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Differential Regulation of Distinct Types of Gap Junction Channels by Similar Phosphorylating Conditions

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Studies on physiological modulation of intercellular communication mediated by protein kinases are often complicated by the fact that cells express multiple gap junction proteins (connexins; Cx). Changes in cell coupling can be masked by simultaneous opposite regulation of the gap junction channel types expressed. We have examined the effects of activators and inhibitors of protein kinase A (PKA), PKC, and PKG on permeability and single channel conductance of gap junction channels composed of Cx45, Cx43, or Cx26 subunits. To allow direct comparison between these Cx, SKHep1 cells, which endogenously express Cx45, were stably transfected with cDNAs coding for Cx43 or Cx26. Under control conditions, the distinct types of gap junction channels could be distinguished on the basis of their permeability and single channel properties. Under various phosphorylating conditions, these channels behaved differently. Whereas agonists/antagonist of PKA did not affect permeability and conductance of all gap junction channels, variable changes were observed under PKC stimulation. Cx45 channels exhibited an additional conductance state, the detection of the smaller conductance states of Cx43 channels was favored, and Cx26 channels were less often observed. In contrast to the other kinases, agonists/antagonist of PKG affected permeability and conductance of Cx43 gap junction channels only. Taken together, these results show that distinct types of gap junction channels are differentially regulated by similar phosphorylating conditions. This differential regulation may be of physiological importance during modulation of cell-to-cell communication of more complex cell systems.

INTRODUCTION

Gap junction channels, which provide a pathway for direct ionic and metabolic intercellular communication (cell coupling), are formed by a group of related proteins, commonly referred to as the connexin (Cx) family (Beyer *et al.*, 1990). Members of this family are differentially expressed in tissues, and one cell type can express several Cx (Bennett *et al.*, 1991; Beyer, 1993). The diversity of Cx expression suggests that the cell-specific distribution of distinct types of gap junction channels may be an important factor for tissue function. This view is supported by the observation that biophysical properties and modulatory mechanisms of gap junction channels in different cell types vary, and that many of these differences are correlated with the Cx isoforms of which the channels are built (Bennett and Verselis, 1992). Analysis of cloned Cx isoforms indicates that they are most divergent in their cytoplasmic loop and in their C-terminal tail

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(Beyer, 1993). These Cx-specific sequences contain phosphorylation consensus sites that may confer unique physiological regulatory mechanisms to the cells in which these channels are expressed.

Support for the latter hypothesis is provided by the demonstration that Cx32 (Sáez *et al.*, 1986, 1990; Takeda *et al.*, 1987, 1989; Traub *et al.*, 1987, 1989), Cx43 (Crow *et al.*, 1990; Musil *et al.*, 1990a,b; Kadle *et al.*, 1991; Laird *et al.*, 1991; Lau *et al.*, 1991; Berthoud *et al.*, 1992), and Cx45 (Laing *et al.*, 1994; Traub *et al.*, 1995) are phosphoproteins. In contrast, Cx26 has no obvious consensus sites for phosphorylation (Zhang and Nicholson, 1989), and is not phosphorylated in hepatocytes or in isolated liver gap junctions incubated with ATP and the catalytic subunit of cAMP-dependent protein kinase, protein kinase C (PKC), or Ca^{2+}/calmodulin-dependent protein kinase II (Traub *et al.*, 1989; Sáez *et al.*, 1990). For Cx43, the correlation between phosphorylation and changes in cell coupling has been extensively studied. Phosphorylation of Cx43 may be involved in various processes including channel insertion in and/or removal from the plasma membrane, changes in single channel conductances, and channel closure (Crow *et al.*, 1990; Musil *et al.*, 1990b; Lau *et al.*, 1991; Oh *et al.*, 1991; Berthoud *et al.*, 1992, 1993; Takens-Kwak and Jongsma, 1992, 1995; Lampe, 1994; Moreno *et al.*, 1994; Kwak *et al.*, 1995). Because of the variable effects of similar phosphorylating conditions on dye and electrical coupling in tissues and cells expressing Cx43 or Cx32, the correlation between phosphorylation level of Cx and degree of cell coupling remained, however, controversial (Sáez *et al.*, 1993).

A possible explanation for the variable effects elicited by similar phosphorylating conditions is the differential and/or multiple expression of Cx and/or kinase isoforms in the preparations studied. In this context, a comparison between the behavior of gap junction channels formed of distinct Cx isoforms within one cell system in which protein kinase isoforms are identical, may provide novel information on the mechanisms underlying changes in cell coupling during phosphorylation. For this purpose, we studied dye coupling and single channel conductances of several types of gap junction channels following stable transfection of their cDNAs into a human hepatoma cell line (SKHep1). This approach allowed us to compare the behavior of gap junction channels formed of phosphorylatable proteins Cx45 and Cx43 with those consisting of Cx26 subunits, which are not phosphorylated. The results presented in this study indicate that permeability and single channel conductances of distinct gap junction channels are differentially regulated under similar phosphorylating conditions.

MATERIALS AND METHODS

Cell Culture

A subclone of the human hepatoma SKHep1 cell line, which was selected for low electrical coupling and absence of dye coupling, was kindly provided by Dr. D.C. Spray (Department of Neuroscience, AECOM, Bronx, NY). This subclone of SKHep1 cells was used for transfection. Parental and all transfected SKHep1 clones were cultured in DMEM supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin in a 5% CO_2/95% air- and humidity-controlled incubator at 37°C. Media, sera, and antibiotics were purchased from Life Technologies (Gaithersburg, MD).

Expression of Protein Kinases

Plastic-grown monolayers of SKHep1 cells were scraped with a rubber policeman and suspended (~5 mg cell protein/ml) in an homogenization buffer containing 20 mM Tris/HCl (pH 7.4), 0.25 M mannitol, 2 mM EGTA, 10 μg/ml trypsin inhibitor, and 5 μg/ml leupeptin. The suspension was homogenized in a Virtis blender.

Protein kinase A (PKA) activity was determined in cell homogenates (10–50 μl) by a Kemptide phosphorylation assay, as described by Glass *et al.* (1978). The specific activity of PKA (defined as the difference in activity in the presence and absence of 10 μM cAMP) is expressed as pmol phosphate transferred per min and per mg protein. The stimulation by cAMP was 340 ± 30% (mean ± SEM; n = 4). The cAMP-dependent activity, but not the basal activity, was inhibited completely by 0.2 μM of the kinase inhibitor peptide. Kemptide and kinase inhibitor peptide (PKI[6–22]-amide) were obtained from Sigma (St. Louis, MO).

The expression of protein kinase Cα (PKCα) and PKCγ was detected by immunoblotting as described earlier for HT29 human colon carcinoma cells (Van den Berghe *et al.*, 1992). The latter cell type served as a positive control. The sole difference with the procedure described previously was the use of the enhanced chemiluminescence method (Amersham Life Sciences, Arlington Heights, IL) for detection of the immunoreactive proteins. Rabbit antibodies against PKCα and PKCγ were purchased from Calbiochem (La Jolla, CA). Molecular mass markers for SDS-PAGE and [γ-^{32}P]ATP were purchased from Amersham Life Sciences.

Purification of protein kinase G (PKG) from SKHep1 cell homogenates was performed by affinity chromatography on cAMP-Sepharose beads exactly as described for intestinal PKG (De Jonge, 1981). The overall yield of enzyme using this procedure is about 20%. Purified samples were separated by 7.5% SDS-PAGE and transferred to nitrocellulose. Blots were blocked overnight at 4°C in 20 mM Tris/HCl (pH 7.5), 500 mM NaCl, and 0.1% Tween-20, and incubated for 1 h at room temperature with antibodies against PKG type I (1:500) or PKG type II (1:3000) in the same buffer. The immunoreactive proteins were detected by the enhanced chemiluminescence method. The amount of purified PKG was quantitated by densitometric scanning of the autoradiogram using purified bovine lung PKG type I as a standard. Purification of cyclic GMP-dependent protein kinase (PKG type I) from bovine lung, and the preparation of specific antibodies against it have been described previously (Walter *et al.*, 1980). Antibodies against PKG type II were raised as described in Jarchau *et al.* (1994).

Transfection of Cx cDNAs

cDNA coding for rat Cx43 and rat Cx26 were subcloned into the eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA). SKHep1 cells were co-transfected with Cx cDNAs and pMCS, a plasmid conferring resistance to the antibiotic G418 (Grosveld *et al.*, 1982), using the DOTAP transfection reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's directions. One day after transfection, cells were exposed to 400 μg/ml G418 (Boehringer Mannheim). Colonies of G418-resistant cells were trans-

ferred to multiwell culture plates (Costar, Cambridge, MA) and grown separately for further analysis. To select for clones expressing functional gap junction channels, all colonies were first screened for dye coupling; then dye coupled colonies were further analyzed for correct Cx expression.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

mRNA was extracted using Pharmacia's QuickPrep Micro mRNA purification kit (Piscataway, NJ). Reverse transcription was carried out using Pharmacia's first strand cDNA synthesis kit and oligo(dT) primers. Single-stranded cDNA was amplified by the PCR using the following primers: for Cx45, sense 5′ TCTCCCATGTACGCT-TCTGG and antisense (degenerate) 5′ TGGG(A/C/G)C(T/G)G-GA(A/C/G)A(T/C/G)GAAGCAGT (Haefliger *et al.*, 1992); for Cx43, sense 5′ AGGAGTTCCACCAACTTTGGCG and antisense 5′ ATCGACAGCTCGATGCTCAAGC; for Cx26, sense 5′ GGGACA-CAGTGCCAACCATC and antisense 5′ GTTGCCTCATCCTCTC-CCTC. The PCR reaction was performed in a final volume of 25 μl containing 25 pmol of each primer, 2 μl of the RT reaction, 0.2 mM of each deoxyribonucleotide (Pharmacia), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01% gelatin, 0.1% Triton X-100, and 0.1 U Super *Taq* (HT Biotechnology). To compare expression levels, 10 pmol of the mouse α-actin primers (sense 5′ AGGTCATCAC-CATCGGCAAT and antisense 5′ CGTCGTACTCCTGCTTGGTG) were included in the PCR reaction. PCR was performed for 35 cycles (94°C, 1 min; 59°C, 2 min; 72°C, 1 min) preceded by 5 min at 94°C and followed by 10 min at 72°C. Five microliters of the PCR reaction was run in parallel with a molecular weight marker (1-kb ladder; Life Technologies) on a 1.5% agarose gel stained with ethidium bromide (Sambrook *et al.*, 1989). To detect Cx45 mRNA in SKHep1 cells, the PCR product was separated by agarose gel electrophoresis and the fragment was purified using the Geneclean II Kit (Bio 101, La Jolla, CA) and subjected to another round of PCR amplification.

Dye Coupling

Subconfluent cultures of parental and transfected cells were rinsed with a control solution containing (in mM): NaCl 136, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, glucose 2.5, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 10 (pH 7.4), and transferred to the stage of an inverted microscope (Nikon Diaphot TMD). Single cells within small clusters were then impaled with microelectrodes backfilled with a 4% Lucifer Yellow solution prepared in 150 mM LiCl (buffered to pH 7.2 with 10 mM HEPES). The fluorescent tracer was allowed to fill the cells by simple diffusion for 3 min. After the injection period, the electrode was removed and the fluorescent cells were counted. Cells were visualized using epifluorescent illumination provided by a 100 W mercury lamp and the appropriate filters. A similar procedure was used for 2′-7′-dichlorofluorescein except that the dye was prepared in 150 mM KCl (buffered to pH 7.2 with 10 mM HEPES). Lucifer Yellow and 2′-7′-dichlorofluorescein were purchased from Sigma. Results are expressed as mean ± SEM and n is the number of injections. Results obtained under the various phosphorylation conditions were compared with control conditions using a two-tailed Mann Whitney U test (SPSS/PC+, SPSS, Chicago, IL) and considered different when $p < 0.05$.

Electrophysiology

Junctional conductance (g_j) was monitored using the dual whole-cell voltage-clamp technique on pairs of cells (Neyton and Trautmann, 1985), which were frequently encountered in low density cell cultures of 1–2 days old. Throughout the experiments, performed at room temperature, cells were superfused with control solution using a gravity-driven perfusion system. Patch electrodes had resistances of 2.5–7 mΩ when filled with a solution containing (in mM): CsCl 135, $CaCl_2$ 0.5, Na_2ATP 5, $MgCl_2$ 1, EGTA 10, HEPES 10 (pH 7.2). CsCl was chosen to replace KCl in these experiments to reduce activity of membrane potassium channels. Both cells of a pair were voltage-clamped at a common holding potential. To measure gap junctional currents (I_j), transjunctional potential differences (V_j) were elicited by changing the holding potential of one member of a cell pair for at least 2 s. I_j was defined as the current recorded in the cell kept at a constant potential. g_j was then calculated by $g_j = I_j/V_j$. Series resistance was not compensated and was less than 2% of the combined junctional and cell input resistances.

In poorly coupled cell pairs or in pairs in which g_j was pharmacologically reduced with 2 mM halothane or heptanol, gating of single gap junction channels could be detected. Current flowing through these channels was discriminated from other nonjunctional membrane current as step-like changes of opposite polarities but identical amplitudes recorded simultaneously in both current traces. All current and voltage signals were acquired at 2 kHz sampling rate using either custom-made software (Scope; kindly provided by Drs. J. Zegers and A.C.G. van Ginneken, Department of Physiology, University of Amsterdam, The Netherlands) or the Pulse+PulseFit package software (Heka Elektronik), and stored on the hard-disk of an Apple Macintosh computer (IIvx or Quadra 660AV) equipped with data aquisition boards (National Instruments NB-MIO-16H9). Digitized current traces were filtered at 0.1–1 kHz for analysis and display of single channel activity using customized software (MacDAQ; kindly provided by Dr. A.C.G. van Ginneken). To determine single gap junctional channel conductance, the amplitudes of single channel transitions were measured and divided by the applied V_j to obtain conductances associated with the current steps. Conductance values were converted into step-amplitude histograms with a bin width of 4 pS (for Cx45 and Cx43 channels) or of 8 pS (for Cx26 channels). A 8-pS bin width was used in Cx26-expressing cells because of the larger error in estimating the current transitions of the very large (140–150 pS) conductance of these channels. To determine single channel conductance sizes, peak values of the single channel conductance distributions were determined by fitting the data to gaussian distributions using KaleidaGraph software. Results obtained from the fits are expressed as mean ± SD, N is the number of experiments, and n is the number of transitions measured.

Phosphorylating Conditions

For dye coupling studies, cells were incubated with 1 mM of the membrane-permeant nucleotide 8-bromo-cAMP (8Br-cAMP) or 8Br-cGMP, or with 12-*O*-tetradecanoyphorbol-13-acetate (TPA) for 15 min (all purchased from Sigma). TPA was first dissolved in dimethyl sulfoxide and then added to the control solution at a final concentration of 100 nM. To verify the specificity of the TPA effects, the inactive phorbol ester 4α-phorbol 12,13-didecanoate (αPDD; Sigma), also prepared in dimethyl sulfoxide, was applied at 100 nM. At the end of the incubation period, cells were microinjected within 30 min, as described above.

For electrophysiological experiments, we took advantage of the whole-cell configuration of the patch-clamp technique to intracellularly apply nonhydrolyzable protein kinase activators Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate (Sp-5, 6DCl-cBIMPS) for PKA and β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphate (PET-cGMP) for PKG (Biolog). These agonists were added to the intracellular solution at concentrations ranging from 20 to 100 μM. Specificity of these compounds was tested by using the protein kinase antagonists: Rp-adenosine-3′,5′-cyclic phosphorothioate (Rp-cAMPS) for PKA and Rp-guanosine-3′,5′-cyclic phosphorothioate (Rp-cGMPS) for PKG (Biolog), added alone or in combination with their respective agonists at a final concentration of 100 μM. After the dual whole-cell configuration had been established, a period of at least 10 min was allowed for diffusion of these agents before starting the recordings. PKC-dependent effects were studied using the active and inactive phorbol esters TPA and αPDD, as described above.

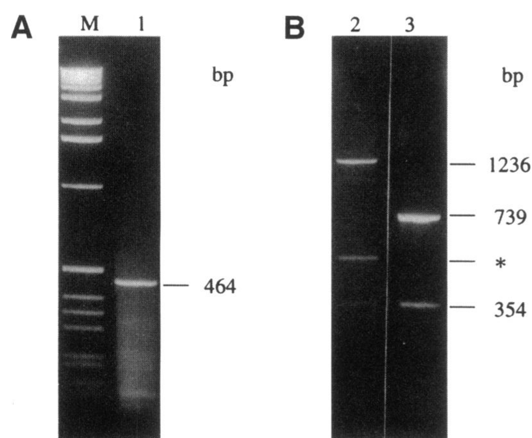

Figure 1. Analysis of Cx expression in parental and transfected SKHep1 cells by RT-PCR. mRNAs isolated from the parental (A) or the transfected (B) cells were used in a RT-PCR reaction using primer pairs specific for the expected Cx. An aliquot of the PCR amplification reaction product was applied directly onto a 1.5% agarose gel. The size of the PCR fragments is given in bp. Lane 1, SKHep1 cells (Cx45); lane 2, SKHep1/Cx43 cells; lane 3, SKHep1/Cx26 cells; and lane M, Lambda DNA 1 kb marker. To compare the levels of connexin expression between the different clones, a 354-bp fragment of the endogenously expressed human α-actin gene was amplified in parallel. The asterisk indicates a molecular species of unknown identity that is only formed in the PCR reaction of SKHep1/Cx43 derived mRNA using the primers specified in the MATERIALS AND METHODS section.

RESULTS

Expression of Cx

SKHep1 cells endogenously express low levels of Cx45 (Laing *et al.*, 1994; Moreno *et al.*, 1995b). To confirm Cx45 expression, mRNA was extracted from SKHep1 cells and reverse transcribed into cDNA. The cDNA was PCR-amplified using primers recognizing Cx45 and analyzed by gel electrophoresis. As shown in Figure 1A, a cDNA fragment of 464 bp was detected, corresponding to the expected size for Cx45 (Figure 1, lane 1). Specificity of the Cx45 fragment was confirmed by restriction digests.

Among the clones of transfected SKHep1 cells that showed resistance to G418 and dye coupling, one clone expressing Cx43 (SKHep1/Cx43 cells) and one clone expressing Cx26 (SKHep1/Cx26 cells) were selected for this study. As shown in Figure 1B, cDNA fragments of the expected size for Cx43 (1236 bp; Figure 1, lane 2) and for Cx26 (739 bp; Figure 1, lane 3) were detected from PCR-amplified reverse-transcribed mRNA of SKHep1/Cx43 and SKHep1/Cx26 cells, respectively. Crosswise control PCRs with distinct primer pairs did not result in an amplification product, neither did a PCR without reverse transcriptase, indicating that our mRNA samples were free of genomic DNA.

Expression of Protein Kinases

To explore a possible role of protein kinases as effectors of cyclic nucleotide- and phorbol ester-modulation on gap junction channels, the expression of endogenous cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), and of several PKC isoforms (PKCα and PKCγ) was evaluated in SKHep1 cells either by kinase activity assays (PKA) or by immunoblotting (PKC and PKG). The specific activity of PKA in SKHep1 cells was 460 ± 18 pmol/min/mg protein (mean ± SEM, n = 4). This is within the range of activities reported for other mammalian cell types including rat liver (Takai *et al.*, 1981). Among the phorbol ester-sensitive PKC isoforms ubiquitously expressed in mammalian tissues, the PKCα and PKCγ isoenzymes were found in considerable amounts in SKHep1 cell lysates (Figure 2, A and B). The level of both PKC isotypes was comparable or higher than that in several human colon carcinoma cell lines (e.g. HT-29cl.19A; see Figure 2, A and B) characterized in a previous study (Van den Berghe *et al.*, 1992). Two PKG isoforms have been reported in mammalian tissues. In SKHep1 cell lysates, the expression of the PKG type I isoform appeared too low to allow detection by immunoblotting; however, as shown in Figure 2C, PKG type

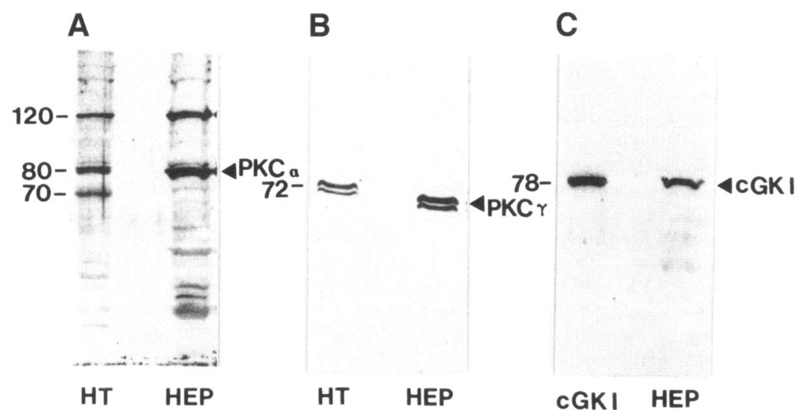

Figure 2. Immunoblots showing expression of protein kinase C and G isoforms in SKHep1 cells. Western blots showing PKCα (A), PKCγ (B), and PKG type I (C) immunoreactivity were prepared as described in MATERIALS AND METHODS. Molecular mass markers (in kDa) are indicated on the left side of each panel. (A and B) SKHep1 (HEP) and HT29cl.19A (HT) cell lysates (20 μg protein/lane). (C) PKG type I standard purified from bovine lung (cGKI; 4 ng) and PKG type I purified from SKHep1 cell homogenates (HEP; 1.6 ng) as described in MATERIALS AND METHODS.

I in SKHep1 cells could be readily detected following its purification and concentration by cAMP-Sepharose affinity chromatography. Assuming a 20% enzyme recovery (see MATERIALS AND METHODS), the level of PKG type I in SKHep1 cells was estimated to be ~30 ng/mg protein. In contrast, no positive immunoreactivity with a PKG type II-specific antibody was observed in SKHep1 cell lysates nor in purified PKG samples. Taken together, these results indicate that SKHep1 cells are equipped with all the protein kinase isoforms necessary to carry out protein phosphorylation in response to activation by phorbol ester, cAMP, or cGMP.

Dye Coupling

The effects of membrane permeant activators of protein kinases were first studied on dye coupling in subconfluent cultures of SKHep1 cells. Under control conditions, Cx45 channels did not transfer molecules as large as the fluorescent dye Lucifer Yellow (Figure 3A). Exposure of the cells to 1 mM 8Br-cAMP, 100 nM TPA, or 1 mM 8Br-cGMP did not change this absence of dye transfer (Figure 3A). Incidentally, diffusion of Lucifer Yellow to one neighboring cell was observed. Under control conditions, Cx45 channels also did not transfer the less polar tracer 2′-7′-dichlorofluorescein

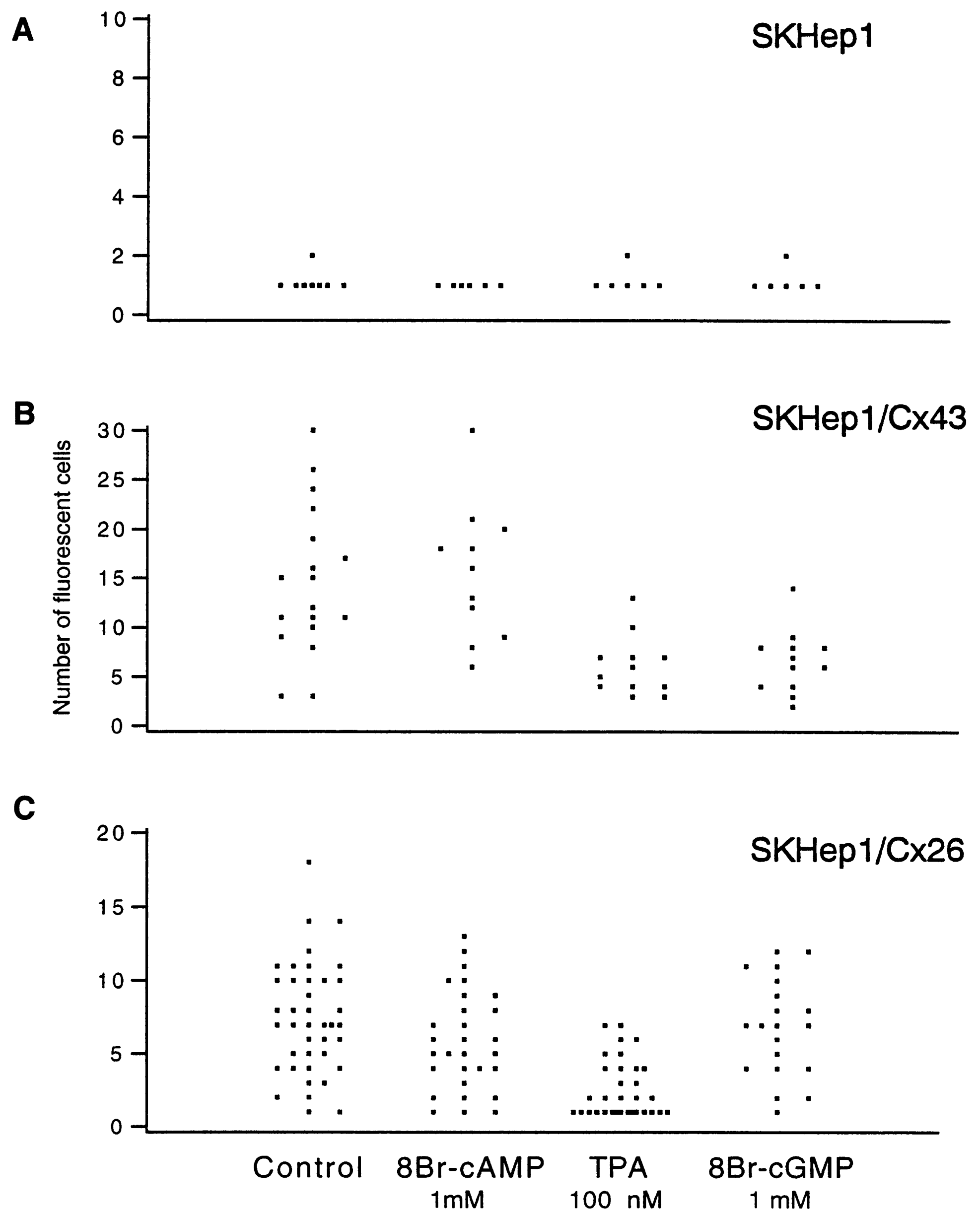

Figure 3. Dye coupling in parental and in transfected SKHep1 cells. Extent of Lucifer Yellow diffusion in parental (A, SKHep1), and in transfected (B, SKHep1/Cx43) and (C, SKHep1/Cx26) cells under control and various phosphorylating conditions. In SKHep1 cells (Cx45), Lucifer Yellow diffusion was restricted to the injected cells. This was not modified by incubating the cells 10–45 min in the presence of either 1 mM 8Br-cAMP or 8Br-cGMP, or 100 nM TPA. In contrast to parental cells, SKHep1/Cx43 and SKHep1/Cx26 cells extensively exchanged Lucifer Yellow. The extent of dye coupling was, however, markedly reduced in SKHep1/Cx43 cells exposed to TPA and 8Br-cGMP (B) and in SKHep1/Cx26 cells exposed to TPA (C).

(n = 10). Exposure of the cells to 8Br-cAMP (n = 3), TPA (n = 6), or 8Br-cGMP (n = 3) did not change this absence of dye transfer.

In contrast to parental cells, cells transfected with Cx43 or Cx26 cDNAs were able to transfer Lucifer Yellow. On average, the fluorescent tracer labeled 14.6 ± 1.8 SKHep1/Cx43 cells (n = 18 injections, mean ± SEM) and 7.4 ± 0.6 SKHep1/Cx26 cells (n = 38). Whereas 100 nM TPA and 1 mM 8Br-cGMP markedly reduced the extent of Lucifer Yellow diffusion in SKHep1/Cx43 cells ($p < 0.01$), 1 mM 8Br-cAMP was without effect (Figure 3B). In SKHep1/Cx26 cells, decreased dye coupling was observed only in cells treated with 100 nM TPA ($p < 0.01$) (Figure 3C). In the presence of 100 nM of the inactive phorbol ester αPDD, the dye labeled 11.4 ± 2.1 SKHep1/Cx43 cells (n = 5) and 8.3 ± 1.6 SKHep1/Cx26 cells (n = 6). These values were not significantly different from those observed under control conditions.

Single Channel Conductances

Despite the absence of dye coupling in clusters of SKHep1 cells, electrical coupling could be detected in cell pairs with macroscopic gap junctional conductance (g_j) averaging 3.1 ± 0.4 nS (n = 22, mean ± SEM). In weakly coupled cell pairs, junctional currents decayed with time to a steady-state level in response to transjunctional voltage pulses (V_j). With increasing V_j's, the current relaxation occurred more rapidly and to a lower steady-state level, indicating strong dependence of Cx45 channels on V_j as previously reported (Veenstra *et al.*, 1994a; Moreno *et al.*, 1995b). At this low steady-state level, openings and closures of single gap junction channels could be observed without the use of uncoupling agents. Properties of Cx45 single gap junction channels were examined under reduced coupling conditions using either high V_j, or bath application of halothane or heptanol. Typical examples of such recordings are shown in Figure 4, A and C. At low V_j (40 and 50 mV) all openings and closures of the channels were of the same amplitudes. At larger V_j (70 and 80 mV), events of smaller amplitudes were observed besides the larger openings and closures. A frequency distribution was constructed from a total of 269 transitions measured at V_j's ranging between 25 and 50 mV in four cell pairs. This distribution of events could be described by one gaussian relation with a mean (± SD) value of 36.5 ± 6.8 pS (Figure 4B). At larger V_j's, an additional conductance state with a smaller amplitude (22.5 ± 4.3 pS; N = 4, n = 388) was detected (Figure 4D). These observations suggest that Cx45 formed V_j-dependent gap junction channels of two conductances.

To investigate the effects of phosphorylating conditions on single Cx45 gap junction channels, cells were either intracellularly perfused via the patch electrodes with specific agonists (Sp-5, 6DCl-cBIMPS, and PET-cGMP) and antagonists (Rp-cAMPS and Rp-cGMPS) of PKA and PKG, respectively, or superfused with the PKC activator TPA. Exposure of the cells to 100 nM TPA led to the detection of another conductance state of ~16 pS, which was recorded along with the 22 and 36 pS channels (Figure 4E). Although the 16 pS channel was clearly observed in recordings, this novel conductance state could not be separated from the 22 pS channels in the frequency distribution of channel transitions (Figure 4F, N = 4, n = 318). However, the overall distribution of smaller events was markedly shifted to lower conductance values as compared with the distribution observed under control conditions (Figure 4D). Addition of the PKA agonist Sp-5,6DCl-cBIMPS (N = 3, n = 159) or of the PKG agonist PET-cGMP (N = 4, n = 907) to the intracellular solution did not alter the frequency distributions of Cx45 conductances. Similarly, the antagonists Rp-cAMPS (N = 2, n = 55) or Rp-cGMPS (N = 2, n = 134) were unable to change the frequency distribution of Cx45 conductances.

Electrical coupling between pairs of SKHep1 cells transfected with Cx43 or Cx26 cDNAs was larger than that observed in parental cells. g_j averaged 25.7 ± 5.6 nS (n = 11, mean ± SEM) and 39.1 ± 8.5 nS (n = 22) in pairs of SKHep1/Cx43 and SKHep1/Cx26 cells, respectively. Under reduced coupling conditions using halothane or heptanol, single channel activity could be resolved in these two clones. As shown in Figure 5A, SKHep1/Cx43 cell pairs exhibited channels of distinct sizes that could be separated in frequency distribution of channel transitions as three conductance states of 30.5 ± 9.1 pS, 61.2 ± 9.8 pS, and 89.1 ± 12.0 pS (Figure 6A; mean ± SD, N = 5, n = 1474). On the other hand, SKHep1/Cx26 cell pairs exhibited a major conductance state of 140–150 pS, although smaller events of ~70 and 110 pS were also detected (Figures 5B and 6B; N = 6, n = 493). Thus, Cx43 and Cx26 channels could be distinguished on the basis of their single channel conductances.

The effects of agonists and antagonists of PKA and PKG, and of TPA and αPDD, on Cx43 and Cx26 single channel conductances were studied. Changes in the frequency distribution of Cx43 conductances were observed in the presence of TPA and PET-cGMP. In SKHep1/Cx43 cells, addition of 100 nM TPA decreased the relative frequency of detecting the 61pS and 89 pS events (Figure 6C; N = 3, n = 648). No changes in the frequency distribution of Cx43 conductances were observed with the inactive phorbol ester αPDD (N = 1, n = 85). A shift to lower conductance values of the frequency distribution of Cx43 conductances was elicited by 20 μM PET-cGMP (Figure 7A; N = 3, n = 937). This change, as shown in Figure 7C, was prevented by 100 μM of

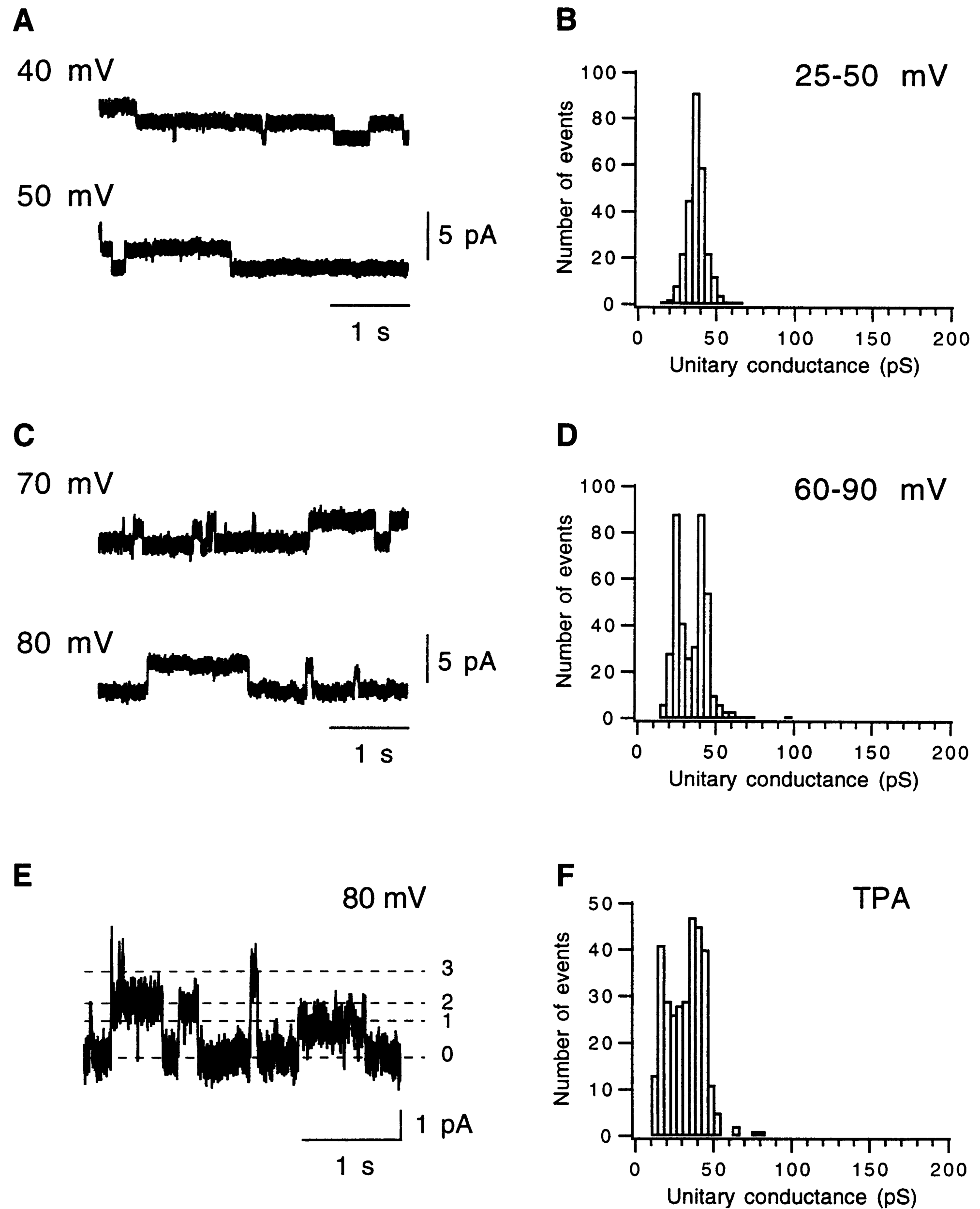

Figure 4. Effect of TPA on single gap junction channels in SKHep1 cell pairs. (A and C) Examples of single gap junction currents recorded in SKHep1 cell pairs that were monitored in the presence of halothane at V_j = 40 mV or 50 mV (A) and at V_j = 70 mV or 80 mV (C). Frequency distribution of conductances calculated from all current transitions were grouped in bins of 4 pS. The distribution of these values revealed one single peak of ~36 pS for V_j's <50 mV (B), whereas a second conductance state of ~22 pS could be observed for V_j's >60 mV (D). (E) Ten-minute exposure of the cells to 100 nM TPA induced a novel conductance state that could be resolved in addition to the 22 and 36 pS conductances. The current levels for these three conductance states are indicated by the dashed lines. Level 1, 16 pS; level 2, 23.5 pS; and level 3, 37 pS. (F) Frequency distribution of transitions measured at V_j's >60 mV in cell pairs exposed to 100 nM TPA for 10–45 min. Under these conditions, the larger peak corresponding to the 36 pS channel population was not affected by the phorbol ester. In contrast, the peak normally observed at 22 pS (see D) was shifted to lower conductance values. This peak probably reflects the transitions of 16 as well as 22 pS, conductance states that do not segregate into two separate peaks because of their very small difference in conductances.

the specific PKG antagonist Rp-cGMPS (N = 2, n = 834), the 89 pS conductance state being predominantly recorded under these conditions. Changes in the frequency distribution of Cx26 conductances were observed in the presence of TPA only. In SKHep1/Cx26 cells, addition of 100 nM TPA decreased the relative frequency of detecting the 140–150 pS events (Figure 6D; N = 3, n = 876). The incidence of recording these conductances decreased with increasing exposure time; these events were no longer detected in cell pairs that were exposed to the phorbol ester >20 min. Consistent with this observation, g_j decreased with time during TPA exposure, an effect that was not observed in cell pairs treated with either αPDD or PET-cGMP. Furthermore, no changes in the frequency distribution of Cx26 conductances were observed with αPDD (N = 2, n = 69) or with PET-cGMP (Figure 7B; N = 2, n = 391). Addition of the PKA agonist Sp-5,6DCl-cBIMPS (20 μM) did not affect the frequency distribution of Cx43 conductances (Figure 8A; N = 2, n = 551) or that of Cx26 conductances (Figure 8B; N = 4, n = 213). Increasing the concentration of the agonist to 100 μM was also without effects.

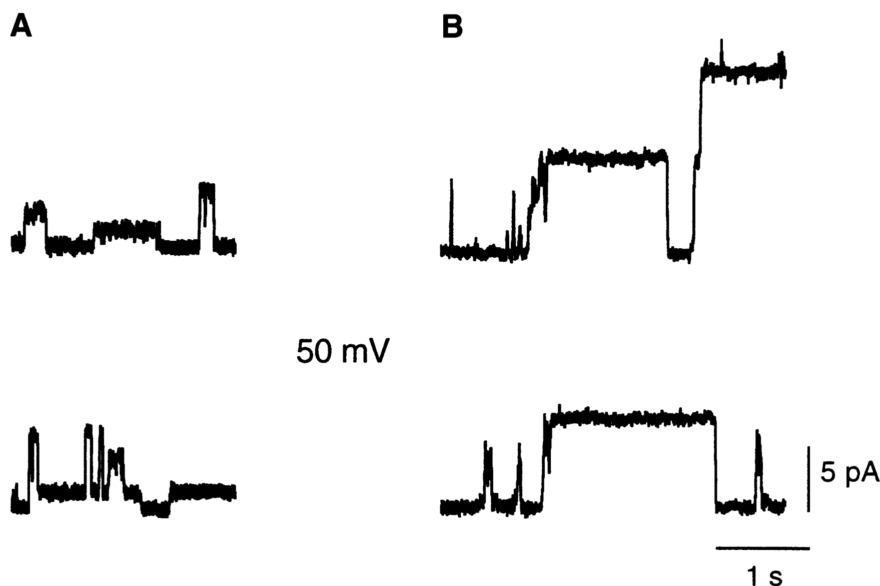

Figure 5. Single gap junctional channels in SKHep1/Cx43 and SKHep1/Cx26 cell pairs. Examples of gap junction channel activity (measured at V_j = 50 mV) in SKHep1 cells transfected with cDNAs coding for Cx43 (A) or Cx26 (B). In SKHep1/Cx43 cells, three conductance states of ~31, 61, and 89 pS were resolved. By comparison, SKHep1/Cx26 cells exhibited larger channels of 140–150 pS with frequent transitions to other current levels. Current traces in panels A and B were selected for illustration because of the absence of endogenous channel activity in these recordings.

DISCUSSION

A large body of evidence suggests that cell-to-cell communication is modulated in response to the activation and/or inhibition of protein kinases. The mechanisms by which distinct gap junction channel types were affected could not be rigorously assessed, however, because of the possible expression of multiple connexins and/or multiple protein kinases in tissues and cell types that have been investigated. In this study, we took advantage of the transfection method to exogenously express the phosphoprotein Cx43 and the nonphosphoprotein Cx26 in SKHep1 cells, a human hepatoma cell line which endogenously expresses Cx45. Using a combination of dye transfer assays and single channel measurements, we observed differences in the regulation of the various gap junction channel types by protein kinase activation within the same cell line.

It has been demonstrated previously that Cx45 is expressed in SKHep1 cells (Laing *et al.*, 1994; Moreno *et al.*, 1995b). We confirmed the presence of endogenous human Cx45 in SKHep1 cells by RT-PCR. Irrespective of the expression of Cx45, SKHep1 cells did not transfer the fluorescent dye Lucifer Yellow (MW = 457) nor the less polar dye 2′,7′-dichlorofluorescein (MW = 401). Absence of dye coupling using Lucifer Yellow or another tracer of smaller molecular weight, 6-carboxyfluorescein (MW = 376), was also reported for chick Cx45 expressed in N_2A cells, whereas 2′,7′-dichlorofluorescein was shown to pass through these channels (Veenstra *et al.*, 1994a). Rat Cx45 endogenously expressed in an osteoblasic cell line also did not pass Lucifer Yellow (Steinberg *et al.*, 1994). In contrast, transfer of Lucifer Yellow was observed in HeLa cells transfected with a cDNA coding for murine Cx45 (Elfgang *et al.*, 1995). Altogether, these observations suggest that Cx45 channels exhibit species-specific differences in permeability. As previously shown for chick and human Cx45 channels (Veenstra *et al.*, 1994a; Moreno *et al.*, 1995b), Cx45 channels exhibited strong dependence on V_j and two single channel conductances of 22 and 36 pS. Because of the contamination of Cx45 channels in recordings of transfected SKHep1 cells, it was necessary to investigate the effects of phosphorylating conditions on dye and electrical coupling between parental cells. The absence of dye transfer using either Lucifer Yellow or 2′,7′-dichlorofluorescein was not modified by incubating the cells for 10–45 min in the presence of either 1 mM 8Br-cAMP, 1 mM 8Br-cGMP, or 100 nM TPA. At the single channel level, a novel conductance state of 16 pS was detected after exposure of the cells to TPA. Although this effect could not be ascribed unambigously to PKC activation, the inactive phorbol ester αPDD, or agonists of PKA and PKG did not change the single channel conductances observed under control conditions. It has been recently reported that Cx45 is a phosphoprotein and that its level of phosphorylation was altered by TPA (Laing *et al.*, 1994). Whether a change in the phosphorylation level of Cx45 is directly related to the detection of the 16 pS conductance state remains to be established. Alternatively, it cannot be ruled out that TPA stimulates the recruitment of a novel gap junction channel type, distinct of Cx45 channels, in SKHep1 cells.

Transfection of cDNAs coding for connexins in SKHep1 cells, and other cell types, have previously been reported (Eghbali *et al.*, 1990; Fishman *et al.*, 1990; Zhu *et al.*, 1991; Beyer *et al.*, 1992; Hennemann *et al.*, 1992; Veenstra *et al.*, 1992). In contrast to parental cells,

cells transfected either with Cx43 or Cx26 cDNAs showed extensive dye transfer of Lucifer Yellow and a marked increase in g_j. In SKHep1/Cx43 cells, single channel conductance states of 31 pS, 61 pS, and 89 pS were observed. These conductances are comparable to those reported previously for Cx43 channels (Fishman *et al.*, 1990; Takens-Kwak *et al.*, 1992, 1995; Veenstra *et al.*, 1992; Moreno *et al.*, 1994; Kwak *et al.*, 1995). In SKHep1/Cx26 cell pairs, a large conductance state of 140–150 pS was often detected together with smaller conductance states of ~70 and 110 pS, as has been recently reported for Cx26 channels expressed in HeLa cells (Bukauskas *et al.*, 1995). Among other possibilities, the multiple channel conductance states recorded in SKHep1/Cx43 and SKHep1/Cx26 cells may result from transitions between substates, or from heterotypic association of exogenous hemichannels with endogenous hemichannels. Observations favoring each of these possibilities have been reported in experiments in which the activity of a few single channels was detected in the absence of uncoupling agents (Rook *et al.*, 1988; Brink and Fan, 1989; Bukauskas and Weingart, 1993; Chanson *et al.*, 1993; Veenstra *et al.*, 1994a,b; Bukauskas *et al.*, 1995; Moreno *et al.*, 1995a). Evidence for assembly of Cx32 and Cx26 into heteromeric hemichannels has been recently provided (Stauffer, 1995).

Specific activators and/or inhibitors of PKA did not change dye coupling or frequency distributions of Cx45-, Cx43-, or Cx26-single channel conductances. This absence of effects cannot be explained by deficiency of endogenous PKA activity in this cell line, because this level appeared to be similar or even higher than that in most other mammalian cell types, including rat liver (Takai *et al.*, 1981). The possibility that PKA changes the open probability of the different gap junction channels, however, could not be investigated in our study because of the use of uncoupling agents. Alternatively, the SKHep1 cells may fail to express one or more regulatory proteins, such as A Kinase Anchor Proteins, which can place PKA in close proximity to the substrate proteins (Rubin, 1994).

Specific activators and/or inhibitors of PKG only affected Cx43 gap junction channels. Dye transfer was

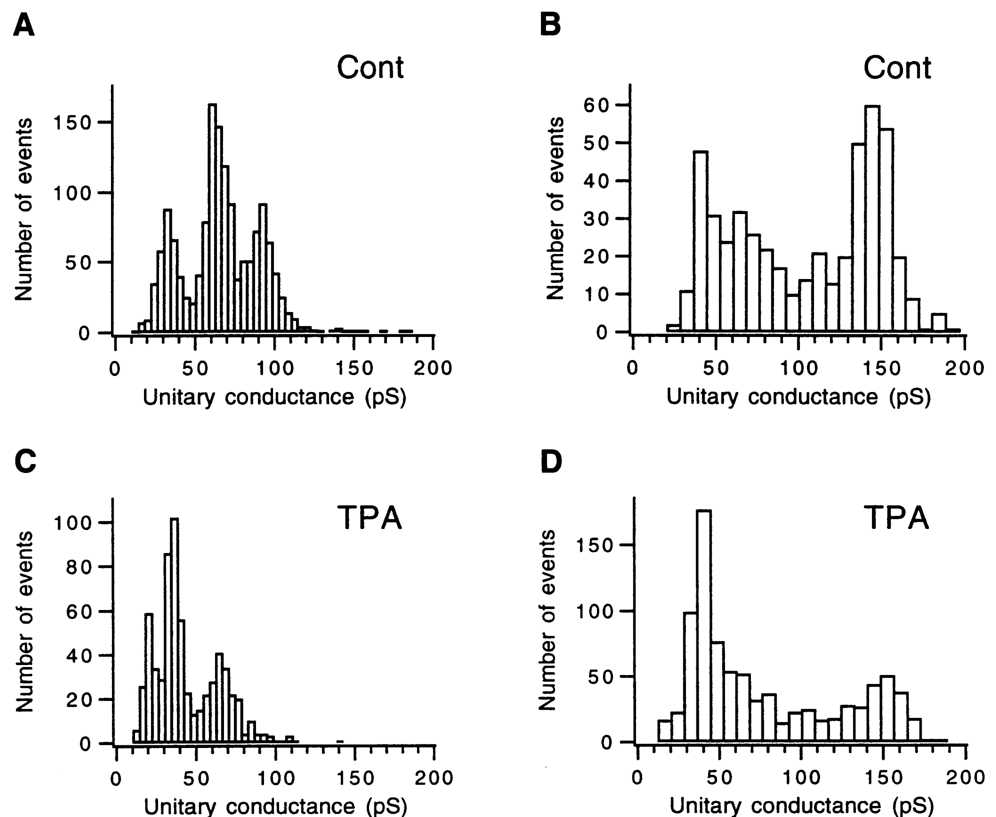

Figure 6. Effect of TPA on single gap junction channels in SKHep1/Cx43 and SKHep1/Cx26 cell pairs. Frequency distributions of transitions measured in SKHep1/Cx43 (A) and SKHep1/Cx26 (B) cell pairs that were monitored under control conditions in the presence of halothane. In SKHep1/Cx43 cells, three distinct peaks of ~31, 61, and 89 pS could be resolved at V_j's of 40–100 mV, the intermediate conductance state being most frequently observed. In SKHep1/Cx26 cells, a major conductance state of 140–150 pS was resolved as well as less frequent transitions of ~70 and 110 pS. Because of the large size of Cx26 channels, these experiments were performed at V_j's of 20–60 mV. The peak at 30–40 pS represents the contribution of the endogenous channel activity (see Figure 4B). In the presence of TPA (100 nM), the frequency distribution of Cx43 channel conductances shifted to lower values, the 89 pS peak being almost no longer detected (C). The additional peak at 10–25 pS probably reflects the endogenous channel activity (see Figure 4F). In SKHep1/Cx26 cells, the frequency of the 140–150 pS events markedly decreased in the presence of TPA (D). Bin width was 4 pS in panels A and C, and 8 pS in panels B and D.

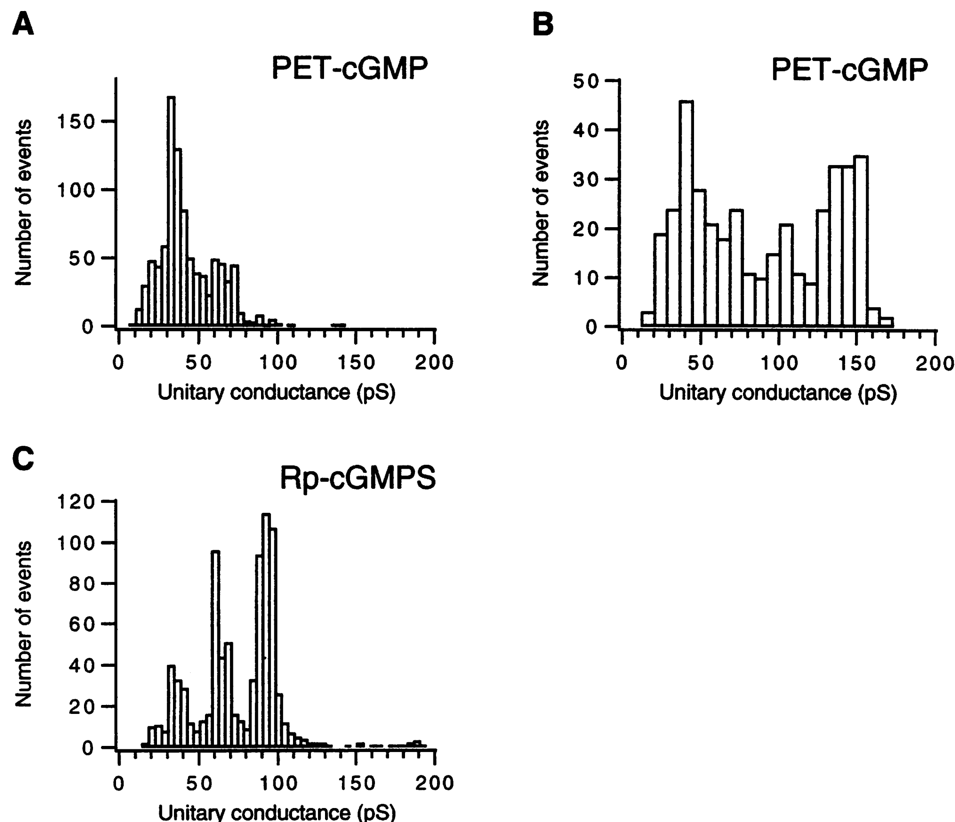

Figure 7. Effect of PKG agonist and antagonist on single gap junction channels in SKHep1/Cx43 and SKHep1/Cx26 cell pairs. Addition of 20 μM PET-cGMP to the intracellular solution shifted the frequency distribution of Cx43 channel conductances to lower values, the 89 pS conductance peak (see Figure 6A) being no longer detected (A). This effect was prevented by supplementing the electrode solution with 100 μM of the PKG antagonist Rp-cGMPS (C). Under these conditions of reduced PKG activity, the 89 pS conductance state was predominant. In contrast, PET-cGMP did not change the frequency distribution of Cx26 channel conductances (B).

reduced and the frequency distribution of single channel conductances was shifted to the lower sizes with elevated intracellular cGMP concentrations. Potential substrates for cGMP in mammalian tissues include cGMP-gated cation channels, cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases, and possibly cAMP-dependent protein kinases (PKA) (Schmidt *et al.*, 1993; Francis and Corbin, 1994; Vaandrager and De Jonge, 1994). In view of the failure of cAMP analogues to change dye coupling and the distribution of single channel conductances in SKHep1/Cx43 cells, cross-activation of PKA is clearly not the mechanism by which cGMP affects Cx43 gap junction channels. The fact that Rp-cGMPS prevented the shift in the distribution of Cx43 conductances induced by PET-cGMP strongly suggests that a G kinase was involved. PKG type I was found in extremely low levels in SKHep1 cells, whereas PKG type II could not be detected. The absolute amount of PKG type I in a tissue may be a poor indicator of its importance in

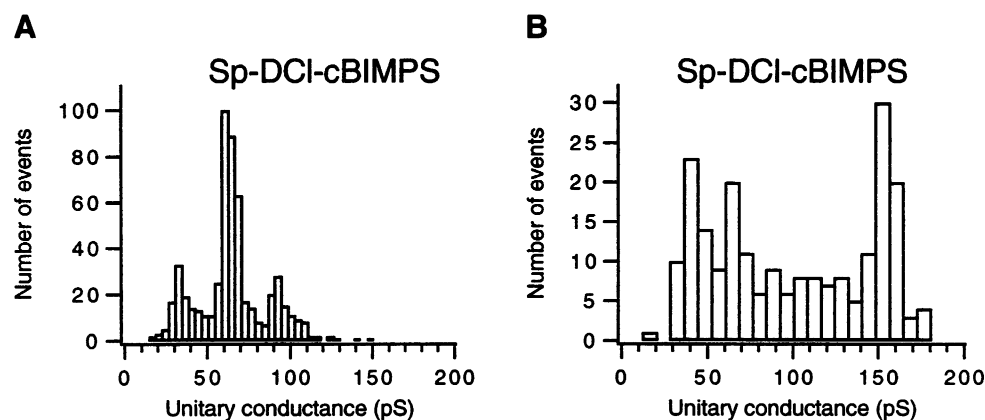

Figure 8. Effect of PKA agonist on single gap junction channels in SKHep1/Cx43 and SKHep1/Cx26 cell pairs. (A) Frequency distribution of Cx43 channel conductances in the presence of 20 μM Sp-5,6DCl-cBIMPS. Under these conditions, the intermediate 61 pS transitions were most frequently detected. This distribution was not markedly different from the distribution observed under control conditions. (B) Similarly, the PKA agonist did not change the frequency distribution of Cx26 channel conductances.

regulating cellular functions, considering the ability of the kinase to shift its intracellular distribution and to accumulate in the micro-environment of its target substrates following cell stimulation (Francis and Corbin, 1994). It has been shown previously that in cells transfected with rat Cx43 cDNA, but not in cells transfected with human Cx43 cDNA, the cGMP-induced shift in channel conductances coincides with a change in the level of phosphorylation of Cx43 (Kwak *et al.*, 1995). Thus, PKG-dependent phosphorylation may represent a mechanism to regulate post-transcriptionally gating properties of Cx43 gap junction channels.

TPA, a powerful activator of PKC, decreased dye coupling and/or altered the frequency distribution of Cx45-, Cx43-, and Cx26-single channel conductances. Because SKHep1 cells express considerable amounts of at least two phorbol ester-sensitive protein kinases, i.e., PKCα and PKCγ, one or more of these kinases are likely to be involved in the TPA-mediated effects on gap junction channels. The observation that another phorbol ester αPDD, despite being of a molecular structure closely related to that of TPA, affected neither dye coupling nor the frequency distribution of Cx45-, Cx43-, and Cx26-single channel conductances, argues against a nonspecific effect of the TPA molecule. Interestingly, the frequency distribution of single channel conductances appeared to be differently affected with each gap junction channel type. In parental cells, as discussed above, an additional conductance state was detected. In contrast, the frequency of recording certain Cx43- and all Cx26-channel conductances decreased in transfected cells. Previous studies have shown an inverse relationship between Cx43 phosphorylation and cell coupling by TPA in various systems, conditions under which the amount of Cx43 protein was unaffected (Musil *et al.*, 1990b; Oh *et al.*, 1991; Berthoud *et al.*, 1992, 1993; Lampe, 1994; Moreno *et al.*, 1994). These observations have led to the suggestion that assembly and/or gating of Cx43 channels are regulated post-transcriptionally by phosphorylation. This mechanism, however, cannot be responsible for the closure or disappearance of Cx26 channels during PKC activation because evidence exists that Cx26 is not phosphorylated (Traub *et al.*, 1989; Sáez *et al.*, 1990). Alternative mechanisms may involve destabilization of Cx26 channels by the endogenous Cx45 channels, particularly if they are in the same gap junction plaque, or modulation of the expression of Cx26 mRNA, as reported for human mammary tumor cell lines (Lee *et al.*, 1992). Anyhow, our results indicate that PKC activation differently regulates cell-to-cell communication depending on the type of gap junction channels expressed. This idea is further strenghtened by preliminary results from experiments performed on SKHep1 cells stably transfected with a cDNA coding for rat Cx32. Although a PKC-mediated phosphorylation of Cx32 has been demonstrated (Takeda *et al.*, 1987; Sáez *et al.*, 1990), TPA did not change the extent of dye coupling nor the frequency distribution of Cx32 conductances (~60 pS) in these SKHep1/Cx32 cells (Chanson, Kwak, and Hermans, unpublished results).

In conclusion, our data show that distinct gap junction channel types expressed in the same cell system exhibit different behavior under similar phosphorylating conditions. Although some of these effects could be ascribed to the activation of specific protein kinases, the precise mechanisms by which these enzymes regulate the different gap junction protein isoforms remain to be elucidated. The differential regulation of distinct gap junction channels may be of physiological relevance in tissues in which multiple Cx are expressed, or in tissues that undergo changes in Cx expression.

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REFERENCES

Bennett, M.V.L., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E., and Sáez, J.C. (1991). Gap junctions: new tools, new answers, new questions. Neuron *6*, 305–320.

Bennett, M.V.L., and Verselis, V.K. (1992). Biophysics of gap junctions. Semin. Cell Biol. *3*, 29–47.

Berthoud, V.M., Ledbetter, M.L.S., Hertzberg, E.L., and Sáez, J.C. (1992). Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{2+}. Eur. J. Cell Biol. *57*, 40–50.

Berthoud, V.M., Rook, M.B., Traub, O., Hertzberg, E.L., and Sáez, J.C. (1993). On the mechanisms of cell coupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. Eur. J. Cell Biol. *62*, 384–396.

Beyer, E.C. (1993). Gap junctions. In: Int. Rev. Cytol., vol. 137, Receptor and Membrane Proteins: Molecular Biology of Membrane Transport, ed. M. Mueckler and M. Friedlander, San Diego, CA: Academic Press, 1–37.

Beyer, E.C., Paul, D.L., and Goodenough, D.A. (1990). Connexin family of gap junction proteins. J. Membr. Biol. *116*, 187–194.

Beyer, E.C., Reed, K.E., Westphale, E.M., Kanter, H.L., and Larson, D.M. (1992). Molecular cloning and expression of rat connexin40, a gap junction protein expressed in vascular smooth muscle. J. Membr. Biol. *127*, 69–76.

Brink, P.R., and Fan, S. (1989). Patch clamp recordings from membranes which contain gap junction channels. Biophys. J. *56*, 579–593.

Bukauskas, F.F., Elfgang, C., Willecke, K., and Weingart, R. (1995). Heterotypic gap junction channels (connexin26-connexin32) violate the paradigm of unitary conductance. Pflügers Arch. *429*, 870–872.

Bukauskas, F.F., and Weingart, R. (1993). Multiple conductance states of newly formed single gap junction channels. Pflügers Arch. *423*, 152–154.

Chanson, M., Chandross, K.J., Rook, M.B., Kessler, J.A., and Spray, D.C. (1993). Gating characteristics of a steeply voltage-dependent gap junction channel in rat Schwann cells. J. Gen. Physiol. *102*, 925–946.

Crow, D.S., Beyer, E.C., Paul, D.L., Kobe, S.S., and Lau, A.F. (1990). Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus–transformed mammalian fibroblasts. Mol. Cell. Biol. *10*, 1754–1763.

De Jonge, H.R. (1981). Cyclic GMP-dependent protein kinase in intestinal brushborders. Adv. Cyclic Nucleotide Res. *14*, 315–333.

Eghbali, B, Kessler, J.A., and Spray, D.C. (1990). Expression of gap junction channels in communication-incompetent cells after stable transfection with cDNA encoding connexin 32. Proc. Natl. Acad. Sci. USA *87*, 1328–1331.

Elfgang, C., Eckert, R., Lichtenberg-Fraté, H., Butterweck, A., Traub, O., Klein, R.A., Hülser, D., and Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. J. Cell Biol. *129*, 805–817.

Fishman, G.I., Spray, D.C., and Leinwand, L.A. (1990). Molecular characterization and functional expression of the human cardiac gap junction channel. J. Cell Biol. *111*, 589–598.

Francis, S.H., and Corbin, J.D. (1994). Progress in understanding the mechanism and function of cGMP-dependent protein kinase. Adv. Pharmacol. *26*, 115–170.

Glass, D.B., Masaracchia, R.A., Feramisco, J.R., and Kemp, B.E. (1978). Isolation of phosphorylated peptides and proteins on ion exchange papers. Anal. Biochem. *87*, 566–575.

Grosveld, F.G., Lund, T., Murray, E.J., Mellor, A.L., Dahl, H.H.M., and Flavell, R.A. (1982). The construction of cosmid libraries which can be used to transform eukaryotic cells. Nucleic Acids Res.*10*, 6715–6732.

Haefliger, J.-A., Bruzzone, R., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Paul, D.L. (1992). Four novel members of the connexin family of gap junction proteins: molecular cloning, expression, and chromosome mapping. J. Biol. Chem. *267*, 2057–2064.

Hennemann, H., Suchyna, T., Lichtenberg-Fraté, H., Jungbluth, S., Dahl, E., Schwarz, J., Nicholson, B.J., and Willecke, K. (1992). Molecular cloning and functional expression of mouse connexin40, a second gap junction gene preferentially expressed in lung. J. Cell Biol. *117*, 1299–1310.

Jarchau, T., Häusler, C., Markert, T., Pöhler, D., Vandekerckhove, J., De Jonge, H.R., Lohmann, S.M., and Walter, U. (1994). Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. Proc. Natl. Acad. Sci. USA *91*, 9426–9430.

Kadle, R., Zhang, J.T., and Nicholson, B.J. (1991). Tissue-specific distribution of differentially phosphorylated forms of Cx43. Mol. Cell. Biol. *11*, 363–369.

Kwak, B.R., Sáez, J.C., Wilders, R., Chanson, M., Fishman, G.I., Hertzberg, E.L., Spray, D.C., and Jongsma, H.J. (1995). Effects of cGMP-dependent phosphorylation on rat and human connexin43 gap junction channels. Pflügers Arch. *430*, 770–778.

Laing, J.G., Westphale, E.M., Engelmann, G.L., and Beyer, E.C. (1994). Characterization of the gap junction protein, connexin45. J. Membr. Biol. *139*, 31–40.

Laird, D.W., Puranam, K.L., and Revel, J.-P. (1991). Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. *273*, 67–72.

Lampe, P.D. (1994). Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. J. Cell Biol. *127*, 1895–1905.

Lau, A.F., Hatch-Pigott, V., and Crow, D.S. (1991). Evidence that heart connexin43 is a phosphoprotein. J. Mol. Cell. Cardiol. *23*, 659–663.

Lee, S.W., Tomasetto, C., Paul, D., Keyomarsi, K., and Sager, R. (1992). Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. J. Cell Biol. *118*, 1213–1221.

Moreno, A.P., Fishman, G.I., Beyer, E.C., and Spray, D.C. (1995a). Voltage dependent gating and single channel analysis of heterotypic gap junction channels formed of Cx45 and Cx43. In: Progress in Cell Research, vol. 4: Intercellular Communication through Gap Junctions, ed. Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, and T. Shimazu, Amsterdam: Elsevier, 405–408.

Moreno, A.P., Laing, J.G., Beyer, E.C., and Spray, D.C. (1995b). Properties of gap junction channels formed of connexin 45 endogenously expressed in human hepatoma (SKHep1) cells. Am. J. Physiol. *268*, C356–C365.

Moreno, A.P., Sáez, J.C., Fishman, G.I., and Spray, D.C. (1994). Human connexin43 gap junction channels: regulation of unitary conductances by phosphorylation. Circ. Res. *74*, 1050–1057.

Musil, L.S., Beyer, E.C., and Goodenough, D.A. (1990a). Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. J. Membr. Biol. *116*, 163–175.

Musil, L.S., Cunningham, B.A., Edelman, G.M., and Goodenough, D.A. (1990b). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J. Cell Biol. *111*, 2077–2088.

Neyton, J., and Trautmann, A. (1985). Single-channel currents of an intercellular junction. Nature *317*, 331–335.

Oh, S.Y., Grupen, C.G., and Murray, A.W. (1991). Phorbol ester induces phosphorylation and down-regulation of connexin43 in WB cells. Biochim. Biophys. Acta *1094*, 243–245.

Rook, M.B., Jongsma, H.J., and van Ginneken, A.C.G. (1988). Properties of single gap junctional channels between isolated neonatal rat heart cells. Am. J. Physiol. *255*, H770–H782.

Rubin, C.S. (1994). A kinase anchor proteins and the intracellular targeting of signals carried by cyclic AMP. Biochim. Biophys. Acta *1224*, 467–479.

Sáez, J.C., Berthoud, V.M., Moreno, A.P., and Spray, D.C. (1993). Gap junctions: multiplicity of controls in differentiated and undifferentiated cells and possible fuctional implications. In: Advances in Second Messenger and Phosphoprotein Research, vol. 27, ed. S. Shenolikar and A.C. Nairn, New York: Raven Press, 163–198.

Sáez, J.C., Nairn, A.C., Czernik, A.J., Spray, D.C., Hertzberg, E.L., Greengard, P., and Bennett, M.V.L. (1990). Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. Eur. J. Biochem. *192*, 263–273.

Sáez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E., Greengard, P., and Bennett, M.V.L. (1986). cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. Proc. Natl. Acad. Sci. USA *83*, 2473–2477.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schmidt, H.H.H.W., Lohmann, S.M., and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim. Biophys. Acta *1178*, 153–175.

Stauffer, K.A. (1995). The gap junction proteins β1-connexin (connexin-32) and β2-connexin (connexin-26) can form heteromeric hemichannels. J. Biol. Chem. *270*, 6768–6772.

Steinberg, T.H., Civitelli, R., Geist, S.T., Robertson, A.J., Hick, E., Veenstra, R.D., Wang, H-Z., Warlow, P.M., Westphale, E.M., Laing, J.G., and Beyer, E.C. (1994). Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. EMBO J. *13*, 744–750.

Takai, Y., Kishimoto, A., Kawahara, Y., Minakuchi, R., Scow, K., Killawa, U., Mori, T., Yu, B., Kaibuchi, L., and Nishizuka, Y. (1981). Calcium and phosphatidyl turnover as signalling for transmembrane control of protein phosphorylation. Adv. Cyclic Nucleotide Res. *14*, 301–313.

Takeda, A., Hashimoto, E., E., Yamamura, H., and Shimazu, T. (1987). Phosphorylation of liver gap junction protein by protein kinase C. FEBS Lett. *210*, 169–172.

Takeda, A., Saheki, S., Shimazu, T., and Takeuchi, N. (1989). Phosphorylation of the 27-kDa gap junction protein by protein kinase C in vitro and in rat hepatocytes. J. Biochem. *106*, 723–727.

Takens-Kwak, B.R., and Jongsma, H.J. (1992). Cardiac gap junctions: three distinct single channel conductances and their modulation by phosphorylating treatments. Pflügers Arch. *422*, 198–200.

Takens-Kwak, B.R., and Jongsma, H.J. (1995). Mechanism of carbachol-induced decrease of cardiac gap junctional conductance. In: Progress in Cell Research, vol. 4: Intercellular Communication through Gap Junctions, ed. Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, and T. Shimazu, Amsterdam: Elsevier, 221–224.

Traub, O., Butterweck, A., Elfgang, C., Hertlein, B., Balzer, K., Gergs, U., Hafemann, B., and Willecke, K. (1995). Immunochemical characterization of connexin31, -37, -40, -43, and -45 in cultured primary cells, transfected cell lines and murine tissues. In: Progress in Cell Research, vol. 4: Intercellular Communication through Gap Junctions, ed. Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, and T. Shimazu, Amsterdam: Elsevier, 343–347.

Traub, O., Look, J., Dermietzel, R., Brümmer, F., Hülser, D., and Willecke, K. (1989). Comparative characterization of the 21-kDa and 26-kDa gap junction proteins in murine liver and cultured hepatocytes. J. Cell Biol. *108*, 1039–1051.

Traub, O., Look, J., Paul, D., and Willecke, K. (1987). Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26-kDa gap junction protein in cultured mouse hepatocytes. Eur. J. Cell Biol. *43*, 48–54.

Vaandrager, A.B., and De Jonge, H.R. (1994). Effect of cyclic GMP on intestinal transport. Adv. Pharmacol. *26*, 253–283.

Van den Berghe, N., Vaandrager, A.B., Bot, A.G.M., Parker, P.J., and De Jonge, H.R. (1992). Dual role for protein kinase C as a regulator of ion secretion in the HT29cl.19A human colonic cell line. Biochem. J. *285*, 673–679.

Veenstra, R.D., Wang, H.-Z., Beyer, E.C., and Brink, P.R. (1994a). Selective dye and ionic permeability of gap junction channels formed by connexin45. Circ. Res. *75*, 483–490.

Veenstra, R.D., Wang, H.-Z., Beyer, E.C., Ramanan, S.V., and Brink, P.R. (1994b). Connexin37 forms high conductance gap junction channels with subconductance state activity and selective dye and ionic permeabilities. Biophys. J. *66*, 1915–1928.

Veenstra, R.D., Wang, H.-Z., Westphale, E.M., and Beyer, E.C. (1992). Multiple connexins confer distinct regulatory and conductance properties of gap junctions in developing heart. Circ. Res. *71*, 1277–1283.

Walter, U., Miller, P., Wilson, F., Menkes, D., and Greengard, P. (1980). Immunological distinction between guanosine 3′;5′-monophosphate-dependent and adenosine 3′;5′-monophosphate-dependent protein kinases. J. Biol. Chem. *255*, 3757–3762.

Zhang, J.-T., and Nicholson, B.J. (1989). Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J. Cell Biol. *109*, 3391–3401.

Zhu, D., Caveney, S., Kidder, G.M., and Naus, C.C.G. (1991). Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. Proc. Natl. Acad. Sci. USA *88*, 1883–1887.