± 7[c]	11
	a_2	54	10	20 to 40	93 ± 4	18
				85 to 100	48 ± 10	16
				253 to 283	43 ± 8	17

[a] Time after TPA application.
[b] Mean ± SE; *n*, the number of injection trials.
[c] Junctional permeability is recovering here from the TPA-induced depression; note that this datum is not significantly different from that in the last test period of sister culture a_2.

Table 3. Vanadate does not prevent recovery of junctional permeability

Treatment	Experiment No.	Concentration[a]	Vanadate (μM)	Test period (hr)[b]	Permeable interfaces (%)[c]	n
Diacylglycerol	1	100 μg/ml	25	4 – 4.5	69% ± 4	18
TPA	$2a_1$	0 ng/ml	50	14 –14.5	76% ± 7	18
	a_2	10	50	12 –12.5	57% ± 9	18
	a_3	10	0	12.5–13	65% ± 6	29

Diacylglycerol was applied 25 min before vanadate; TPA and vanadate were applied together.
[a] Of diacylglycerol or TPA.
[b] hr after application of diacylglycerol or of TPA and/or vanadate.
[c] Mean ± SE; n, the number of injection trials.

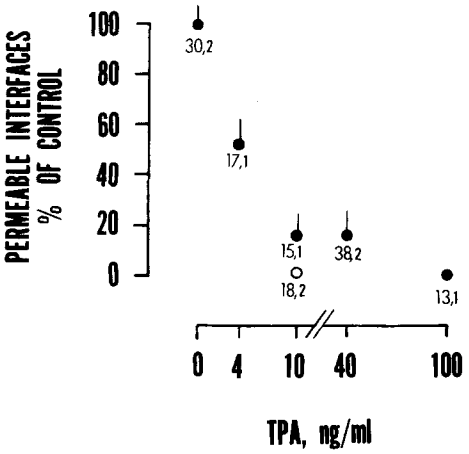

Fig. 11. Effect of TPA. Solid circles, confluent cultures; open circle, sparse culture. The data are expressed as percent of control. Control is the mean incidence (72 ± 6%) in cultures treated with 0.4% DMSO alone. (Only for 100 ng/ml was DMSO concentration higher, namely 0.5%.) Data were pooled from the periods 20–50 min and 10–40 min after application of 4 and 10 ng/ml, or 40 and 100 ng/ml TPA, respectively

Discussion

Dual Regulation of the Cell-to-Cell Channel

The present results show that junctional permeability is reduced by exogenous diacylglycerol. This widens our perspective of the regulation of junctional cell-to-cell communication: the finding carries the suggestion that the cell-to-cell membrane channel may be physiologically controlled by the diacylglycerol messenger branch of the phosphoinositide transmembrane route. So far we only knew it to be controlled by the G-protein transmembrane route, namely the cyclic AMP-messenger extension of that route (*cf.* Loewenstein, 1981; 1985). Thus, the notion of a dual control of cell-to-cell communication emerges, the cyclic AMP route upregulating and the diacylglycerol route downregulating it.

This notion spins a connecting thread with our knowledge about the *src* genes and their effects on cell-to-cell communication. The products of these oncogenes—both of the viral and the cellular genes—cause reduction of communication (Atkinson et al., 1981; Azarnia & Loewenstein, 1984*a*,*b*; R. Azarnia & W.R. Loewenstein, *unpublished work*). The *src* proteins enhance inositol-lipid kinase activity (Sugimoto & Erikson, 1985) causing the steady-state level of diacylglycerol to increase (Macara, Marinetti, Livingston & Balduzzi, 1985). The resulting enhancement of the signal flow in the diacylglycerol route would thus explain the downregulatory action on cell-to-cell communication.

Is Ca^{2+} Involved in the Downregulation?

In considering the question of the downregulatory mechanism, we may reasonably assume that, as in other functions of the diacylglycerol messenger pathway (Nishizuka, 1984), the action of diacylglycerol is relayed by protein kinase C. Beyond that, we have a few clues: (*i*) TMB-8 counteracts the downregulation, as found in the present study; (*ii*) TMB-8 blocks intracellular Ca^{2+} mobilization (Chiou & Malagodi, 1975; Mix et al., 1984); and (*iii*) intracellular Ca^{2+} mobilization causes downregulation of junctional permeability (Loewenstein & Rose, 1978). (*iv*) Ca^{2+} is required for protein kinase C activation (Takai, Kishimoto & Nishizuka, 1982; Wolf, LeVine, May, Cuatrecasas & Sahyoun, 1985); and (*v*) TMB-8, $\geq$400 μM, inhibits protein kinase C *in vitro*, competitively with phospholipid (Y. Nishizuka, *personal communication*) (although

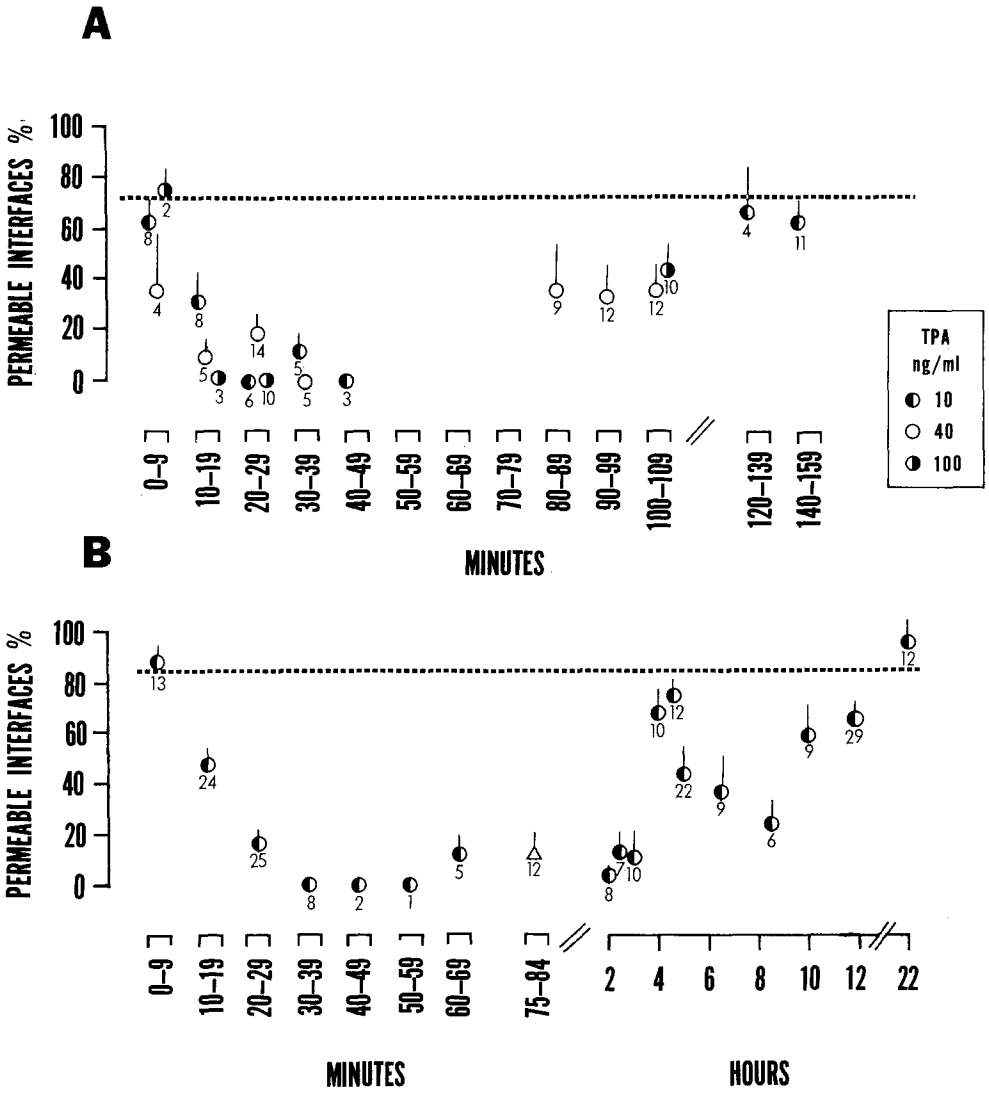

Fig. 12. Time course of the effect of TPA. (*A*) Confluent, (*B*) sparse cultures. Mean incidence of permeable interfaces ± SE; the number of injection trials is given below the data points. In *A*, the points for different TPA concentrations within the same time brackets were slightly offset horizontally from one another to avoid overlap. The triangle datum in *B* is from an experiment in which a culture was treated first with 50 μg/ml diacylglycerol (incidence 2% ± 3, $n = 10$), tested for recovery 2.5 hr later (78% ± 8, $n = 11$), and then treated with 10 ng/ml TPA at 3 hr ($t = 0$ for TPA). *See* inset, for TPA concentrations used in *A* and *B*. The dashed lines represent the mean incidences for control cultures: *A*, 72 ± 6%, $n = 30$ (0.4% DMSO); *B*, 85 ± 5%, $n = 20$ (0.3% DMSO)

this concentration is an order of magnitude higher than that needed for counteracting the diacylglycerol effect on junctional permeability, one must reckon with the possibility that TMB-8 could be more potent in the intact membrane than *in vitro*).

The first four clues all implicate Ca^{2+} in the diacylglycerol downregulatory mechanism of communication. But while *iv* together with *v* indicate a role of Ca^{2+} at the level of protein kinase C, *i* through *iii* suggest a Ca^{2+} role further down the pathway, closer to the channel. We will consider both possibilities below.

A role of H^+ does not seem likely here. This ion has been implicated in the control of the channel because intracellular acidification causes fall of junctional conductance (Turin & Warner, 1977; Rose & Rick, 1978; Spray, Harris & Bennett, 1981). But activation of the diacylglycerol signal pathway powerfully stimulates the Na^+/H^+ antiport exchange across the plasma membrane of many types of cells (*cf.* Grinstein & Rothstein, 1985), lowering the intracellular H^+ concentration (Burns & Rozengurt, 1983; Moolenaar, Tsien, van der Saag & de Latt, 1983; Rosoff, Stein & Cantley, 1984; Grin-

Table 4. Diacylglycerol sensitivity and the number of cells

Culture	Cells/dish	Diacylglycerol (μg/ml)	Incidence of permeable interfaces		
			Relative to control (%)	(%)[a]	*n*
A. Special	2×10^5	0	100	73 ± 7	18
		50	63	46 ± 8	23
B. Confluent	1×10^6	0	100	72 ± 6	30
		50	104	75 ± 12	8
		50	85	61 ± 8	9
		50	85	61 ± 6	17
		50	88	63 ± 11	11

A. Cells were seeded only onto center of dish (*see* text). Junctional transfer was tested in cells in the confluent central area where the cell density was comparable to that in *B*.
B. Cells seeded uniformly over the entire dish (the usual confluent cultures).
[a] Mean ± SE; *n*, the number of injection trials. Data collected 10–40 min after diacylglycerol application.

stein, Rothstein & Cohen, 1985)—precisely the opposite direction to the change needed for downregulation of junctional permeability.

We present two general models in which Ca^{2+} intervenes in the diacylglycerol signal pathway to control the channel. By control we mean control of the open state of the channel or of channel formation.

In the first model, Ca^{2+} is the coupling element between the diacylglycerol pathway and the channel. The information in this model flows:

$$\text{Diacylglycerol pathway} \rightarrow Ca^{2+} \rightarrow \text{channel}. \quad (1)$$

The ion here mediates the action of the diacylglycerol pathway. The signal flow in the pathway starts at the surface membrane receptor (or at a later stage if the receptor is bypassed) and Ca^{2+} is the signal proximal to the channel.

This type of model is merely an extension of the original Calcium Hypothesis of channel regulation (Loewenstein, 1966). It adds an information input stage—a transmembrane stage—to the regulatory mechanism by Ca^{2+}. There may be other inputs to that mechanism besides the diacylglycerol pathway (Loewenstein, 1985), but for all of them Ca^{2+} would be the "end" signal for the channel.

In our simplest conception of the model, the Ca^{2+} signal is an elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the junctional locale in response to diacylglycerol. This idea is not too far-fetched: the phorbol ester TPA, which mimics the diacylglycerol effect, causes (oscillatory) elevation of the $[Ca^{2+}]_i$ in mammalian oocytes (Cuthbertson & Cobbold, 1985).

There are several ways how such a Ca^{2+} signal could be generated by activity in the phosphoinositide pathway. For example, we may envision it to be produced by an action of diacylglycerol on the Na^+/H^+ membrane antiport, in two steps: first, $[Na^+]_i$ rises due to activation of the Na^+/H^+ exchange; and second, $[Ca^{2+}]_i$ rises as a result of that. This idea is guided by the knowledge that a rise in $[Na^+]_i$ causes enhancement of Ca influx and diminution of Ca efflux across the plasma membrane (Baker, 1972; 1978) and release of Ca^{2+} from intracellular stores (Crompton, Capano & Carafoli, 1976; Carafoli & Crompton, 1978).

In our second model, the action of Ca^{2+} is farther removed from the channel. The ion is an early signal in the diacylglycerol pathway, and the information flows:

$$Ca^{2+} \rightarrow \text{diacylglycerol pathway} \rightarrow \text{channel}. \quad (2)$$

Here Ca^{2+} drives the diacylglycerol pathway, and the regulatory action on the channel is exerted by a pathway product.

This model represents a more substantial modification of the Calcium Hypothesis. We will descend to particulars and specify the model for the example-case where protein kinase C is the site of the Ca^{2+} action.

This key enzyme in the diacylglycerol pathway binds to plasma membrane at Ca^{2+} concentrations of $1–5 \times 10^{-7}$ M and above (in the presence of 3 mM Mg) (Wolf et al., 1985). Such binding, that is, translocation of the enzyme from the cytoplasm to the membrane, is probably a prerequisite for its activation by transmembrane signals. The enzyme is activated *in vitro* by Ca^{2+} concentrations of $0.5–5 \times 10^{-5}$ M in the absence of diacylglycerol (Takai et al., 1982; Wolf et al., 1985). Therefore, in the cell, we may expect the post-protein kinase C segment of the pathway to be active in the absence of an endogenous diacylglycerol signal input when $[Ca^{2+}]_i$ rises to that concentration range. The model thus becomes:

$$Ca^{2+} \rightarrow \text{protein kinase C} \rightarrow s_1 \rightarrow s_n \rightarrow \text{channel} \quad (2a)$$

where s_1 and s_n are intermediary stages along the pathway to the channel.

In this form 2*a*, the model would fit situations where the transmembrane segment of the pathway is silent and where $[Ca^{2+}]_i$ is sufficient to drive the

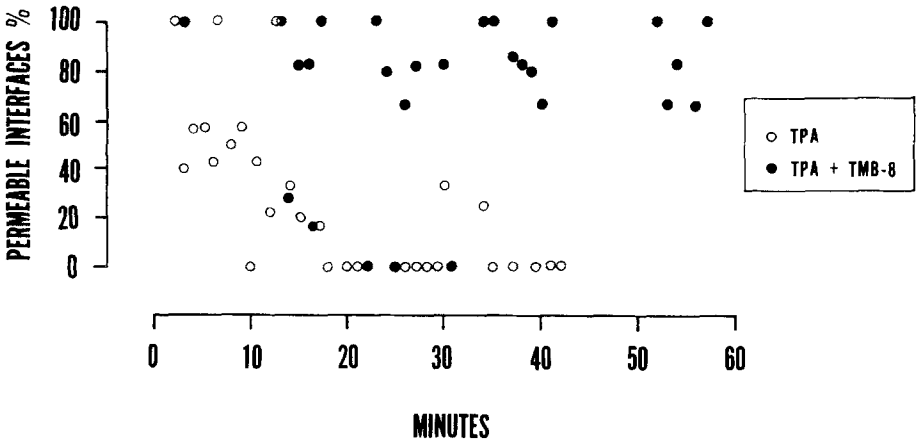

Fig. 13. TMB-8 counteracts the effect of TPA. Confluent sister cultures were treated with 10 ng/ml TPA at time zero. 54 μM TMB-8 was added to one of the cultures (●) 12 min earlier

intracellular segment by itself. Such situations may arise when a cell is injured or when its Ca outflux is slowed by a depressed energy metabolism. Indeed, cell injury and inhibition of energy metabolism cause junctional permeability to fall, if (and only if) the $[Ca^{2+}]_i$ rises in the junctional locale (Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1976).

But of more immediate interest to the present work are situations where Ca^{2+} and diacylglycerol are both present in sufficient concentrations. Then, the pathway would be expected to operate at lower $[Ca^{2+}]_i$. In the presence of diacylglycerol the Ca^{2+} requirement for activation of protein kinase C *in vitro* drops to below 10^{-6} M (Takai et al., 1982). This Ca^{2+} requirement, as well as that for the enzyme's translocation to the membrane, is then within the concentration range of physiological Ca^{2+} signalling.

So, we arrive at the more general form of the model where conflux of Ca^{2+} and diacylglycerol leads to downregulation of cell-to-cell permeability:

$$\begin{matrix} Ca^{2+} \\ \qquad\searrow \\ \qquad\quad \text{protein kinase C} \rightarrow s_1 \rightarrow s_n \rightarrow \text{channel.} \\ \text{diacyl-}\quad\nearrow \\ \text{glycerol} \end{matrix} \qquad (2b)$$

In this light, the channel regulation would be one of many instances of synergism between Ca^{2+} and the diacylglycerol pathway.

As to the origin of the Ca^{2+} signals here, we may imagine the signals to be produced by the diacylglycerol-stimulated Na^+/H^+ antiport exchange, as described above, and/or by the inositol triphosphate messenger branch (Berridge & Irvine, 1984). In the first mechanism, diacylglycerol would be both the cause of the Ca^{2+} signal and its co-actor at protein kinase C; the diacylglycerol here would have to linger at least as long as it takes to produce the Ca^{2+} signal. The second mechanism would come into play when the transmembrane branch of the phosphoinositide pathway is active—a good possibility, for example, in the case when the *src* genes are expressed.

The aforegoing are tentative models, pending an elucidation of the relative magnitudes of the effects TMB-8 exerts on mobilization of intracellular Ca^{2+} and directly on protein kinase C in the present conditions.

As to the nature or identity of the intermediary stages, s_1, s_n, there is not much to guide us. But dealing with a pathway segment containing tyrosyl kinases, it is hard to escape the thought that conformational switching of proteins by tyrosine phosphorylation might be critically involved. In this context it is interesting that $pp60^{c\text{-}src}$ (and $pp60^{v\text{-}src}$), a protein-tyrosyl kinase (Bolen et al., 1984), is itself phosphorylated (at serine 12) by diacylglycerol activation of protein kinase C in the cell (Gould, Woodgett, Cooper, Buss, Shalloway & Hunter, 1985). This fact, plus the evidence suggesting that $pp60^{c\text{-}src}$ is negatively regulated (Cooper, Hunter & Shalloway, 1985; Courtneige, 1985) are guideposts for future work.

Diacylglycerol Sensitivity of Confluent and Sparse Cultures

Sparse (growing) cultures were more sensitive to diacylglycerol than confluent (growth-arrested) ones (Fig. 2), and they recovered more slowly from the TPA-induced depression of junctional permeability (Fig. 12). Is this difference related to the growth cycle of the cell, or merely to the number of

cells, that is, to the number of diacylglycerol-metabolizing (or TPA-metabolizing) cells per dish? The second is a very real possibility, because endogenous and exogenous diacylglycerol are rapidly metabolized (Kaibuchi et al., 1983; Sugimoto et al., 1984) and because of the result of the experiment of Fig. 9 revealing that such metabolization probably is the factor that limits the duration of the junctional-permeability depression produced by exogenous diacylglycerol. We have no definitive answer to this question, but the following experiments shed some light.

In these experiments we compared the diacylglycerol sensitivity of special cultures in which the total number of cells per dish were different, but where the cell density in the test region was like that in confluent culture. To this end, we seeded only onto the center of the dish, depositing a dense suspension of cells there, and allowed the cells to grow. After about 2 days the culture covered about three quarters of the dish, its central portion having about the same cell density as the usual confluent culture. From earlier studies of radioactive thymidine incorporation, we know that in the central region of the special culture most cells are growth-arrested, as are most cells in confluent culture.

We tested junctional permeability in the central region of diacylglycerol-treated special cultures and compared it with that in diacylglycerol-treated confluent cultures. The reduction of junctional permeability in the former was more pronounced (Table 4).

The difference in sensitivity also manifested itself in the effect of TMB-8: this agent was not as potent in counteracting diacylglycerol in the special culture as it was in confluent ones. For example, 70 μM TMB-8 failed to protect junctional permeability against 100 μg/ml diacylglycerol in the special culture, whereas it was highly effective in confluent cultures.

These differences in sensitivity, thus, appear to relate to the number of the cells rather than to their growth. The result can be explained by invoking nothing more than a difference in the number of diacylglycerol-metabolizing cells.

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