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# Downregulation of Cell-to-Cell Communication by the Viral *src* Gene is Blocked by TMB-8 and Recovery of Communication is Blocked by Vanadate

Birgit Rose, Toshihiko Yada, and Werner R. Loewenstein
Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33101

**Summary.** The viral *src* gene downregulates junctional communication, closing cell-to-cell membrane channels presumably by way of the phosphoinositide signal route. We show that TMB-8 [8-N, N-(diethylamino) octyl-3,4,5-trimethoxybenzoate] counteracts this downregulation in cells transformed by temperature-sensitive mutant Rous sarcoma virus: TMB-8 (36–72 $\mu$M) raises junctional permeability when applied during activity of *src* protein kinase, i.e., at steady permissive temperature; and TMB-8 inhibits the fall of junctional permeability, when the activity of *src* protein kinase gets turned on. TMB-8 also (reversibly) inhibits the growth of the cells at permissive temperature and reverses the morphological changes associated with transformation. The morphological reversal lags several hours behind the junctional-permeability reversal. Communication recovers within a few minutes when the activity of the *src* protein kinase is turned off (in absence of TMB-8). Sodium orthovanadate (20 $\mu$M) prevents this recovery, but it has no major effect on junctional permeability on its own. We discuss possible modes of action of these agents on critical stages of the signal route, related to intracellular $Ca^{2+}$ and protein kinase C.

**Key Words** junctional permeability · gap junction · *src* gene · *src* protein · Rous sarcoma virus · calcium ion · TMB-8 · vanadate · phorbol esters

## Introduction

The viral *src* gene downregulates junctional cell-to-cell communication (Atkinson et al., 1981; Azarnia & Loewenstein, 1984*a*; Atkinson, Anderson & Sheridan, 1986). This action of the gene is independent of that on cytoskeleton (Azarnia & Loewenstein, 1984*b*). It involves a modification of the cell-to-cell membrane channels producing a reduction in junctional permeability (Azarnia & Loewenstein, 1984*a*) that depends on the tyrosine-specific protein kinase activity of the gene product, $pp60^{v\text{-}src}$ (R. Azarnia and W.R. Loewenstein, *unpublished*). The channel modification ensues rapidly (within 10 min) when the protein kinase activity of (mutant) $pp60^{v\text{-}src}$ is switched on. It is one of the earliest detectable changes of cell transformation. Its reversal upon switching off $pp60^{v\text{-}src}$ activity is similarly fast.

There are reasons to suspect that the viral *src* gene causes cell transformation by activation of the phosphoinositide transmembrane signal route, particularly its diacylglycerol branch (Gilmore & Martin, 1983; Berridge & Irvine, 1984; Sugimoto, Whitman, Cantley & Erikson, 1984; Macara, Marinetti, Livingston & Balduzzi, 1985; Sugimoto & Erikson, 1985; Jackowski, Rettenmier, Sherr & Rock, 1986; Kaplan et al., 1986; Macara et al., 1986). This also may be the pathway by which the gene causes modification of the cell-to-cell channels, since administration of diacylglycerol to normal cells produces reduction of junctional communication (Enomoto & Yamasaki, 1985; Gainer & Murray, 1985; Yada, Rose & Loewenstein, 1985). Thus, we explored here whether the *src* action on junctional communication could be altered by perturbation of the diacylglycerol pathway.

We asked whether the downregulation of junctional communication by *src* could be blocked by 8-N,N-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of intracellular $Ca^{2+}$ mobilization (Chiou & Malagodi, 1975; Mix, Dinerstein & Villereal, 1984; Sawamura, 1985) and of protein kinase C (Kojima, Kojima & Rasmussen, 1985; Sawamura, 1985). Protein kinase C is the key enzyme in the diacylglycerol pathway and $Ca^{2+}$ is known to play a crucial role in its activation; the free ionized $Ca^{2+}$ concentration in the cytoplasm must rise to $10^{-6}$ M for activation of protein kinase C (Kishimoto et al., 1980). Apart from such *a priori* considerations, the possibility of blocking the downregulatory action of *src* was suggested by our recent finding that TMB-8 prevents the reduction of junctional permeability induced by exogenous diacylglycerol (Yada et al., 1985). This finding prompted the present work.

We further asked whether the recovery from *src*-induced downregulation, namely the restoration of communication following the switching off of $pp60^{v\text{-}src}$ activity, could be blocked by vanadate.

Vanadate is an inhibitor of phosphatases that dephosphorylate at tyrosine (Leis & Kaplan, 1982; Swarup, Cohen & Garbers, 1982). Tyrosine residues are the main targets of protein phosphorylation when cells are transformed by the *src* gene (Collett, Purchio & Erikson, 1980; Hunter, 1980) or when their diacylglycerol pathway is activated otherwise (Cooper et al., 1982; Gilmore & Martin, 1983). Thus, on the assumption that the reversal of the *src*-induced channel modification depends on tyrosyl phosphatases, one would expect that the recovery from downregulation be slowed.

We used vertebrate cells infected with temperature-sensitive mutant Rous sarcoma viruses. The $pp60^{v\text{-}src}$ coded by these viruses has thermolabile protein kinase activity. The activity is high at 34°C, the "permissive" temperature, and low at 41°C, the "nonpermissive" temperature. Thus, the transforming activity of this protein could be switched on or off by merely shifting the temperature of the cultures by a few degrees. Junctional permeability of the cells was probed with the fluorescent tracer Lucifer Yellow (443 daltons).

We show that TMB-8 counteracts the *src*-induced reduction of junctional permeability and that, concurrently, other manifestations of cell transformation are inhibited. We show, furthermore, that vanadate retards the return to normal junctional permeability when the *src* function is turned off.

## Materials and Methods

### Cell Culture

Mouse 3T3 fibroblasts infected with temperature-sensitive RSV-LA90 (Schmidt-Ruppin group D), 3T3-*ts*LA90 (a gift of Dr. Joan Brugge), and quail embryo fibroblasts infected with temperature-sensitive RSV-NY68 (Schmidt-Ruppin group A, QEF-*ts*NY68, were grown in DMEM (GIBCO) with 10% fetal bovine serum (Hyclone) and 40 $\mu$g/ml gentamycin on 35 mm plastic dishes (Nunc) in an atmosphere of 95% air/5% $CO_2$ at 33–35°C (Azarnia & Loewenstein, 1984*a*). Special temperature regimens are described in Results. Rat epithelioid liver cells, clone 12-B (Yada et al., 1985) were grown in BME (GIBCO) with the same serum supplement and antibiotic at 37°C. The medium (2.5 ml) was renewed every other day and on the day before the experiments on junctional transfer. For the measurements of growth on solid substrate the medium was renewed every day, once the cultures had reached a density above $4 \times 10^4$ cells/cm$^2$.

For growth in suspension, autoclaved agar (1.9%) and methylcellulose (2.2%; Fischer) were mixed 1 : 1 with double-strength DMEM plus 10% fetal bovine serum (final agar, 0.86%; final methylcellulose, 1%). 4 ml agar medium was plated in 60-mm plastic dishes; the cells were suspended in 4 ml methylcellulose medium ($10^3$ cells/ml) and layered over the agar. The cultures were at 34°C in 95% air/5% $CO_2$. For colony counts 10 areas were randomly chosen in each dish.

Culture dishes from the same passage with the same cell density and maintained under strictly parallel conditions are referred to as *sister cultures*.

### Temperature Shifts and Regulation

For temperature shifts, the cultures were moved from their incubator at 33–35°C (the permissive temperature for the virus) to one at 40–41°C (the nonpermissive temperature), and vice versa. Before downshifts, the cultures were kept at 40–41°C for 14–24 hr.

During the testing of junctional transfer outside the incubators the temperature of the culture dishes was controlled by a feedback-regulated heater coil submersed in the medium of the dish (Azarnia & Loewenstein, 1984*a*).

### Treatments

TMB-8 (Calbiochem), sodium orthovanadate (Sigma) and D-600 (a gift of Dr. C. van Breemen) were applied from stock solutions in phosphate-buffered saline or distilled water; chlorpromazine (Sigma) from stock solutions in DMSO. Synthetic diacylglycerol, 1-oleoyl-2-acetyl-rac-glycerol (Sigma), dissolved in DMSO (25 or 50 mg/ml) was added to 0.5 ml medium withdrawn from the experimental culture or its sister culture; the mixture was sonicated 5 min at room temperature, added to the culture and mixed by swirling.

For treatment of suspension cultures in methylcellulose TMB-8 was dissolved in DMSO, and 1 ml of TMB-8-containing medium was added to the 4 ml methylcellulose medium (final concentrations: 36 $\mu$M TMB-8, 0.02% DMSO). The control sister culture contained the same concentration of DMSO.

For the experiments with Ca-free medium (*see* Table 3) serum was omitted. The Ca-free phosphate buffered saline (Ca-free PBS) contained (g/liter): KCl, 0.2; $KH_2PO_4$, 0.2; NaCl, 8.0; $Na_2HPO_4$, 1.15; $MgSO_4$, 0.0592. The cultures were washed three times with Ca-free PBS and then Ca-free PBS containing 1 or 5 mM EGTA was applied. In the experiments with diacylglycerol (*see* Table 3), 10 mg/ml egg albumin (Sigma) was added to Ca-free PBS to serve as a carrier, in lieu of serum, for the hydrophobic diaclyglycerol.

### Determination of Junctional Transfer

Junctional permeability was probed with Lucifer Yellow CH (Molecular Probes; 10% aqueous solution). The dye was injected into the cells with the aid of a micropipette by pulses of pneumatic pressure controlled by a solenoid valve. For observation and microinjection of the cells, we used a television camera (DAGE MTI65 SIT) coupled to a Leitz Diavert microscope equipped with phase contrast and fluorescence optics (100 W mercury arc lamp). Cells and microinjection pipette were viewed on a television monitor. The experiments were videotaped and played back for evaluation of junctional transfer.

We determined the *incidence of permeable interfaces*, i.e., the proportion of fluorescent first-order neighbors of the injected cell. The incidence was scored at a fixed interval from the injections, timed by a clock displayed on the TV monitor. The interval was 20 sec for 3T3-*ts*LA90 and QEF-*ts*NY68 cells, and 10 sec for rat liver cells (which had a higher junctional permeability). In all cases analyzed, the first-order neighbors were clearly distin-

guishable in both the untransformed and transformed condition. (Regions with excessive cell overlap were not microinjected.) The video taping of the spread of fluorescence during and after injection contributed to the accuracy of the determinations of incidence of permeable interfaces.

The cultures were at room air during the testing of junctional transfer. We generally limited the testing period to 30 min in order to avoid effects of the lack of $CO_2$ on junctional permeability. This period left a good safety margin; in untreated cultures, the incidence of permeable interfaces was not affected by this condition over periods of 50 min.

The cell density of cultures examined for junctional transfer was $3–10 \times 10^4$ cells/cm$^2$ for 3T3-*ts*LA90 and QEF-*ts*NY68, and $\approx 1 \times 10^5$ cells/cm$^2$ for rat liver cells.

## Results

### TMB-8 Impedes *src*-Induced Downregulation of Junctional Permeability

Figures 1 and 2 (open circles) show the typical *src* effect on junctional communication in mouse 3T3-*ts*LA90 cells: junctional transfer of the tracer molecules decreased upon shifting the temperature of the cultures from the nonpermissive to the permissive level for the virus; the incidence of Lucifer-permeable interfaces fell from about 100 to near 0%. As shown before, such fall in junctional transfer is a response to pp60$^{v\text{-}src}$, not to the temperature change itself, and represents a fall in junctional permeability (nonjunctional membrane permeability is unaltered) (Azarnia & Loewenstein, 1984*a*). The response set in within 10 min of the temperature downshift and was usually complete by 30–40 min.

TMB-8 counteracted this response. In medium containing 36–180 $\mu$M TMB-8 (administered 5–17 min before the temperature step), the incidence of permeable interfaces remained high for several hours after the temperature shift (Figs. 1,*I* and 2), and even after one day it was >35% (Fig. 2).

Similar results were obtained with quail QEF-*ts*NY68 cells. Here, after 40–60 min at permissive temperature, the incidence of permeable interfaces was 51 ± 7% in the TMB-8-treated condition against 18 ± 5% in the untreated controls.

The effect of TMB-8 was dose dependent (Fig. 3).

A variant of the aforegoing experiments showed that TMB-8 also can undo an established *src*-induced response. Here TMB-8 was administered in the steady phase of the permissive temperature while junctional permeability was at its steady minimum (Fig. 4). Then, within 15 min of TMB-8 (72 $\mu$M) application, the incidence of permeable interfaces rose, reaching a level of about 35%. This level is comparable to that eventually reached in the pretreated cells in Fig. 2, following downshift of temperature.

Thus, TMB-8 can prevent to some extent the *src*-induced loss of junctional communication, as well as restore to some extent communication in *src*-transformed cells.

### TMB-8 Impedes Diacylglycerol-Induced Downregulation of Junctional Permeability

Implicit among the considerations prompting this study (*see* Introduction) was the notion that the downregulatory effect of diacylglycerol on junctional communication and the counteraction of this effect by TMB-8 found in liver epithelial cells (Yada et al., 1985) also operated in the present cell type. The validity of this notion, essential to the interpretation of the present results, was tested in the following experiments.

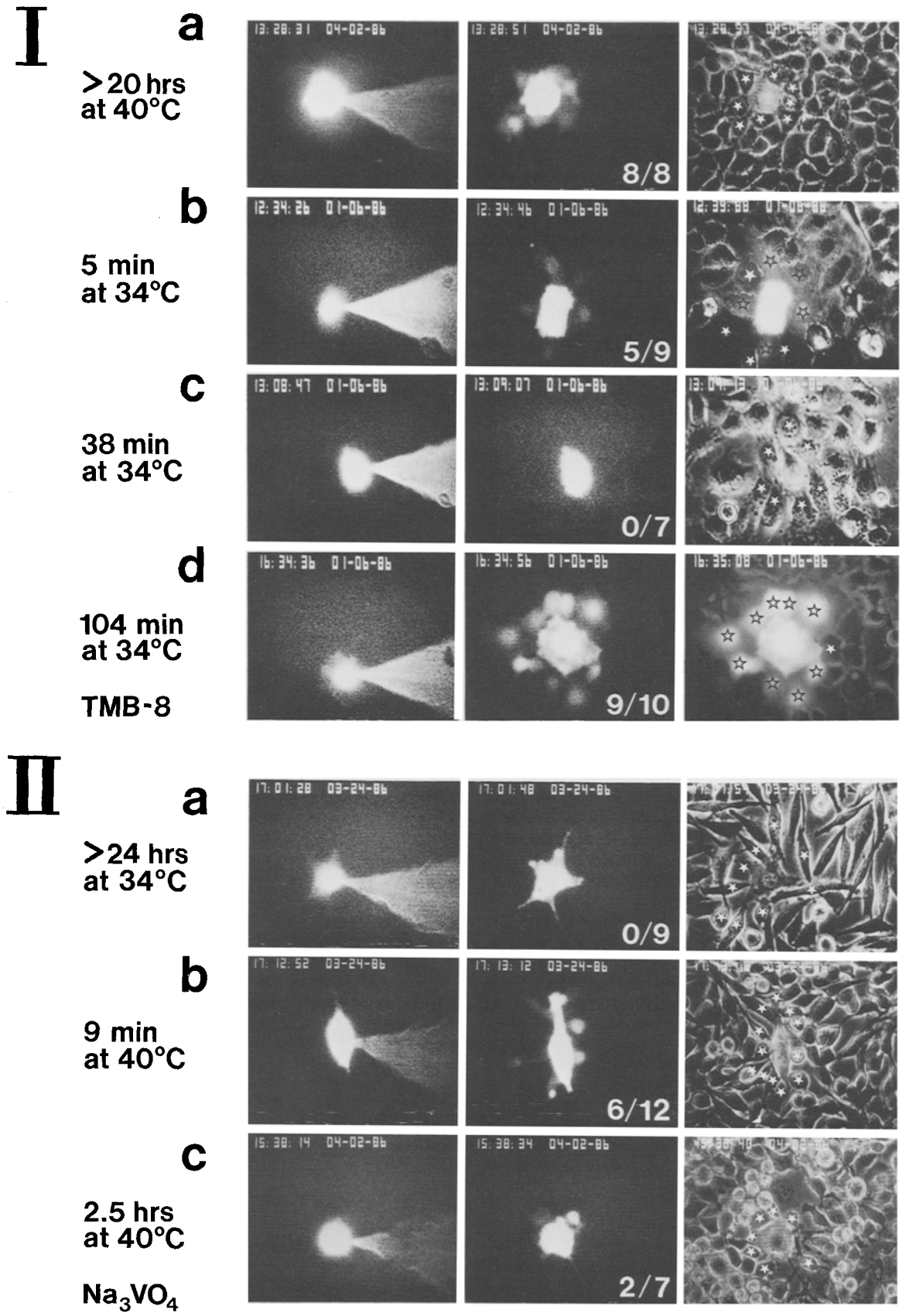
The synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol was administered (25–50 $\mu$M) to confluent 3T3-*ts*LA90 cultures kept at the nonpermissive temperature. This promptly produced the expected response: within 2 min of the diacylglycerol application, the incidence of permeable interfaces fell to 0% (Fig. 5*A*, open circles). The cells here were more sensitive than the (uninfected) liver epithelial cells in our earlier work; 50 $\mu$M diacylglycerol sufficed to completely block junctional transfer (Fig. 5*B*), whereas 75–100 $\mu$M were needed in (confluent) liver cells (Yada et al., 1985). But otherwise the response was similar to that of the liver cells.

Also the TMB-8 action was similar. TMB-8 blocked the effect of diacylglycerol on junctional transfer. In the presence of 72 $\mu$M TMB-8, the incidence of permeable interfaces stayed relatively high; it was still above 50% in most cells 20 min after diacylglycerol treatment (Fig. 5).

### Vanadate Impedes Restoration of Junctional Permeability

To study the effect of vanadate on the recovery of junctional communication from the *src*-induced downregulation, we used 20 $\mu$M sodium orthovanadate. The vanadate enters cells by the transport route of phosphate (Cantley, Resh & Guidotti, 1978), and at that concentration there were no signs of toxicity (*see also* Klarlund, 1985).

We treated 3T3-*ts*LA90 cells with the vanadate, and after 9–90 min shifted their temperature from the permissive to the nonpermissive level. The ef-


I
a
>20 hrs
at 40°C
8/8
b
5 min
at 34°C
5/9
c
38 min
at 34°C
0/7
d
104 min
at 34°C
TMB-8
9/10
II
a
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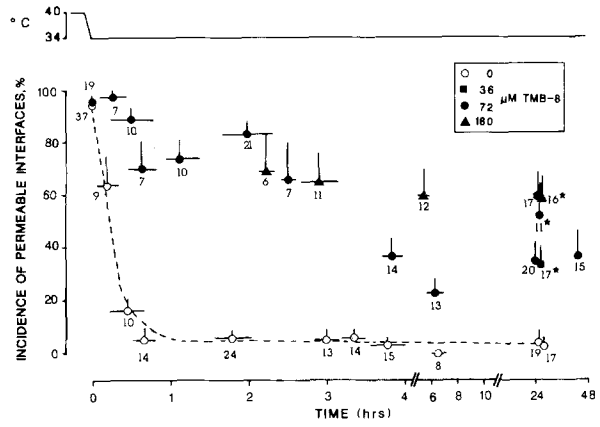


**Fig. 2.** TMB-8 counteracts *src*-induced loss of junctional communication. The temperature of 3T3-*ts*LA90 cultures was stepped (time 0) from 40 to 34°C, the permissive temperature for the *src* virus. The graph gives the time course of the ensuing change in junctional transfer of Lucifer Yellow in untreated cultures (open circles) and sister cultures treated with TMB-8 (filled symbols). TMB-8 was added 5–17 min before time 0, except for the data with stars where the addition was 40–50 min before time 0. Data points are the mean incidences of permeable interfaces ± SE; horizontal bars give the sampling (trial) period; next to each point, the number of injection trials. The cultures had been at 40°C for 19–25 hr before time zero. (The data point for untreated cultures at time 0 (37 trials) was pooled from three dishes; all other points are from one dish each.) Note changes in scale on abscissa

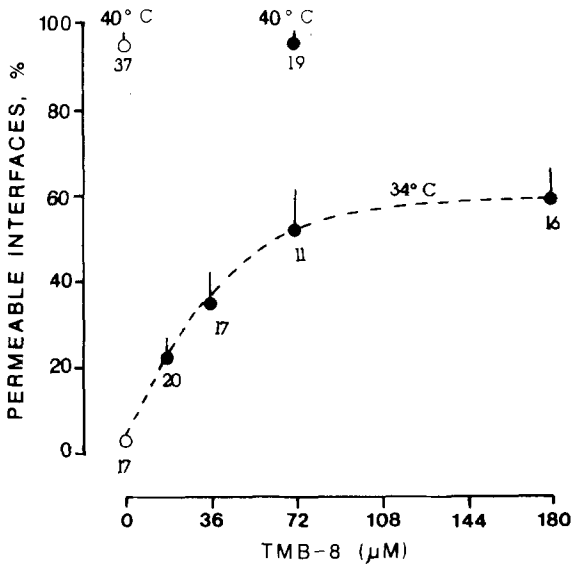


**Fig. 3.** Dose dependence of the TMB-8 counteraction. The incidence of permeable interfaces (±SE) 26–28 hr after downshifting TMB-8-treated 3T3-*ts*LA90 cells from 40 to 34°C. TMB-8 was applied 40–50 min before the temperature shift. All data on the curve are from sister cultures. Also shown are the data points for 0 and 72 μM TMB-8 at 40°C

fect of vanadate was striking. Whereas in the untreated condition the incidence of permeable interfaces rose to a high level within a few minutes from the temperature shift (by 12 min the mean incidence was about 75%), in the vanadate-treated condition it stayed near zero for more than 30 min (Fig. 1,*IIc*), and even after 24 hr it was still depressed (Fig. 6).

Vanadate did not have a major effect on junctional communication in cells with inactive $pp60^{v\text{-}src}$. The incidence of permeable interfaces stayed high when cells that had been kept at the nonpermissive temperature for 17–23 hr were treated with vanadate (Table 1).

**Table 1.** Vanadate treatment at the nonpermissive temperature. 3T3-*ts*LA90 cells at 40°C

| μM | Time[a] (min) | Permeable interfaces[b] (%) |
|---|---|---|
| 0 | — | 94 ± 2 (37) |
| 20 | 23–40 | 76 ± 9 (13) |
| 20 | 75–90 | 86 ± 9 (11) |

Cultures had been at 40°C for 17.5 or 23 hr before application of the sodium orthovanadate.

[a] Time after vanadate application when junctional transfer was tested.

[b] Mean incidence of permeable interfaces ± SE; in parentheses, the number of injection trials.

**Fig. 1.** (*Facing page*) Video pictures of sample Lucifer-Yellow microinjections into 3T3-*ts*LA90 cells. The fluorescence images in the left column were photographed at the time of injection and those in the middle column 20 sec later, at the time of scoring of fluorescent first-order neighbors of the injected cell. Time is displayed in hr:min:sec in upper left corners. The right column shows the cells in brightfield. Cells considered first-order neighbors are marked by stars. The scores "number of fluorescent first-order neighbors/total number of first-order neighbors" are given in the lower right corners. (*I*) Changes of junctional transfer upon temperature downshift from the nonpermissive (40°C) to the permissive level (34°C). The junctional transfer (*a*) at the nonpermissive temperature (cells had been for 20 hr at that temperature), (*b*) 5 min and (*c*) 38 min after the downshift; (*d*) 104 min after downshift in the presence of TMB-8, 72 μM. (*II*) Changes of junctional transfer upon temperature upshift from permissive to nonpermissive. Junctional transfer (*a*) at the permissive temperature (cells had been at that temperature for >24 hr), (*b*) 9 min after upshift; (*c*) 2.5 hr after upshift in the presence of sodium vanadate, 20 μM

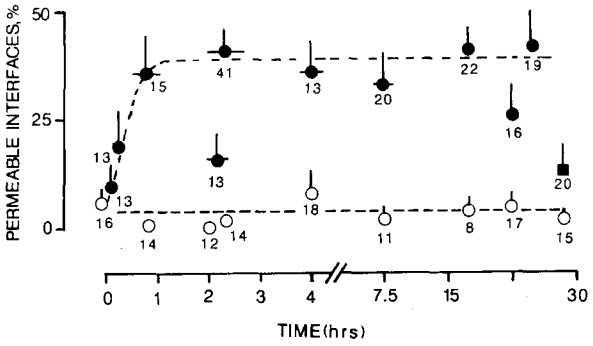


**Fig. 4.** TMB-8 restores junctional communication in *src*-transformed cells. TMB-8 was applied (time 0) to 3T3-*ts*LA90 cultures kept at 34°C. TMB-8 concentration was 72 μM, except for ■ where it was 36 μM. The mean incidence of permeable interfaces was determined at various times on individual pairs of sister cultures, TMB-8-treated (filled symbols) and untreated (open circles), except for the point at time zero and the first two points in the treated condition where each datum was pooled from four sister cultures used in obtaining the time course

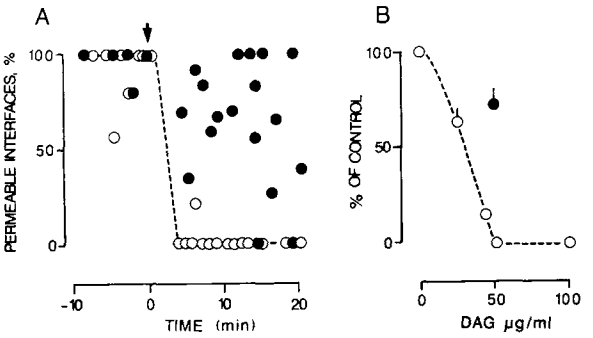


**Fig. 5.** TMB-8 prevents diacylglycerol-induced loss of junctional communication. (*A*) Diacylglycerol (50 μg/ml) is applied (arrow at time 0) to 3T3-*ts*LA90 cells at 41°C. Plotted is the response of junctional transfer in two untreated cultures (○) and in their two sister cultures treated with 72 μM TMB-8 (●). The TMB-8 treatment started 24–36 min before the diacylglycerol application. The points are individual incidence values of permeable interfaces (each from one injection trial). (*B*) Dose response curve for diacylglycerol (*DAG*). The mean incidence of permeable interfaces (± SE) is normalized with respect to the control in the absence of diacylglycerol (17–30 trials in two cultures for each point). The absolute mean incidence value of the control (before diacylglycerol treatment) was 81 ± 3% ($n$ = 25). The filled circle is the normalized mean incidence value ± SE of the TMB-8-treated data in *A*

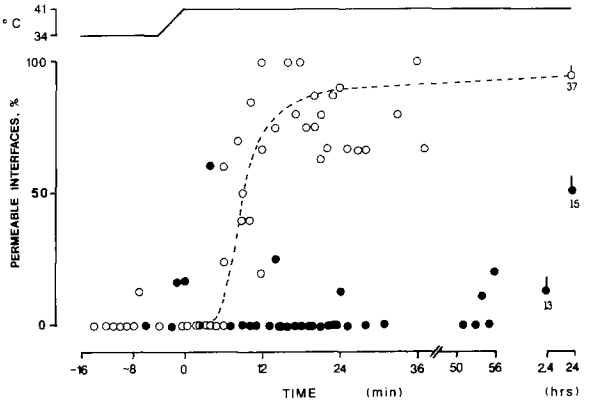


**Fig. 6.** Vanadate blocks recovery of junctional communication. 3T3-*ts*LA90 cells growing at 34°C were treated with 20 μM vanadate 9 to 90 min before shifting to 41°C (time 0). The graph gives the incidences of permeable interfaces of the vanadate-treated cultures (●; 6 dishes) and of the untreated controls (○, 4 dishes). The data on the min scale are individual values; on the hr scale, mean values ± SE

## Changes in Morphology

### *TMB-8*

The 3T3-*ts*LA90 and QEF-*ts*NY68 cells underwent the morphological changes characteristic of *src*-transformed cells when their *src* protein kinase activity was turned on. At the nonpermissive temperature the cells had the flat, polygonal appearance of normal cells (Figs 1,*Ia* and 7*e*). When they were shifted to the permissive temperature, the cells acquired, after a day or so, a transformed morphology: they were more refractile and spindle-shaped (Figs. 1,*IIa* and 7*a*). These morphological changes were prevented by TMB-8.

Figure 7 illustrates examples in which a series of 3T3-*ts*LA90 sister cultures were exposed to various concentrations of TMB-8, starting 50 min before shifting to the permissive temperature. The photographs were taken 26 hr after the temperature shift. The morphology tended to stay normal at concentrations of 72 μM and higher. At 180 μM TMB-8 the cells looked like those at the nonpermissive temperature (Fig. 7*d*). (The TMB-8 effect on junctional transfer shown in Fig. 3 was obtained in these same cultures.)

When applied at the steady permissive temperature TMB-8 caused partial normalization of morphology. This morphological recovery lagged several hours behind the recovery of junctional permeability.

### *Vanadate*

Treatment with vanadate tended to enhance the transformed morphology of cells steadily maintained at the permissive temperature; more cells tended to round up. (All these morphological changes were reversible, however, upon washout of vanadate and temperature upshift.)

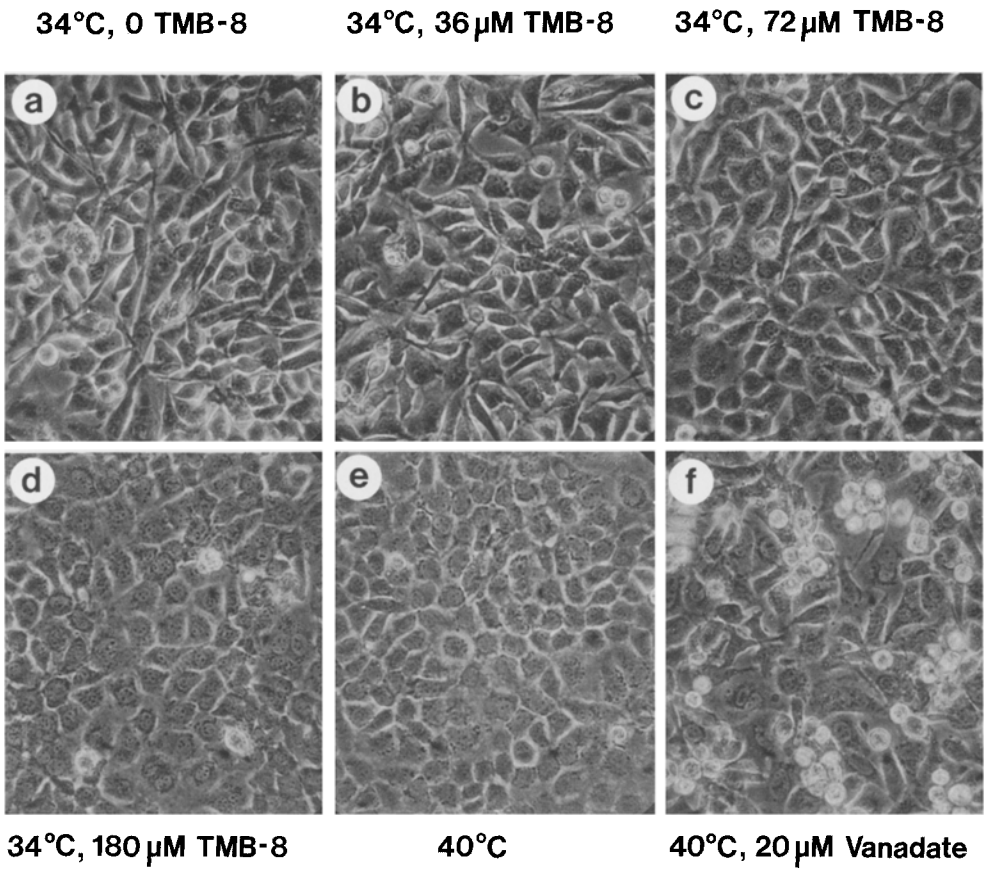


**Fig. 7.** (*a–e*) TMB-8 normalizes morphology of cells at the permissive temperature (34°C). Phase contrast photomicrograph of 3T3-*ts*LA90 cells (sister cultures) at the permissive temperature (34°C): (*a*) untreated; (*b*, *c*, *d*) treated with 36, 72 and 180 μM TMB-8; (*e*) untreated at the nonpermissive temperature. Protocol: cells were seeded and grown at 34°C (day 0). On day 2 the temperature was shifted to 40°C for 24 hr and on day 3, for dishes *a–d*, down again to 34°C. TMB-8 was added 50 min before the temperature downshift. The photographs were taken 26 hr after the temperature downshift. Dish *e* remained at 40°C. On these dishes the junctional transfer of Fig. 3 was determined. (*f*) Vanadate blocks restoration of normal morphology on return to nonpermissive temperature (there are many 'rounded-up' cells when cultures at the permissive temperature are treated with vanadate). Protocol: cells were grown at 34°C. On day 3 after seeding, the cultures were exposed to 20 μM sodium orthovanadate and, 1 hr later, the temperature was shifted to 40°C. The photomicrograph was taken ~24 hr after the temperature upshift. The incidence of permeable interfaces in a sister dish at that time was 51% ± 5; Fig. 6

Furthermore, vanadate prevented the return to normal morphology following temperature shifts from the permissive to the nonpermissive level. Figure 7*f* gives an example of a culture (sister of the cultures in the measurements of junctional transfer of Fig. 6) whose treatment with 20 μM vanadate had begun 1 hr before the temperature shift. The culture still exhibited a transformed morphology 24 hr later.

## Changes in Growth

### *Growth on Solid Substrate*

As a further expression of their transformation, the cells at the permissive temperature continued to grow at densities well above those at which they grow at the nonpermissive temperature (Fig. 8*A*). Such growth was inhibited by TMB-8.

Figure 8*B* shows this for an experiment in which the cells (at permissive temperature) were treated with TMB-8 (36 μM) in their exponential growth phase. This caused marked inhibition of growth: by day 9 after the seeding, the cell density was less than one fourth (curve *3*) that of the untreated controls (curve *1*).

The effect was fully reversible. After removal of TMB-8 the cells resumed their fast growth (curve *2*).

There was no indication of TMB-8 causing cell death in the aforegoing experiments. This was so, too, when TMB-8 was applied in the stationary

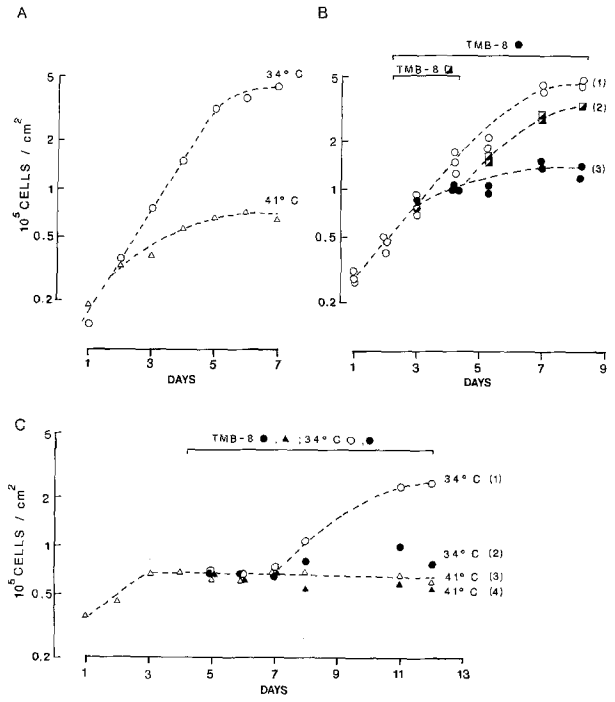


**Fig. 8.** (*A*) Growth curves of 3T3-*ts*LA90 cultures at the permissive (34°C) and nonpermissive (41°C) temperatures. (*B*) TMB-8 reversibly inhibits growth at the permissive temperature (exponential growth phase). Growth of TMB-8-treated cells (curve 3), TMB-8 treated cells after TMB-8 removal (*2*), untreated controls (*1*); data from 35 sister cultures. Protocol: 35 dishes were each seeded with $3 \times 10^5$ cells on day 0. Seventeen of these dishes were exposed to 36 $\mu$M TMB-8 on day 2 (●), and the exposure was terminated on day 4 in a subgroup of 5 dishes (◪). 18 dishes were left untreated from the start (○). (*C*) TMB-8 treatment in the stationary growth phase at the nonpermissive temperature. Growth of treated cultures (*4*), untreated controls (*3*) at the permissive temperature and after shifting to permissive temperature in the treated cultures (*2*), and untreated controls (*1*); data from 68 sister cultures. Protocol: 68 dishes were seeded on day 0 and allowed to grow to confluence at 41°C. Of these, 28 dishes were treated with 72 $\mu$M TMB-8 on day 4 (▲, ●) and 28 dishes were left untreated (△, ○); 14 untreated dishes (○) and 14 treated dishes (●) were shifted to 34°C on day 4, 30 min after treatment. Plotted are the means of 3 dishes (8 hemocytometer counts/dish) in each category from day 1 through 6, and of 2 dishes from day 7 through 12. The SE was ≤5% in all but three cases, where it was ~10%

phase of growth at the nonpermissive temperature. There were then no major changes in cell density even after 8 days of continuous treatment (Fig. 8*C*,*4*). Moreover, the treated cells were evidently still capable of growing when the temperature was shifted to the permissive level (*2*), and the cells looked healthy even at 180 $\mu$M concentration for, at least, 3 days (Fig. 7*d* illustrates an example for 1 day).

For these experiments of growth, which lasted several days, we chose conditions minimizing effects of toxicity. We used submaximal doses of TMB-8 and treated the cultures only after they had reached densities of $>4 \times 10^4$ cells/cm$^2$. At high doses (72 and 180 $\mu$M) or when applied to cultures of lower densities, TMB-8 caused cell death over the long term. Our 3T3-*ts*LA90 cells were particularly useful for long-term experiments at high temperature. Unlike many other 3T3 lines they survived at 41°C for many days (*see* Fig. 8*c*).

### *Growth in Suspension*

TMB-8 also inhibited growth at the permissive temperature when the cells ($10^3$/ml) were suspended in methylcellulose. Four days after their suspension, the cells were treated with 36 $\mu$M TMB-8. At this time, many cells had formed small colonies of 2–8 cells. The number of colonies continued to rise in the treated condition, but their size stayed small (<17 cells). By contrast, in the untreated condition the colonies continuously increased in number and size (Fig. 9).

## Discussion

### Mechanisms of Action of TMB-8

Our results show that TMB-8 blocks the downregulation of junctional permeability by the viral *src* gene. We will discuss some possible modes of action of the drug, predicated on the fact that mobilization of $Ca^{2+}$ to the cytosolic locale of cell junctions causes closure of the cell-to-cell channels (Rose & Loewenstein, 1976) and on the idea that the *src* effect on the cell-to-cell membrane channels is mediated by the diacylglycerol-protein kinase C pathway of the phosphoinositide transmembrane signal route (Yada et al., 1985).

This route is stimulated by the *src* protein and other oncogene products with protein tyrosine kinase activity (Macara et al., 1984, 1985; Sugimoto et al., 1984; Sugimoto & Erikson, 1985; Jackowski et al., 1986; Kaplan et al., 1986; Macara et al., 1986). Continuity of the diacylglycerol pathway requires mobilization of $Ca^{2+}$ to the locale of protein kinase C; translocation of this enzyme from the cytoplasm to the plasma membrane requires $2–5 \times 10^{-7}$ M $Ca^{2+}$ (Wolf et al., 1985) and its activation requires (besides diacylglycerol) $>10^{-6}$ $Ca^{2+}$ (Kishimoto et al., 1980), concentrations higher than that normally in the cytosol (Baker, 1976). Since TMB-8 blocks $Ca^{2+}$ mobilization to cytosol (Mix et al., 1984; Sawamura, 1985; but *see also* Kojima et al., 1985), we are immediately drawn to protein kinase C as a possible site where such a blockade might critically in-

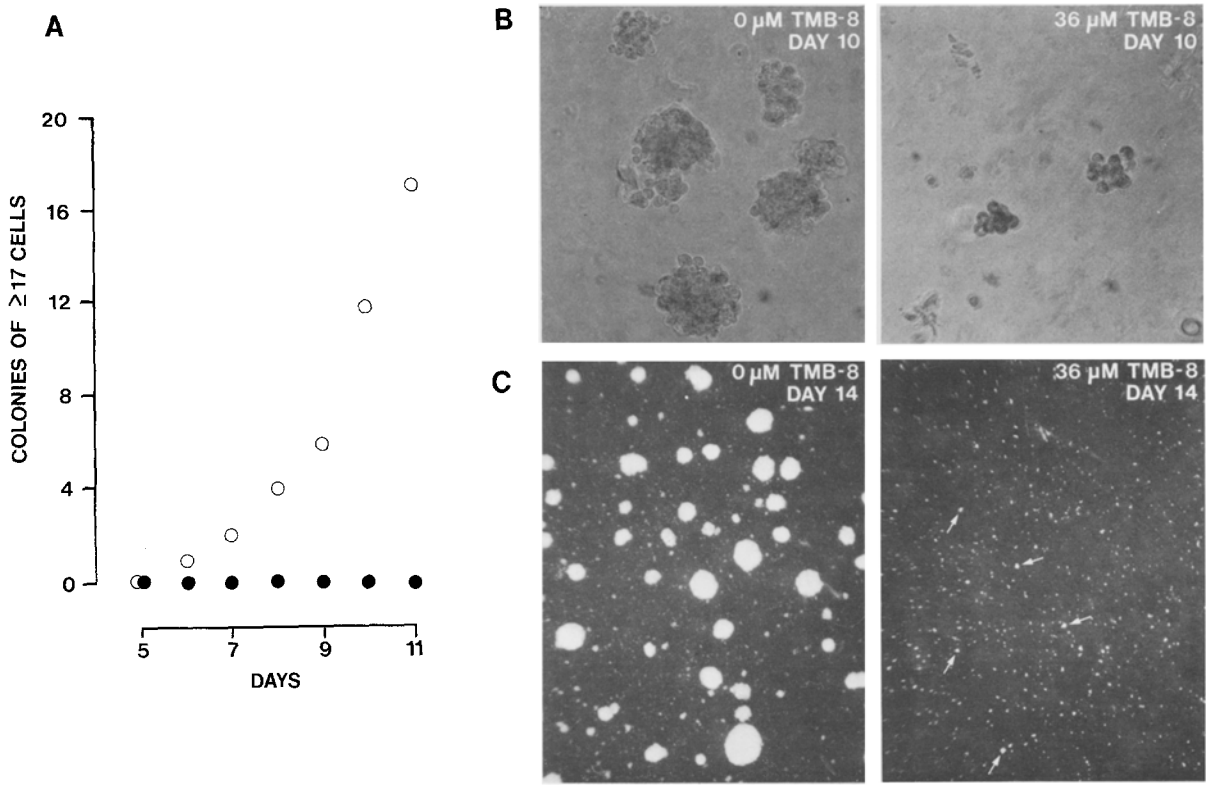


**Fig. 9.** Growth in methylcellulose suspension. 3T3-*ts*LA90 colonies at the permissive temperature. (*A*) Number of colonies *vs.* time of growth. Two cultures were seeded ($10^3$ cells/ml) on day 0 and one of them was treated with 36 μM TMB-8 from day 4 onward. Plotted is the number of colonies with ≥17 cells in the treated (●) and untreated (○) condition. (*B*) *top*, brightfield micrographs showing typical colony sizes of treated and untreated cultures at day 10. (*C*) Darkfield micrograph (at lower magnification) of cultures at day 14. The largest white spots in the treated culture (arrows) are colonies of ≈10 cells, and the largest in the untreated culture are colonies of ≥100 cells

terfere with the pathway. Alternatively or additionally, the blockade may occur further down the pathway at a critical $Ca^{2+}$-dependent step closer to the channel or at the channel itself, as proposed and discussed in more detail elsewhere (Yada et al., 1985).

In either case the necessary mobilization of $Ca^{2+}$ could be driven by inositol triphosphate (Streb, Irvine, Berridge & Schulz, 1983). Extracellular $Ca^{2+}$ seems not important; the downregulation of junctional permeability by *src* could be elicited in the presence of Ca chelators. We tested this point on 3T3-*ts*LA90 cells for downshifts of temperature in (growth) medium containing 1 or 1.5 mM EDTA, or 2 or 3 mM EGTA. Junctional permeability fell with a similar time course as in control medium, and the fall was reversible upon temperature upshift (Fig. 10). Moreover, the reduction of junctional permeability produced by exogenous diacylglycerol, as tested on untransformed rat liver cells, was independent of extracellular $Ca^{2+}$: it ensued in Ca-free saline containing 1 or 5 mM EGTA, or in the presence of D-600, a blocker of Ca channels in plasma membrane (Table 2). Besides, D-600 alone or in combination with chlorpromazine did not protect cells from *src*-induced downregulation of junctional permeability, as tested for temperature shifts in 3T3-*ts*LA90 cells (Table 3).

A further possibility is a direct action of TMB-8 on protein kinase C. Indeed, TMB-8, by interaction with phospholipids, inhibits protein kinase C in vitro. That inhibition requires relatively high TMB-8 concentrations. A 50% inhibition, in the presence of 12.5 μg/ml phospholipid (and 150 μM $Ca^{2+}$), requires between 100 to 250 μM TMB-8 in one in vitro system (Kojima et al., 1985) and 300 μM in another (Sawamura, 1985; Y. Nishizuka, *personal communication*), and in the presence of higher lipid concentrations (160 μg/ml) even 400 μM TMB-8 produced only a <10% inhibition (Sawamura, 1985). These TMB-8 concentrations are much higher than those needed for our effect, but one cannot off-hand dismiss such a mechanism because the TMB-8 concentration in the membrane lipid, rather than that in the aqueous bulk phase, might matter here. Besides, TMB-8 could be more potent in the intact cell membrane than in vitro.

Further weighing against such a direct action on

**Table 2.** Reduction of junctional communication by exogenous diacylglycerol in epithelial liver cells. Effects of Ca-free medium, chlorpromazine, D-600, and TMB-8

| Treatment | Concentration ($\mu$M) | Pretreatment[a] (min) | Test period | Permeable interfaces[b] |
|---|---|---|---|---|
| | | | min | % |
| — | — | — | 4–30 | 6 ± 4 (13) |
| — | — | — | 12–20 | 0 ± 0 (6) |
| — | — | — | 10–50 | 2 ± 2 (7) |
| EGTA (Ca-free saline) | 1000 | 3 | 20–40 | 2 ± 1 (13) |
| | 5000 | 4 | 13–43 | 0 ± 0 (15) |
| D-600 | 10 | 54 | 10–26 | 0 ± 0 (10) |
| Chlorpromazine | 20 | 18 | 17–40 | 0 ± 0 (10) |
| TMB-8 | 72 | 7 | 10–40 | 61 ± 11 (10) |

The synthetic diacylglycerol 1-oleoyl-2-acetyl glycerol (100 $\mu$g/ml) was administered to the cells (time zero), and the incidences of permeable interfaces were determined at the times indicated (test period). The mean incidence of permeable interfaces before diacylglycerol administration was about 75%. For all treatments, except for those with EGTA, cells were in serum-containing growth medium.

[a] Time of application before time zero.

[b] Mean incidence of permeable interfaces ± SE; in parentheses, the number of trials.

**Table 3.** Incidence of permeable interfaces (%) following shift of temperature from nonpermissive to permissive level in 3T3-*ts*LA90 cells. Effects of chlorpromazine, D-600 and TMB-8

| Drug | Concentration ($\mu$M) | Pretreatment[a] (min) | Test period[b] | | | |
|---|---|---|---|---|---|---|
| | | | 0.5 hr | 3 hr | 19 hr | 24 hr |
| — | — | — | 17 ± 8 (10)[c] | 6 ± 3 (13) | | 4 ± 5 (19) |
| Chlorpromazine | 20 | 50, 100 | 26 ± 11 (11)[c] | | 0 ± 0 (8) | |
| | 20 | 20 | 3 ± 3 (7) | | | |
| | 20 | 50 | 2 ± 2 (12) | | | |
| D-600 | 10 | 20 | 11 ± 8 (11)[c] | | | |
| Chlorpromazine | 20 | | | | | |
| + D-600 | 10 | 70 | | 0 ± 0 (12) | | |
| TMB-8 | 72 | 20, 6 | 78 ± 9 (10)[c] | | | 35 ± 8 (20) |
| | 180 | 30 | | 65 ± 10 (11) | | |

Mean incidence of permeable interfaces (%) ± SE. In parentheses, the number of injection trials.

[a] Time of drug application before the temperature shift. Where two values are given, they refer to the first and second experiment in the row, respectively.

[b] Time after temperature shift when the testing of incidence of permeable interfaces was begun; test periods were about 30 min.

[c] Sister cultures.

protein kinase C, however, are results we obtained with chlorpromazine. This is another phospholipid-interacting inhibitor of protein kinase C, but it has no apparent action on $Ca^{2+}$ mobilization. Chlorpromazine inhibits protein kinase C almost completely in vitro at 20 $\mu$M concentration (Mori et al., 1980). (TMB-8, at one order-of-magnitude higher concentration, inhibits only by 30 or 50% in vitro; Y. Nishizuka, *personal communication*; Kojima et al., 1985.) We tested the effect of 20 $\mu$M chlorpromazine on the 3T3-*ts*LA90 cells for various times of treatment and at various times after shifting their temperature to the permissive level. Chorpromazine was ineffective in protecting the cells from down-regulation of junctional permeability (Table 2) (and it did not change permeability on its own; *data not shown*). By contrast, TMB-8 was effective at concentrations at which there is little or no protein kinase C inhibition in vitro (Table 3 and Figs. 2 and 3).

This discussion certainly does not exhaust the possibilities for TMB-8 mechanisms, but it seems fruitless to pursue speculations into interactions at

other stages of the pertinent intracellular signal route before a clear picture is on hand on how the *src* protein hooks up with that route. Although there is a body of evidence indicating that the *src*- and other oncogene protein tyrosine kinases cause activation of the phosphoinositide route, it is obscure how they are coupled to it.

## Mechanism of Action of Vanadate

Vanadate inhibited the recovery of junctional permeability following cessation of *src* protein kinase activity. This is the result one would expect if removal of phosphate groups from phosphotyrosine residues is a necessary step in the restoration of junctional permeability. Vanadate specifically inhibits tyrosyl phosphatases in vitro (Leis & Kaplan, 1982; Swarup et al., 1982). Thus, the result is most simply accounted for if vanadate or the vanadyl ion [the form in which vanadium probably enters cells and binds to proteins (Cantley & Aisen, 1979)] also exerts this effect in vivo. There is a cascade of tyrosine phosphorylating events along the phosphoinositide-protein kinase C line, starting with pp60$^{v\text{-}src}$ itself (Collett et al., 1980; Hunter, 1980; Cooper et al., 1982; Gilmore & Martin, 1983; Macara et al., 1985; Sugimoto & Erikson, 1985). Vanadate, at concentrations comparable to those used here, causes marked elevation in the overall level of phosphotyrosine in mammalian cells (Klarlund, 1985). It seems plausible, therefore, that, somewhere along the line, the vanadate treatment inhibited a dephosphorylating function critical for normal junctional permeability.

Apart from inhibiting phosphatase, vanadate inhibits $Na^+$, $K^+$/ATPase in plasma membrane (Cantley et al., 1977, 1978; Bowman, Mainzer, Allen & Slayman, 1978; North & Post, 1984). Thus, we may envision a vanadate effect on the recovery of junctional permeability by way of interference with $[Ca^{2+}]_i$ regulation. $[Ca^{2+}]_i$ may be expected to rise as $Na^+$ accumulates in the cytosol (Baker, 1976; Crompton, Capano & Carafoli, 1976) by stimulation of the $Na^+/H^+$ antiport, a sequel of activation of the diacylglycerol pathway (Burns & Rozengurt, 1983; Moolenaar, Tsien, van der Saag & deLatt, 1983; Rosoff, Stein, & Cantley, 1984; Grinstein & Rothstein, 1986). To restore a low $[Ca^{2+}]_i$, the excess $Na^+$ must be removed. Ordinarily, when the diacylglycerol pathway is turned off, the removal is rapidly achieved by the Na pump. But this mechanism is blocked by ATPase inhibition, and so in the presence of vanadate, $[Ca^{2+}]_i$ would stay elevated even after the *src* protein kinase activity is stopped, impeding the recovery of junctional permeability.

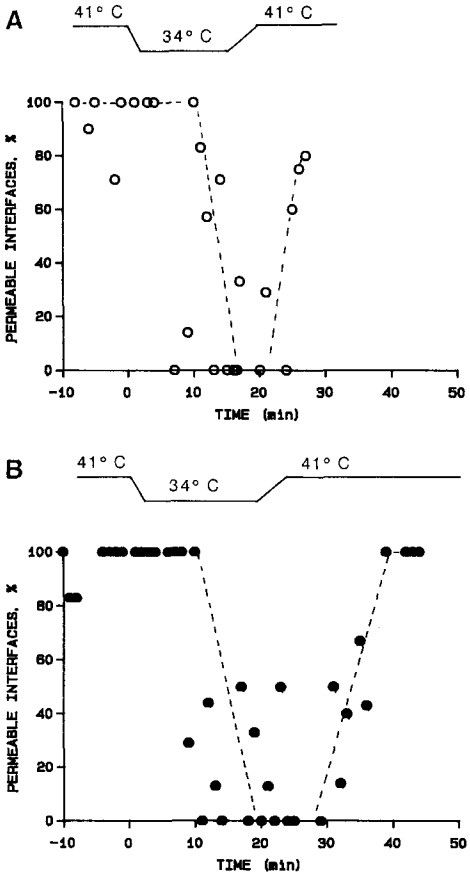


**Fig. 10.** Extracellular calcium is not required for *v-src*-induced loss of junctional communication. Response to shifts of temperature of 3T3-*ts*LA90 cells. (*A*) Control: Cells in growth medium. (*B*) Sister culture in growth medium containing 3 mM EGTA. (The EGTA was added 15 min before the temperature downshift.) The data are individual incidence values of permeable interfaces, each from one injection trial. The cultures were 24 hr at 41°C before the start of the experiment. Note that the period at 34°C in *A* is shorter than in *B*

Considering that junctional permeability stays depressed for more than a day (Fig. 6), it would not be surprising if this mode of vanadate action combined with that on dephosphorylation in the inhibition of the recovery of communication.

## Downregulation of Communication and the Cytoskeleton

Among the effects of the *src* protein kinase activity are the changes in cell shape. These probably reflect alterations of the cytoskeleton and may relate to tyrosine phosphorylation of vinculin, the protein that anchors the cytoskeletal filaments to plasma membrane (Sefton, Hunter, Ball & Singer, 1981; Chen & Singer, 1982; but *see also* Antler, Greenberg, Edelman & Hanafusa, 1985). The effects of *src* protein kinase on junctional communication are

independent of these morphological effects. This has been shown with the aid of a mutant (revertant) clone with high *src* protein kinase activity but normal cytoskeleton. These cells exhibited the usual low junctional permeability in the absence of morphological alterations (Azarnia & Loewenstein, 1984*b*).

The converse is found upon termination of the transformed condition in the present cells: high junctional permeability in the presence of morphological alterations; in the example of Fig. 1,*IIb* even cells completely rounded up exhibit transfer. Clearly, the coarse morphology of the cells does not determine the presence or absence of junctional transfer. Similarly, the communication changes and the morphological changes in *src*-transformed rat NRK cells were found to be out of the phase during onset and termination of the transforming condition (Atkinson et al., 1981), during onset of the response to exogenous diacylglycerol in rat liver cells (Yada et al., 1985) and, as observed here, during TMB-8-induced recovery. There is dichotomy also between the effects on communication and morphology of transforming polyoma middle T antigen (Azarnia & Loewenstein, 1986) and of certain transforming DNA viruses (Loewenstein, 1985).

### Restoration of Communication and Growth Inhibition

Besides restoring communication, TMB-8 inhibited growth. Are these events linked or are they completely independent of each other? This question is important for our understanding of the role of junctional communication in cellular growth control (Loewenstein, 1979), but our work offers no answer at this time. Our results are beclouded in this regard by the fact that TMB-8 proved toxic at low cell densities. It is true that in our experiments of TMB-8 treatment at cell densities $>4 \times 10^4$ cells/cm$^2$ there was no evidence of toxicity: the growth inhibition was then fully reversible, cells treated in the exponential growth phase were capable of growing (Fig. 8*B*), the cells looked healthy (even at 180 $\mu$M; Fig. 7*d*), and there was no large-scale cell death. But because of the toxicity at lower cell densities, we do not discount the possibility that TMB-8 affects growth independently of junctional communication.

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