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ARTICLES

Regulation of Cell-Cell Communication in Rat Bone Cells: The Effect of Phorbol Esters

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Abstract The skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent inhibitor of gap junctional intercellular communication. In the present study, the inhibition of cell-cell communication by TPA has been investigated in primary bone cells from newborn rat calvaria, with an emphasis on the involvement of intracellular pH (pH_i) and cytosolic calcium ($[Ca^{+2}]_i$) in this process. The results show that TPA (5×10^{-8} M) caused a complete inhibition of intercellular communication within 40–60 min. The intercellular communication was fully restored after overnight incubation in the presence of TPA. This effect was found to be associated with an elevation of pH_i. However, neither an increase of pH_i alone nor exposure to TPA, under conditions preventing pH_i-shift, were found to affect intercellular communication. It is suggested that the inhibition of intercellular communication, in the presence of TPA, depends on the pH_i-shift itself rather than on the absolute value of pH_i. In addition, elevation of cytosolic calcium by ionomycin led to the termination of intercellular communication after 30 min. This inhibitory effect was abolished when the cells were incubated for overnight with TPA and then intracellular calcium was elevated by the addition of ionomycin. These results indicate that shift of pH_i and the increase of intracellular calcium are involved in repression of intercellular communication by TPA. J. Cell. Biochem. 74:349–356, 1999.

Key words: phorbol esters; rat; bone

Cell-cell communication via gap junction channels, connexins, is considered an important cellular mechanism for regulating cell growth, tissue development, and cellular differentiation. In many tissues and cell lines gap junctions permit cell to cell transfer of signal molecules and metabolites [Loewenstein, 1979; Schults, 1985; Lo, 1985; Pitts and Finbow, 1986; Guthrie and Gilula, 1989; Loewenstein and Rose, 1992]. Morphological and electrical analysis have shown the existence of functional gap-junctions in bone cells [Jeansonne et al., 1979; Doty, 1981]. These gap junction proteins were shown to consist of 43kD connexin (Cx43) and 45kD connexin (Cx45) [Civitelli et al., 1993; Steinberg et al., 1994], where the first type possess a junctional conductance of 50nS [Schirrmacher et al., 1992].

The level of gap junctional communication was shown to be modulated by different physiological conditions that affect the extent of connexin's expression or the gating of the channels [Spray et al., 1988; Saez et al., 1990]. Many of these effects are mediated by second messenger pathways [Saez et al., 1990; Bennettet al., 1991]. Phosphorylation of connexins by different protein kinases was proposed to be one mechanism by which the gap junctional channels are gated [Spray et al., 1988; Saez et al., 1990]. One of these protein kinases, protein kinase C (PKC), plays a significant role in intracellular signal transduction [Nishizuka, 1988]. Different exogenous and endogenous substances, including phorbol esters, growth factors, diacylglycerols, etc., are known to activate PKC [Nishizuka, 1992]. It has been shown that activators of PKC, for example, phorbol esters, induce strong cell uncoupling and a rapid Cx43 phosphorylation [Oh et al., 1991].

The mechanism for regulation of gap junctional communication in bone cells is not fully understood. It was previously shown that intracellular alkalization increases the cell-cell coupling in primary rat newborn calvaria cells, whereas intracellular acidification strongly inhibits dye coupling in these cells [Massass et al., submitted]. The present study explores the regulation of gap junctional communication by

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the tumor promoter phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), in primary newborn rat calvaria bone cells.

MATERIALS AND METHODS

Cell Tissue Culture

Calvaria removed from newborn rats were placed in minimum essential medium (MEM; Biological Industries, Bet-Haemek, Israel). The media supplemented with 10% fetal calf serum (Biological Industries), penicillin (100 u/ml)-streptomycin (0.1 mg/ml; Biological Industries), and 1% glutamine (Biological Industries). The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was replaced by a new one every 3 days. The cells were prepared by an isolation technique based on the ability of bone cells to migrate from bone tissue on to glass support as previously described [Jones and Boyde, 1977]. The characterization of these cells has been described previously [Nijweide et al., 1981; Massass et al., 1990]. Stripped parietal bones were fragmented and transferred on to glass cover slips (either 22 × 22 mm or 10.5 × 22 mm) which were placed in a 35 mm petri dish (Falcon, England) containing 2 ml of a nutrient medium. After 5–7 days the bone fragments were surrounded by a zone of extended outgrowth of cells. At this phase of growth, the cells were in contact with each other and divided to three distinct types: round cells were found to be in the closet region to the explant, the polygonal cells were located farther away from it, while the fibroblast-like cells were found at the farthest periphery. Bone cell cultures were investigated 5–7 days later when cells were in contact with each other but did not reach complete confluence.

Measurement of Intracellular pH

Intracellular pH (pH_i) was monitored using the pH-sensitive fluorescent probe biscarboxyethyl carboxyfluorescein (BCECF) [Rink et al., 1982]. Cells on a cover glass were loaded with BCECF by preincubation with 2–3 µM BCECF-AM (Molecular Probes, Inc., Eugene, OR) for 20 min at 37°C. The cell were washed twice in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffered standard solution (control) containing (mM):NaCl 145.0; KCl 5.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; glucose 10.0; Hepes 10.0; pH adjusted to 7.3 with NaOH. The measurement was carried out in a 10 × 10 mm rectangular cuvette which contained a triangular adaptor to which monolayer of bone cells on a coverglass (10.5 × 22 mm) was attached. Ratio mode fluorescence of BCECF-AM (excitation 440 and 500 nm; emission 530 nm; slits 4 nm) was monitored by a SLM8000 spectrofluorimeter. The data was recorded and analyzed on a PC computer. Fluorescence background, under the conditions of BCECF-AM experiments, was $<5\%$.

Intracellular pH (pH_i) calibration was performed, in all experiments, by using the nigericin based method [Thomas et al., 1979]: Nigericin (potassium-hydrogen ionophore; Sigma, St. Louis, MO) at a concentration of 10 µM was added to cells kept in an isosmotic standard buffered high KCl solution containing (mM): NaCl 2.0; KCl 130.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; glucose 10.0; Hepes 10.0. The pH of the solution was adjusted to different pH values, in the range of 6.5–8.5, by KOH or HCl. This calibration is based on the fact that at high external potassium concentration, in the presence of nigericin, the external and internal pH are nearly equal (pH clamping).

Measurement of Cytoplasmic Free Ca^{+2}

The preparation of the cells and cytoplasmic free Ca^{+2} ($Ca^{+2}{}_i$) measurement with calcium-sensitive fluorescent probe, Fura2-AM, were performed essentially as described for pH_i measurement. The cells on a cover glass were loaded with 2 µM Fura 2-AM (Molecular Probes, Inc.) for 30 min in Hepes buffer standard solution at 37°C. The cells were washed carefully. Hepes buffer standard solution. The measurement was carried out as described for pH_i measurement. Ratio mode fluorescence of Fura 2-AM (excitation 340 and 380 nm; emission 510 nm; slits 4 nm) was monitored by a SLM8000 spectrofluorimeter.

Calibration of the Fura 2-AM signal was performed by methods described for Quine 2 (fluorescent intracellular probe) [Tsien et al., 1982]. Briefly, the cells were lysed with digitonin (25 µg/ml) to obtain the maximum fluorescence. Next, 10 mM EGTA and sufficient NaOH to elevate the pH to 8.5 were added to obtain the minimum fluorescence. The dissociation constant for Ca^{+2}-Fura 2-AM was assumed to be 220 nM [Grynkiewicz et al., 1985]. Calculation of $[Ca^{+2}]_i$ was similar to that described by Tsien et al. [1982].

Measurement of Intercellular Communication

The intercellular communication via gap junctions was measured by a method based on the microinjection of a fluorescent dye, Lucifer Yellow CH (Sigma), into a single cell in cell monolayer by a convential micropipette (nonpolished). Micropipettes were filled at their tips with 4% (w/v) aqueous Lucifer Yellow CH [Stewart, 1978]. The cultured bone cells on the coverslip (22 × 22 mm) were transferred to the experimental chamber and perfused with standard Hepes solution (see above). After cell impalement, hyperpolarizing pulses (20 nA) were applied for 20 sec in order to eject dye out of the pipette and into the cell. Spreading of the dye from the impaled cell into its neighboring cells was examined under epifluorescence illumination counting the number of fluorescent cells coupled to the microinjected cell within 2 min following the voltage pulses. The results are given in terms of mean ± standard deviation (S.D) and n designates the number of independent microinjection experiments.

Statistical Analysis

The data were summarized as mean ± S.D. The data were analyzed using double-sided *t*-test to study the significance of the differences in the number of coupled cells.

RESULTS

The Effect of TPA on Intercellular Communication

Primary bone cells were incubated with TPA at concentration 5×10^{-8} M. Following incubation in a standard buffer solution (see Materials and Methods; $pH_0 = 7.3$) at room temperature the uncoupling effect of TPA was variable and it occurred following incubation for 40–60 min at 37°C. The number of the stained cells per injection decreased from 32 ± 7 in control group to less than 6 ± 4 in cells exposed to TPA. The inactive derivative of TPA, 4-α-phorbol ester (5×10^{-8} M), had no effect on cell uncoupling. The uncoupling effect of TPA was independent of the presence of serum in the medium. In order to examine the origin of the variable response we have correlated the cellular response with cell morphology. Three classes of cell morphologies which have been previously reported to exist in this type of primary culture preparation included polygonal, fibroblast-like and round cells. The polygonal subpopulation of bone cells, which constitutes ≈70% of whole cell population, showed the highest sensitivity towards uncoupling as reflected by the low number of 2 ± 2 coupled cells following exposure to TPA. The fibroblast-like subpopulation, which composes ≈20% of whole cell population, showed somewhat lower sensitivity towards uncoupling, possessing 6 ± 4 coupled cells following TPA treatment. The subpopulation of round cells, which composes ≈10% of whole cell population, exhibited the lowest sensitivity towards uncoupling by TPA showing a partial uncoupling of 17 ± 6 cells. Differences in the number of coupled cells per injection between all these groups and between each group and control cells were statistically significant (Fig. 1).

The time course and the level of the uncoupling effect were similar following incubation with TPA in the concentration range of 5×10^{-9} M to 10^{-8} M. After an overnight incubation with TPA (5×10^{-8} M) the coupling in bone cells was fully restored (Fig. 1). At this time, new dose of TPA had no uncoupling effect even after 90 min of incubation. A 30 min preincubation with polymixin B (10^{-6} M), an inhibitor of PKC,

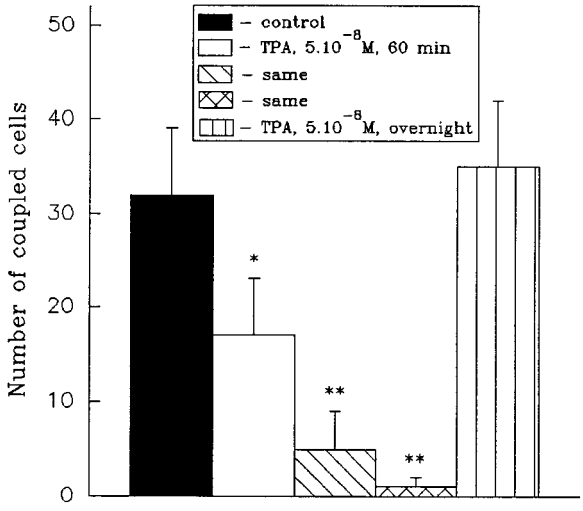

Fig. 1. Effect of TPA on cell-cell coupling in primary rat calvaria bone cells. First column: Represents the mean level of cell-cell coupling in a standard Hepes buffered solution of pH_0 7.3. Second, third, and fourth columns: Represent the level of cell-cell coupling of bone cells following 60 min exposure to 5×10^{-8} M TPA; The second, third, and forth columns represents cell-cell coupling in round, fibroblast-like, and polygonal cells, respectively. Fifth column: Represents the mean level of cell-cell coupling after overnight exposure to 5×10^{-8} M TPA. Results are expressed in terms of number of stained cells per single microinjection (mean ± S.D.). Each experimental condition was an average of 50–60 microinjections. $^*P < 0.001$, $^{**}P < 0.0001$.

was found to abolish the uncoupling effect of TPA.

Dependence of TPA Induced Uncoupling on Intracellular pH

Stimulation of primary bone cells by 5×10^{-8} M TPA induced the elevation of pH_i from a basal level of 7.23 ± 0.06 up to a value of 7.55 ± 0.06, during a period of 4 min (Fig. 2A). This alkalization was followed by a slow decrease of pH_i for further 15 min, until it leveled off at the range of its normal basal level. To find out whether the increase of pH_i and inhibition of gap junction permeability are interrelated, we stabilized the pH_i of primary bone cell cultures at a desired level by clamping pH_i via the addition of nigericin, a K^+/H^+ ionophore exchanger. In the presence of a high-K^+ medium, where the intracellular concentration of K^+ is equal to the extracellular one, the addition of nigericin leads to the clamping of pH_i to the medium pH, by equilibrating the intracellular and extracellular proton concentrations [Thomas et al., 1979]. High-K^+ medium, in the absence of nigericin, did not affect gap-junctional permeability in control cells, and did not interfere with the inhibitory effect of TPA on cell-cell communication (Fig. 3). TPA inhibited gap junction permeability in high-K^+ serum-free medium by 80–90% similar to its effect in serum-supplemented media. In both media the pH_i increased by 0.1–0.3 units following TPA addition (5×10^{-8} M, 5–10 min). Neither the clamping of pH_i at 7.25 or at 7.55, (in a high-K^+ medium containing 10^{-6} M nigericin) nor its shift between these two values reduced gap junction permeability in the absence of TPA (Fig. 3). The inhibitory effect of TPA on cell-cell communication was completely lost when intracellular pH was

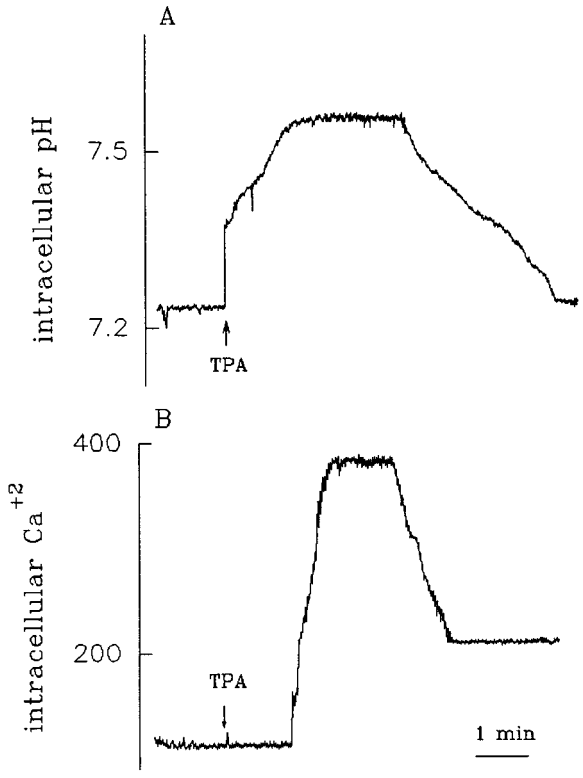

Fig. 2. Effect of TPA on the level of intracellular pH and cytosolic Ca^{+2}. **A**: Trace of time dependent intracellular pH change following exposure to 5×10^{-8} M TPA. **B**: Trace of time dependent changes of intracellular calcium following exposure to 5×10^{-8} M TPA.

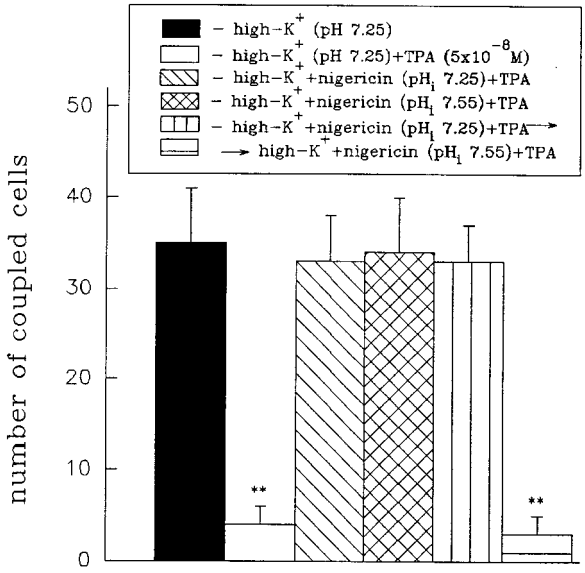

Fig. 3. Effect of TPA on cell-cell coupling as a function of pH. First column: Represents the mean level of cell-cell coupling in a high-K^+ solution at pH 7.25. Second column: Represents the mean level of cell coupling after exposure to 5×10^{-8} M TPA in high-K^+ solution at pH 7.25. Third column: Represents the mean level of cell coupling after clamping pH ($pH_i = pH_0$) in the presence of 1 μM nigericin and a high-K^+ solution at pH 7.25. Fourth column: Represents the mean level of cell coupling after clamping pH in the presence of 1 μM nigericin and high-K^+ solution at pH 7.55. Fifth and sixth column: Represent the change in the mean level of cell-cell coupling after being exposed to 5×10^{-8} M TPA in a medium containing a high-K^+ solution and 1 μM nigericin at pH 7.25 (fifth column) followed by change of the medium to one consisting of a high-K^+ solution, 1 mM nigericin, and 5×10^{-8} M TPA at pH 7.55 (sixth column). Results are expressed by the number of stained cells per single microinjection (mean ± S.D.). Each experimental condition was an average of 50–60 microinjections. $^{**}P < 0.0001$.

clamped. However, gap junctional permeability was inhibited by TPA only by transferring the cell cultures from a nigericin-containing high-K^+ medium at pH of 7.25 to a similar medium at pH of 7.55 (Fig. 3).

Preincubation of the primary bone cell cultures in a 20 mM NH_4Cl-medium for 10 min leads to a rapid intracellular alkalization (pH_i 7.25 to a pH_i 7.61). When this alkalization was followed by the addition of 5×10^{-8} M TPA, the time duration needed to achieve complete inhibition of intercellular communication was reduced from 40–60 min down to 15 min.

Treatment of downregulated bone cells (by an over night incubation of the cultures with 5×10^{-8} M TPA) with 10^{-6} M nigericin at pH_0 6.5, for 30 min, caused full inhibition of gap junctional permeability (Fig. 4). Under these conditions the measured intracellular pH was 6.75 ± 0.02.

Dependence of TPA Induced Uncoupling on Intracellular Ca^{+2}

Exposure of the cells to 5×10^{-8} M TPA in the presence of 2 mM $[Ca^{+2}]$ in the standard solution leads within 70 sec, to a $[Ca^{+2}]_i$ increase from a basal level of 115 ± 12 nM up to a peak of 385 ± 14 nM followed by a decline to a level of 203 ± 15 nM (Fig. 2B). To test the influence of $[Ca^{+2}]_i$, we used the calcium ionophore, ionomycin, in the presence of 2 mM extracellular calcium. Preincubation of primary bone cells for 30 min with 10^{-6} M ionomycin caused inhibition of cell -cell communication (Fig. 5). However, an over night preincubation of primary bone cells with 5×10^{-8} M TPA followed by incubation for 30 min with 10^{-6} M ionomycin did not altered the level of cell-cell communication (Fig. 5).

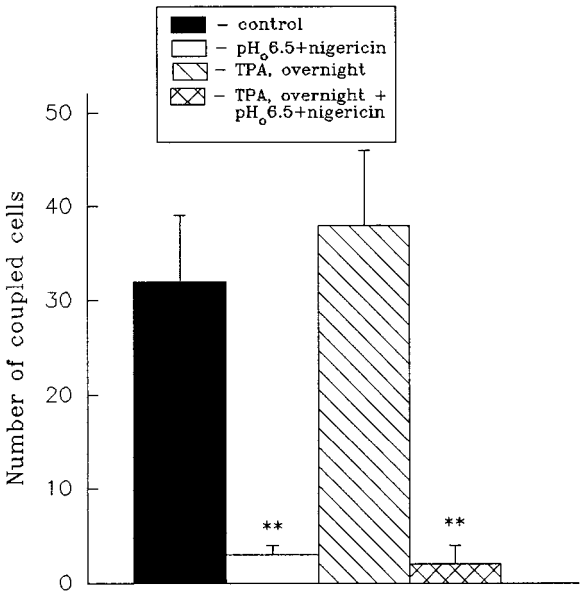

Fig. 4. Effect of acidification on cell-cell coupling in PKC downregulated rat calvaria bone cells. First column: Represents the mean level of cell coupling in a standard Hepes buffered solution of pH_0 7.3. Second column: Represents the mean level of cell coupling following acidification by exposing the cells to a standard Hepes buffered solution of pH_0 6.5 in the presence of 1 μM nigericin. Third column: Represents the mean level of cell-cell coupling after overnight exposure to 5×10^{-8} M TPA. Fourth column: Represents the mean level of cell-cell coupling of cells which were preincubated overnight with TPA after acidification by exposing the cells to a standard Hepes buffered solution of pH_0 6.5 in the presence of 1 μM nigericin. Results are expressed by the number of stained cells per one microinjection (mean ± S.D). Each experimental condition was an average of 50–60 microinjections. $^{**}P < 0.0001$.

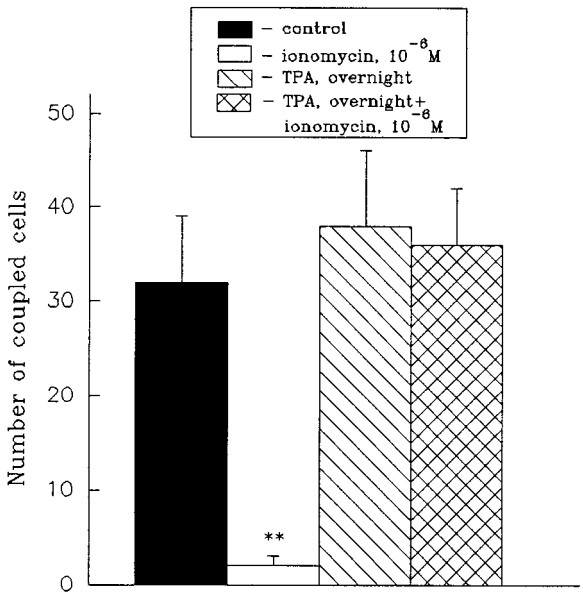

Fig. 5. Effect of ionomycin and TPA on cell-cell coupling. First column: Represents the mean level of cell coupling in a standard Hepes buffered solution at pH 7.25. Second column: Represents the mean level of cell-cell coupling after 30 min exposure to a standard Hepes buffered solution of pH_0 7.25 in the presence of 2 mM Ca^{+2} and 1 μM ionomycin. Third column: Represents the mean level of cell-cell coupling in down regulated cells (by exposure the cells to 5×10^{-8} M TPA for overnight). Fourth column: Represents the mean level of cell-cell coupling of cells which were preincubated overnight with TPA after exposure to a standard Hepes buffered solution of pH_0 7.25 in the presence of 2 mM Ca^{+2} and 1 μM ionomycin. Results are expressed in terms of the number of stained cells per single microinjection (mean ± S.D.). Each experimental condition was an average of 50–60 microinjections. $^{**}P < 0.0001$.

DISCUSSION

In some cell types, short term exposure (up to 1 h) to skin tumor promoter, TPA, was demonstrated to be a potent inhibitor of cell-cell communication [Yamasaki et al., 1985; Mesnil et al., 1986; Asamoto et al., 1991; Berthoud et al., 1992; Chaudhuri et al., 1993; Fitzgerald et al., 1993] while in others it was shown to increase junctional conductance [Saez et al., 1990; Bennett et al., 1991]. Primary calvaria derived bone cell cultures, as well as osteoblastic cell line [Shiokawa-Sawada et al., 1997] belong to the first class of cell types where TPA induces intercellular uncoupling. This inhibitory effect of TPA on intercellular communication in primary bone cells, similarly to other cells [Yamasaki et al., 1985; Mesnil et al., 1986; Asamoto et al., 1991; Berthoud et al., 1992; Chaudhuri et al., 1993; Fitzgerald et al., 1993], was found to be transient, and the cells regained their initial level of intercellular communication after more than 10 h, while becoming refractory to additional exposure to TPA. However, the primary bone cell population showed a heterogeneous inhibitory response towards the TPA effect. The three distinct subpopulations, as reflected by cell morphology, showed different response towards TPA. The location of the different subpopulations goes along with their differentiation process, where the round cells are the least differentiated ones and the polygonal cells is the most differentiated population. The results clearly demonstrate that the effect of TPA on cell uncoupling increases with bone cell differentiation. The three differentiation states described, were previously shown to be associated with three distinct ranges of membrane potentials [Massass et al., 1990]. Thus, the quantitative response to TPA exposure of this primary culture differs from a more homogeneous response when cell lines were used [Yamasaki et al., 1985; Mesnil et al., 1986; Asamoto et al., 1991; Berthoud et al., 1992; Chaudhuri et al., 1993; Fitzgerald et al., 1993].

The proposed mechanism for the inhibitory effect of intercellular communication is based on the activation of protein kinase C (PKC) [Enomoto and Yamasaki, 1985; Gainer and Murray, 1985] by diacylglycerol produced in the plasma membranes due to agonist-induced hydrolysis of phospholipids [Nishizuka, 1992]. It has been suggested that the strong uncoupling in different cell types induced by PKC activators, such as phorbol esters, is accompanied with a simultaneous rapid increase of connexin phosphorylation [Brissette et al., 1991; Oh et al., 1991; Berthoud et al., 1992; Budunova et al., 1993]. Taking into consideration that Cx43 is phosphorylated on serine [Crow et al., 1990; Musil et al., 1990; Brissette et al., 1991], one could suggest that the extent of Cxs43 phosphorylation and, accordingly, the basal level of gap junctional permeability in cell lines bearing Cx43, at least partially depends on PKC, which is serine-threonine protein kinase [Nishizuka, 1988]. The long time treatment of cells with TPA leads to downregulation of this enzyme [Chida et al., 1986] and to a temporary loss of the cell sensitivity to TPA and other protein kinase C-activators.

This suggested mechanism is in full agreement with the observed response of bone cells towards TPA. The initial downregulation of protein kinase C in bone cells was seen as early as 150–200 min following incubation with TPA. The exposure of bone cells to TPA for a long period (of 10–12 h) caused the regain of full cell coupling in all cell subpopulations and a refractory response towards the addition of a new dose of TPA. This differs from the situation in the osteoblastic cell line MC3T3-E1, where long period exposure to TPA leads to a partial recovery of communication due to the decrease in Cx43 transcripts [Shiokawa-Sawada et al., 1997]. The level of Cx43 phosphorylation is expected to be lower in PKC depleted cells which may suggest a higher level of cell coupling. However, PKC depleted cells showed a degree of cell coupling which was not statistically different than that in control undepleted bone cells. This suggests that intercellular coupling is not a linear function of connexin's phosphorylation and may involve a critical amount of phosphorylation so that cell-cell uncoupling could take place.

TPA was shown to increase pH_i by 0.1–0.3 units in a bone cell line and in hamster fibroblasts. This effect can be attributed to the activation of PKC which phosphorylates membrane antiporters which affect pH_i [Nishizuka, 1988; Sardet et al., 1990] and gap junction proteins [Gainer and Murray, 1985; Takeda et al., 1987]. To find out whether the TPA induced increase of pH_i and the inhibition of gap junction permeability are interrelated, we have artificially clamped the intracellular pH or shifted it in a controlled manner by employing a

high-K^+ medium containing nigericin [Budunova et al., 1991]. Thus, the effect of pH_i shift was studied in the absence of TPA, and under conditions where the TPA-induced pH_i-shift was prevented. Our findings show that gap junction permeability was inhibited only if intracellular pH was shifted up by 0.1–0.3 units simultaneously with TPA treatment. Thus concomitant upward pH_i-shift is necessary for TPA-induced inhibition of gap junction permeability. A plausible mechanism that may explain these observations requires the connexin to undergo a conformational change due to the pH shift. During the conformational change the protein exposes, otherwise unexposed protein sequence that is phosphorylated by PKC. It should be stressed that this exposure is a transient one and does not occur under steady state conditions either at the lower or at the higher intracellular pH. In addition, one should note that unlike the TPA-induced inhibition of gap junction permeability, the abolishment of cell-cell communication by lowering the intracellular pH, down to 6.75, is independent on PKC.

The uncoupling effect of calcium ionophore, ionomycin, which increases intracellular concentration of Ca^{+2} by orders of magnitude in bone cells, may be explained by activation of PKC which is strongly dependent on calcium [Kikkawa, 1986]. Similar mechanism of uncoupling by A23187 (a calcium ionophore) was previously shown to exists in hamster fibroblasts [Mittelman et al., 1987]. Indeed, following preincubation of PKC-depleted (downregulated) bone cells for 30 min with ionomycin (10^{-6} M), cell coupling remained at the same level as in downregulated cells before incubation with ionomycin. These findings, in bone cells, suggest that high intracellular concentration of calcium inhibits gap-junctional permeability via activation of PKC. The elevation of intracellular calcium *per se* can not attenuate intercellular coupling. Thus, a synergistic effect of Ca^{+2} on PKC activation by TPA observed in epithelial rat liver cells[Yada, 1985] is necessary to obtain intercellular uncoupling. This is supported by the finding that the effect of uncoupling effect of TPA was impeded by addition of TMB-8 [Yada, 1985], an inhibitor of intracellular Ca^{+2} mobilization [Chiou and Malagodi, 1975].

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