LacSwitch® II Regulation of Connexin43 cDNA Expression Enables Gap-Junction Single-Channel Analysis

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ABSTRACT

Metabolic and electrical coupling through gap junction channels is implicated in cell differentiation, tissue homeostasis, and electrotonic propagation of signals in excitable tissues. The characterization of gating properties of these channels requires electrophysiological recordings at both single- and multiple-channel levels. Hence, a system that is able to control connexin expression by external means would provide a useful tool. To regulate the expression of connexins in cells, plasmids encoding a transactivator and/or a lac-operon IPTG response-dependent Cx43 target gene were transfected into communication-deficient N2a neuroblastoma cells. Immunoblotting, dye coupling, and electrophysiological methods revealed that expression of Cx43 in selected clones could be tightly regulated. After 15–20 h of acute induction with IPTG, cell-to-cell communication reached its peak with junctional conductances of 15–30 nS. Chronic induction at specific doses of IPTG produced constant, controlled levels of Cx43 expression, which were reflected by predictable junctional coupling levels. These conditions allowed prolonged recordings from either lowly or highly coupled cells, making lac operon an ideal regulatory system for channel gating studies at a single-channel level.

INTRODUCTION

Gap junctions are associations of channels that span the cytoplasmic membranes between adjacent cells to establish a pathway for direct intercellular communication. The relative abundance of these channels, as well as their permeability and gating properties, provides mechanisms to regulate the movement of ions and small molecules between cells.

In mammals, gap junction channels are formed by the association of 12 protein subunits known as connexins (1). These proteins are encoded by a family of at least 19 genes in the mouse and 22 in humans (2). Despite the homology of connexin sequences, strong differences exist in their gating and permeability properties. Connexin channels are sensitive to transjunctional voltage, as shown in studies performed in cellular systems that permit the expression of exogenous genes, like *Xenopus* oocytes (3,4) or transfected cells (5,6). To elaborate a complete gating model, it is necessary to study the behavior of single-channel events (7). The transfection of cDNAs that encode specific connexins into communication-deficient tumor cell lines has become a standard procedure; however, the expression of connexins is uncontrolled after these transfections. To observe single-channel events, junctional coupling levels have been reduced with toxic substances that apparently do not modify the unitary conductance of the channel (8) but are suspected of modifying their open and closed times, hindering the possibility of studying their gating properties. Another approach to observing single-channel events is to force the formation of junctions *de novo* by mechanically pushing together isolated cells (6). Here, substantial effort is required to obtain single-channel recordings from channels that last only a few minutes, because of rapid recruitment of other channels into the junctions. Co-expressing connexin clones with a cell fluorescent marker like GFP has become a good indicator of connexin expression level, although it is not regulatable (9).

The lack of ability to control connexin expression also produces other problems. If the expression level is too high, then junctional resistance becomes similar to the resistance of micropipets required to voltage-clamp the cells. Under these circumstances, the "series resistance" prevents precise voltage clamping of the junction and accurate quantification of its gating properties (10).

The LacSwitch® II highly stringent expression system was implemented by

our laboratory to control expression of Cx43 in N2a cells. A similar system (11) based on the bacterial tet-operon system was also used to regulate expression of Cx32 (12). However, their study and results from our laboratory indicated that the tet-regulatory system was not stringent enough to provide consistent expression of a low number of channels in the cells. In our hands, the bacterial lac-operon system provided tighter regulation of connexin expression, allowing us to control more efficiently the number of channels in the junctions. While the expression of a low number of channels allows studies of single-channel gating, expression of intermediate or large numbers of channels can be utilized to determine macroscopic voltage dependency and is foreseen to help in studying the effects of connexin expression in target tissues. Here we present data that show that the expression of connexins can be effectively regulated in the communication-deficient tumor cell line (N2a) using a system of vectors that produces cells with expression levels low enough to record gap-junctional single-channel currents.

MATERIALS AND METHODS

LacSwitch II-Inducible Mammalian Expression System

In the *E. coli* lac operon, the Lac repressor binds as a homotetramer to the Lac operator, blocking transcription of the *lacZ* gene. Physiological or synthetic inducers, such as allolactose or IPTG, bind to the Lac repressor, causing a conformational change and effectively decreasing the affinity of the repressor to the operator; this allows transcription from the lac operon to resume. In the LacSwitch II system (Stratagene, La Jolla, CA, USA), the transactivator maintains inhibition until it is released by the addition of IPTG, allowing tighter control of protein synthesis, although achieving lower total expression.

The LacSwitch-inducible mammalian expression system utilizes a vector system wherein several elements of the lac operon have been modified for use in eukaryotic cells to control gene expression. This method for inducible expression of exogenous genes consists of a Lac-repressor-expressing vector, pRSV-Lac1, and a lac-operator-containing vector, pOPRSV1, into which the gene of interest is cloned. These vectors are co-transfected into a cultured cell line to produce cells in which expression of the inserted gene is suppressed until an inducer is added to the media and expression of the inserted gene resumes.

The pOPRSV1 vector contains the Rous sarcoma virus-LTR promoter, intron sequences from SV40 small t intron and VP1 intron, a *Not*I site for insertion of a reporter gene, and ideal operator sequences from the lac operon inserted at various positions. The system uses a nontoxic, fast-acting inducer, IPTG, that permits induction of protein expression in 4–8 h, which is partly due to the rapid transportation of IPTG into the cell.

To maintain selective pressure for both vectors in cells, each has a distinct antibiotic resistance gene. N2a-Cx43 LacSwitch cells were selected by inclusion of 1 mg/mL geneticin (G418 sulfate; pOPRSV1 vector) and 0.1 mg/mL hygromycin (Hyg pRSV-Lac1 vector) in growth media.

Protocol for Transfection of Plasmids

N2a (rat neuroblastoma; ATCC) cells were transfected with the pCMVLac1-repressor using the TransFast method (Promega, Madison, WI, USA). Stable clones were obtained by selective pressure using the antibiotic, hygromycin, in the culture media (100 µg/mL). Clones were isolated and expanded, and the cells were then lysed for Western hybridization with a polyclonal antibody to the Lac repressor, to assure expression of the 38-kDa Lac repressor monomer.

Those cells stably transfected with

the repressor were transfected with the pOPRSVI operator vector containing Cx43, also using the TransFast method. Stable clones were chosen by selection of doubly transfected cells in media containing 1 mg/mL G418 sulfate in addition to 100 µg/mL hygromycin. Clones were expanded and then induced with IPTG to allow expression of Cx43. As a control, stable and transient transfection of the operator vector containing Cx43 into N2a cells, but without the Lac1 gene, confirmed that the Cx43 gene was functional in the absence of repressor. Expression of Lac1 was ensured by Western blotting with specific polyclonal antibodies (Stratagene).

Induction of Protein Expression

After selection of clones positive for both pCMVLac1 and the operator-Cx43 constructs, the level of expression of the gene of interest (Cx43) was controlled by the addition of IPTG.

Stably co-transfected N2a cells were divided into 60-mm culture dishes at 60% confluence and treated with different concentrations of IPTG. Plates without IPTG were used as repressed samples. For time-dependent expression experiments, cells in sister dishes were incubated at 37°C for 5–30 h with 5 mM IPTG. Plates of cells were lysed at different time points to extract proteins for Western blotting. To determine Cx43 expression during chronic application of IPTG at various concentrations, dishes at 60% confluence were treated with IPTG concentrations of 0.001, 0.01, 0.1, 1, and 5 mM IPTG for 20 h. Cells with no IPTG added were considered as controls.

Cells were harvested and lysed, and extracted proteins were run on a Western blot, using antibodies against Cx43 (BD Transduction Laboratories, Franklin Lakes, NJ, USA). This antibody recognizes various phosphorylation states of Cx43. A Coomassie®-based protein assay was used to determine total protein levels in each harvested sample. Ten micrograms of total protein were loaded into each well of a 12% acrylamide-SDS gel. Proteins were transferred to PVDF membrane and then blocked with a solution of 5% dry milk/2% BSA in PBS + 0.1% Tween® 20. Blots were exposed to the primary anti-Cx43 antibody in blocking solution overnight, washed, and incubated for 1 h with Donkey-anti-mouse-HRP antibody (Amersham Biosciences, Piscataway, NJ, USA) in blocking solution. Blots were processed using an ECL® Detection Kit (Amersham Biosciences) and then exposed to film. HeLa cells transfected with rat Cx43 [kindly provided by Dr. Klaus Willecke (13)] were used as controls for Cx43 protein expression and detection during the initial cloning of LacSwitch Cx43 clones.

Immunocytochemistry

LacSwitch Cx43 N2a cells were air-dried on glass microscope slides overnight at room temperature. This procedure was preferred to fixation because it preserves the conformation of epitopes. Slides were washed for 15 min in 0.01 M PBS, pH 7.2, incubated for 15 min in a moisture box with 25 µL (dilution 1:100) of primary antibody, rinsed in PBS, and washed 3 times for 5 min in a glass reservoir (7.5 × 10 × 12.5 cm) filled with PBS and agitated with a magnetic stirrer. After the last wash, slides were incubated for 15 min with 25 µL (dilution 1:200) Alexa Fluor 488®-labeled $F(ab')_2$ anti-species antibody (Molecular Probes, Eugene, OR, USA) to the isotype of the first antibody. After the final wash, slides were coverslipped with gel/mount (Biomeda, Foster City, CA, USA).

The primary antibody used for high magnification was a rabbit polyclonal to Cx43 (14). For low magnification, the primary antibody used was a monoclonal for Cx43 (BD Transduction Laboratories, San Diego, CA, USA). Immunocytochemical studies were performed by epi-illumination in a Leitz DMRB microscope fitted with interference optics and an HBO-100 mercury-arc lamp. The epi-illuminator contained an I-3 type fluorescein isothiocyanate filter complex consisting of a 450- to 490-nm excitation filter with a 510-nm dichromic mirror and a 515-nm barrier filter allowing wavelengths greater than 515 nm to

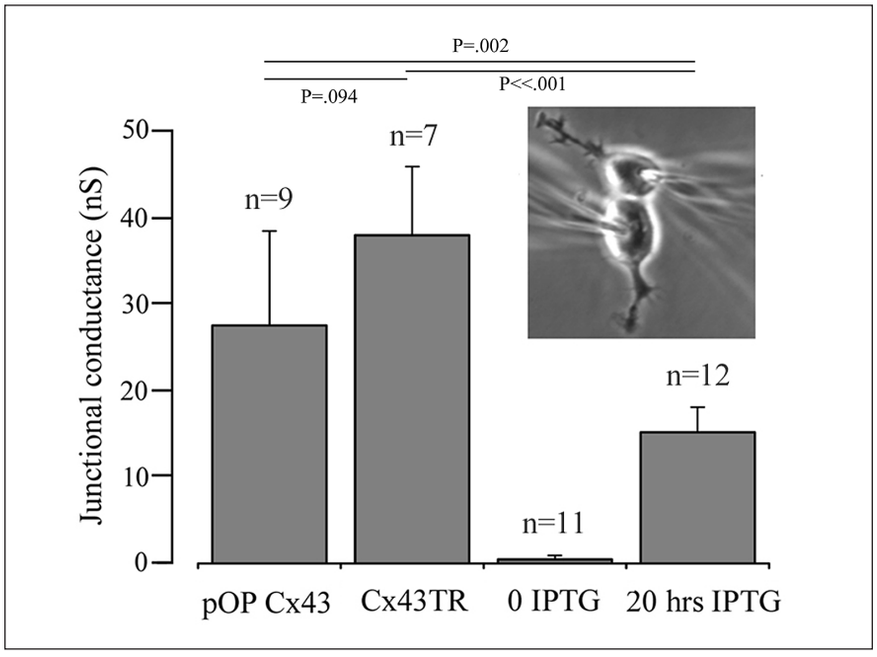

Figure 1. Junctional conductance calculated in cell pairs of various transfected N2a clones. pOPCx43 cells were transfected only with the operator plasmid of the LacSwitch system, which only induces expression of Cx43. Cx43TR corresponds to N2A cells transfected with the non-regulatable plasmid pCDNA3.1Cx43. These cells are compared with clone 6-30 (third and fourth column) with the LacSwitch system when they were not incubated with IPTG or treated with 5 mM IPTG for 20 h before recording junctional conductance. Junctional conductance was calculated using 10-mV pulses. Top, statistical comparison with their correspondent observed significance (*P*). Inset, a cell pair with two patch micropipets.

pass. A Leitz camera that contained ASA 200 daylight 35-mm Ektachrome film was used.

Dye Coupling Analysis

As a rapid assay to test cell coupling, 5% Lucifer Yellow in 150 mM LiCl was injected iontophoretically into cells in 30%–60% confluent dishes through a glass micropipet of 30–50 MΩ. Negative polarity pulses of 100 mV and 20 ms in duration were applied at a frequency of 10 Hz. Coupling was considered successful when the fluorescent dye, observed under epifluorescence, spread to two or more surrounding cells within 30 s after injection. Results are expressed as the percentage of successfully coupled cells from the total number of cells injected. To find the time dependence of dye coupling during the application or removal of IPTG, we fitted the increase (ON) and decrease (OFF) coupling data with single exponential growth curves.

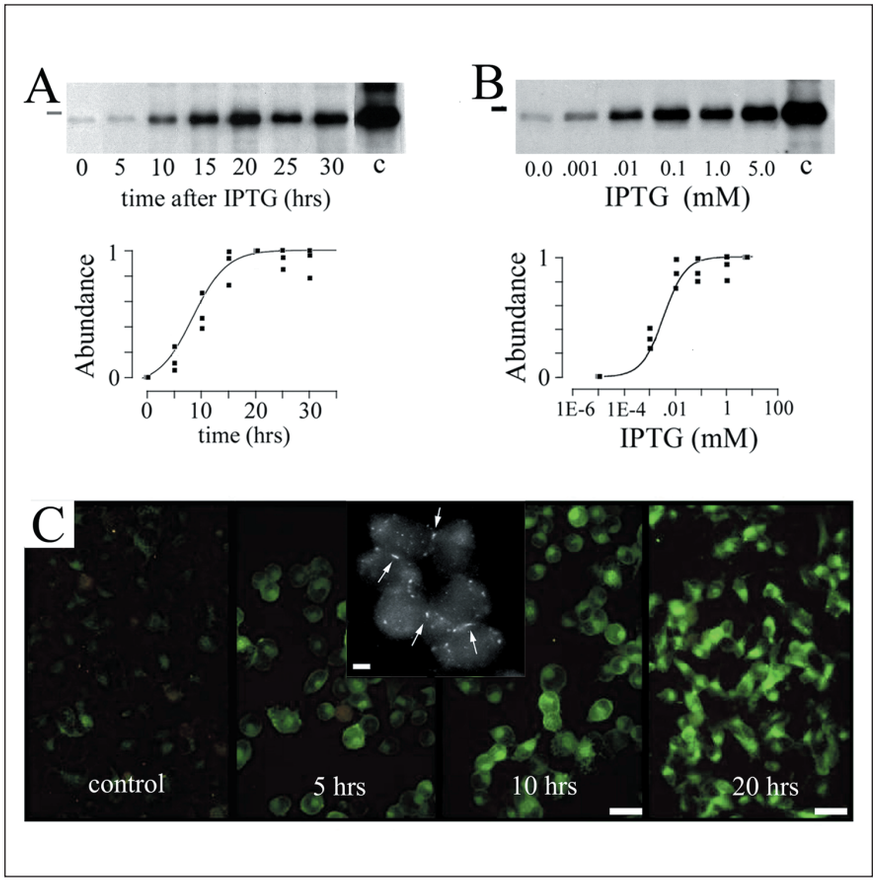

Figure 2. Regulation of Cx43 expression in N2a cells transfected with the LacSwitch system. (A) Western blots showing time dependence. Cells were grown to 60% confluence and incubated with 5 mM IPTG for 5–30 h before being harvested for total protein determination and Western blot analysis. In the clone presented here (6-30), a small amount of Cx43 was expressed at time 0 and became readily detectable after 5 h of IPTG exposure and reached maximum expression after 15 h of induction. "c" corresponds to N2a cells transfected with pCDNA3.1Cx43. The plot below shows the relative abundance of Cx43 expression in three clones after IPTG induction. All values were normalized to 15 h of exposure to IPTG. (B) Western blots showing concentration dependence. Expression of Cx43 was induced for 15 h with different concentrations of IPTG. The plot below corresponds to the relative abundance in the Western blot presented normalized to 5 mM IPTG. The fitted curve indicates that at concentrations of 0.1 mM or larger, the expression of Cx43 becomes maximal. (C) Immunostaining showing time dependence. LacSwitch Cx43 N2a cells incubated for 0, 10, and 20 h with 5 mM IPTG and reacted with mouse monoclonal antibody to Cx43. Note the increasing expression of Cx43 with prolonged incubation with IPTG. The calibration bar at 10 h corresponds to 50 µm; at 20 h, it corresponds to 100 µm. The inset on the middle indicates cells at 5 h after IPTG where junctional plaques are located (inset bar = 10 µm) using a rabbit polyclonal antibody.

Electrophysiology

Junctional conductance (gj) between two cells of distinct pairs was calculated using the dual whole-cell voltage-clamp technique through micropipets connected to a dual HEKA EPC9 voltage clamp amplifier (HEKA, Lambrecht, Germany). Access to the cytoplasm was achieved by using a brief negative pressure pulse after gig-ohm seal formation between polished glass micropipets (2–3 MΩ in 80% of all experiments) and the cell membrane. The resistance of the electrodes was compensated after whole cell configuration was reached, to 70%–80% of its value measured in solution (15). To increase input resistance, the micropipets were filled with a patch solution containing cesium (130 mM CsCl, 0.5 mM $CaCl_2$, 10 mM HEPES, pH 7.2, 10 mM EGTA, titrated with CsOH). During recording, cells were kept at room temperature in a cesium-containing solution (160 mM NaCl, 7 mM CsCl, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, pH 7.4, titrated with NaOH). To determine the transjunctional voltage dependence of junctional channels (Vj), transjunctional current (Ij) was measured in one of the cells (held at zero transmembrane voltage), while a protocol of 10-s voltage steps (ranging from -100 to 100 mV, increasing by 10- or 20-mV increments) was applied to the contiguous cell (HEKA). Junctional conductance at the steady state (gss) was determined for each voltage pulse applied and normalized to the conductance at the beginning of the pulse (gi). The normalized values of Gss (gss/gi) were plotted against Vj in a relation fitted with a two-state Boltzmann function where the parameters Vo (the voltage at which the voltage-dependent conductance reaches half of its initial value) and Gmin (the minimal non-voltage-dependent conductance reached at high voltages) were determined (16).

Currents from cells transfected with the LacSwitch system were recorded from dishes treated with different concentrations of IPTG. The unitary current levels were determined using all-point histograms from 1- to 2-min traces where 60-mV transjunctional pulses were applied. All-point distribution histograms were fitted to a multi-

ple Gaussian curve. The corresponding conductances were determined by calculating the difference between the means of Gaussian curves obtained at the open, residual, and closed states and dividing it by the transjunctional voltage applied.

Statistics

All grouped data have been expressed as $\bar{x} \pm$ SE. Significant differences between populations were determined by a two-sample *t* test where the means of two populations were or were not equal. The two-sample *t* test was performed on two sample datasets that were assumed drawn from populations that follow a normal distribution with constant variance. The preestablished significance level was 0.05.

RESULTS

Independent Expression of the LacSwitch Components

For the LacSwitch regulatory system to be appropriate for use in N2a cells, the independent expression of both plasmid products had to be assured. Stable pRSV-Lac1-transfected cells (selected with 50 µg/mL hygromycin) were shown to express the repressor molecule Lac1 that was identified in various stably transfected N2a cell clones by Western blot analysis using a rabbit polyclonal antibody provided by Stratagene. Those clones expressing the highest levels of Lac1 were selected for additional transfection with the pOPRSV-Cx43 (pOPCx43) construct.

The regulatable plasmid containing Cx43 cDNA (pOPCx43) was transfected independently to obtain various clones of stably transfected N2a cells, and then Cx43 was detected by Western blotting with specific antibodies to Cx43. Dye transfer using microinjection of Lucifer Yellow confirmed the functional expression of Cx43, and junctional conductance was later quantified by double whole-cell voltage-clamp experiments (Figure 1, first column). Eighty percent of the cells tested were coupled ($n = 12$), and their coupling level varied from 2 to 40 nS with an average of 26 ± 8 nS. This coupling level was not significantly different ($P = 0.094$) from cells transfected with pCDNA3.1Cx43 (TR), used as a control where the average coupling was 37 ± 7 nS (Figure 1, second column).

In the first set of experiments that attempted to quantify junctional coupling after IPTG induction, we compared cells transfected with the LacSwitch system, but not treated with IPTG, to those cells treated with IPTG 5 mM for 20 h (Figure 1, columns 3 and 4). It was evident that, for a clone with low basal expression, like 6-30, gj was negligible where the expression of single channels was only present in 5% of the cell pairs tried ($n = 20$). After IPTG was applied, gj reached values close to 20 nS. This level of conductance was lower than that recorded from N2a cells transfected with only the Cx43-regulatable expression vector (pOP Cx43; Figure 1, column 1) or N2a cells transfected with pCDNA Cx43 (Cx43TR; Figure 1, column 2). Statistically, at the 0.05 level, the difference of the population means of Cx43TR and pOP Cx43 was not significant ($P = 0.094$), whereas the mean for IPTG-treated cells was significantly different from both Cx43TR and pOP Cx43 means ($P < 0.002$).

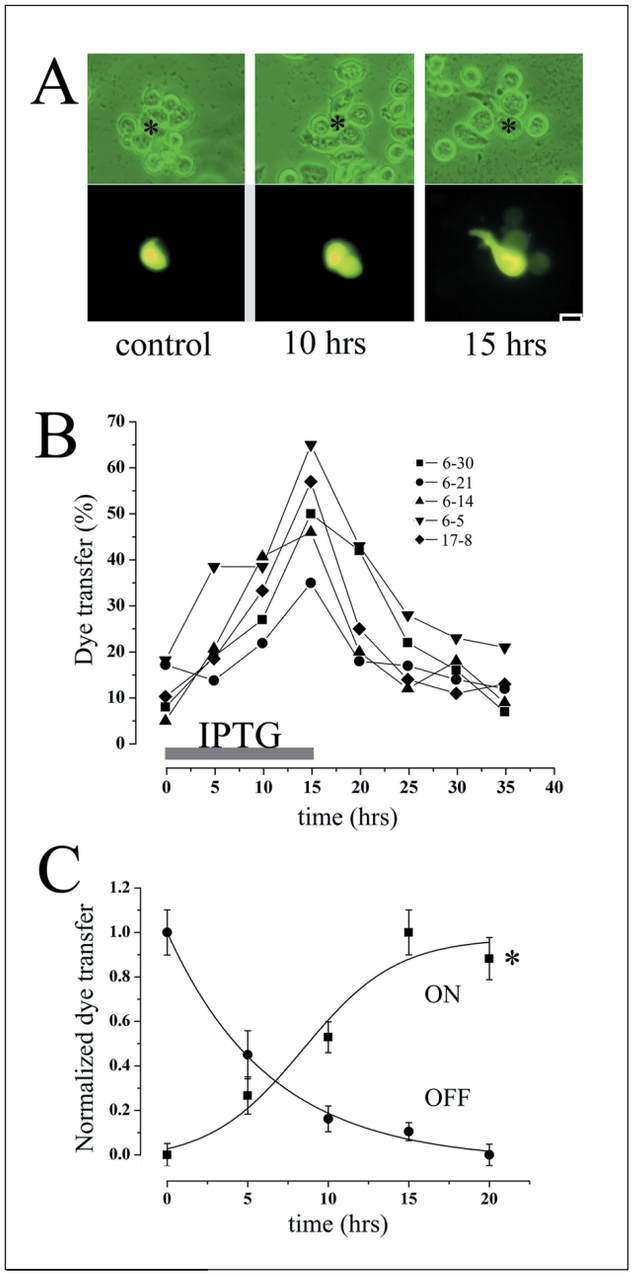

Figure 3. Functional quantification of the expression of Cx43 during IPTG induction in N2a cells with the LacSwitch system. (A) Digitized micrographs of N2a cells observed under phase contrast (top) or under epifluorescent UV light (bottom) in control conditions or 10 or 15 h after 5 mM IPTG was applied. Cells marked with an asterisk correspond to the injected cells. (B) Averaged data from five individual clones injected with Lucifer Yellow at different times after 5 mM IPTG application ($n = 20$–30 for each point). (C) Plots of normalized data from the average of all clones shown in part B ($n = 5$ for each point). The best fit for dye coupling induction (ON) using a Boltzmann relation indicates that half of the permeability increase is reached in 9.4 h. The asterisk in this part indicates the coupling level after 20 h of continuous exposure to IPTG. The extinction of dye coupling (OFF) was best fitted using an exponential function with a time constant of 4.5 h.

Regulation of Cx43 Expression by IPTG

To determine the effectiveness of our clones in producing the encoded protein Cx43 during an appropriate time frame for measuring junctional conductances, it was essential to determine the time course for expression and disappearance of Cx43 during IPTG stimulation. Studies of ON and OFF regulation were performed with stably transfected N2a clones containing both the Cx43 expressing vector (pOPCx43) and pRSV-Lac1 by inducing the cultures with IPTG.

The expression levels of Cx43 protein were not exactly the same in all clones obtained. From 30 clones tried after one set of transfections, 30% of the clones showed very low expression of Cx43, while 20% of the clones with high expression showed no clear regulation of Cx43 expression after the addition of IPTG. When Cx43 expression was robust and regulated by IPTG, maximal expression of Cx43 occurred at levels similar to those shown by HeLa Cx43 TR cells. The best clones were those that showed high expression levels of Cx43 after IPTG induction and good control by the Lac1 plasmid whereby little Cx43 protein would be detected without IPTG. Twenty percent of the clones tried behaved in this ideal manner.

Time and Dose Dependence of Cx43 Expression

IPTG is a potent and stable molecule capable of inactivating the Lac1 transactivator and allowing the promoter of the pOPCx43 vector to initiate expression of Cx43 mRNA; this inactivation of the transactivator and subsequent expression of Cx43 reached its maximum effect after approximately 15 h of induction. To determine the rates of onset and offset of expression, we measured the relative expression of Cx43 from sister dishes of LacSwitch Cx43 N2a cells (clone 6-30) during induction with 5 mM IPTG. Dishes were sampled at 5-h intervals after the addition of IPTG. The data for the onset of Cx43 expression are presented in Figure 2A as a Western blot. This figure shows clear expression of Cx43 5 h after application of IPTG and increases with time to reach a plateau of expression after 15 h. The various phosphorylation states of Cx43 were not resolved in this blot because the acrylamide percentage of the gel and the running time did not allow sufficient separation of the phosphorylated bands. Besides, the exposure time required to display the lower expression levels of connexin also caused very close bands to merge. Other experiments not shown here indicate that there is no difference between phosphorylation levels using the LacSwitch expression system when compared to native expression. The lower plot represents the relative abundance of Cx43 from three different clones of LacSwitch Cx43 cells at different times after IPTG was added. Protein measurements from these three clones were pooled and showed that maximal expression was reached after 15–20 h of IPTG application. Induction of expression was fitted to a sigmoid relation shown as a continuous curve. Even though we did not determine the total amount of Cx43 in these cells, this relationship indicates to us that half of the maximal protein expression was reached in 9.2 ± 2 h.

We also tested expression in the Cx43 LacSwitch system using IPTG at various concentrations (1 μm to 5 mM) for 20 h. Figure 2B shows that high expression levels of Cx43 protein could be reached at a chronic concentration of 1 μM IPTG, and, as in the time-dependent plot, the maximal amount of Cx43 expressed reaches a saturation level.

Immunocytochemical Detection of Cx43 after Treatment with IPTG

Various clones were tested using Cx43 monoclonal and rabbit polyclonal antibodies to determine the increase in expression and changes in distribution of Cx43 after induction with 5 mM IPTG. After 5 h of induction, there was a clear increase in the detection of Cx43 (Figure 2C). Initially, the monoclonal antibody appears to detect abundant Cx43 in the cytoplasm. During the following hours, more connexin appears to be produced, and an increase in fluorescence is detected in sites of intercellular contacts. This was confirmed by using rabbit polyclonal anti-

bodies, as shown by the higher-magnification micrograph shown in the central inset of Figure 2C.

Functional Expression of Cx43: Dye Coupling Experiments

For the LacSwitch system to be useful in determining gating properties at a single-channel resolution, it was important to determine whether there is a close correlation between the number of functional channels and the dose and duration of IPTG exposure.

Our first approach was to detect cell-to-cell communication in partially confluent cells where one cell was injected iontophoretically with Lucifer Yellow, a fluorescent dye that diffuses rapidly across cells expressing Cx43. Using sister cells, we could sample dye coupling every 5 h for each clone treated with 5 mM IPTG. The number of cells injected per clone at each time interval was 20–30. The results from these experiments were expressed as a percentage of cells coupled and are compiled in Figure 3. In Figure 3A, the injected control cells barely allowed transfer of dye. After several hours of IPTG treatment, there was a large increase in the number of incidents in which the dye spread to more than one cell. The percentage of coupling was dependent on the clone used and the duration of IPTG treatment. As shown in Figure 3B, the basal coupling (time = 0) and the maximum coupling reached after 15 h were different for each clone and were sustained after 20 h (not shown here, but see asterisk in Figure 3C). With another set of sister dishes, IPTG was applied for 15 h and then washed out. Within a few hours, dye coupling returned to a basal level. To quantify the ON and OFF curves for the change in dye coupling, we averaged and normalized the dye transfer percentage to 15 h after the addition or removal of IPTG. Figure 3C shows the results. Here, a sigmoid curve was fit to the onset of coupling. The parameters for this fitting indicate that at 9.4 h, half of the maximal coupling was reached. Notice that experiments at 20 h with IPTG (marked with an asterisk) correspond to a different measurement than that presented in the extinction curve 5 h after removing IPTG. The extinction of coupling occurred in an exponential time course, with a time constant of 4.5 h. Here, the time 0 point represents maximum coupling after 15 h of IPTG.

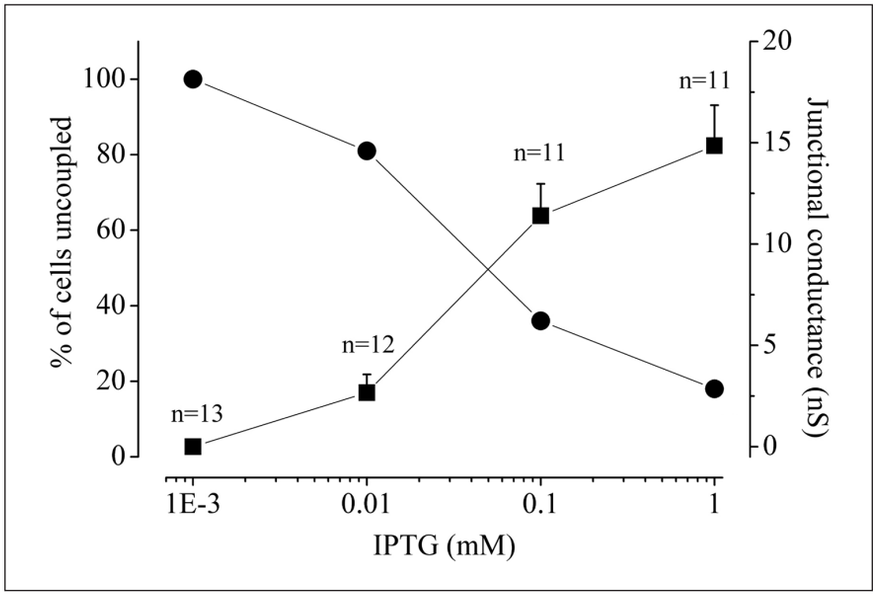

Figure 4. Dose-response curve of IPTG on junctional conductance. This plot presents the averaged data of a series of junctional coupling measurements from N2a cells transfected with LacSwitch Cx43 and treated for 15–20 h with different concentrations of IPTG. In this series, the percentage of cells uncoupled (with no conductance) from the corresponding times decreased as the IPTG concentration increased (●). Junctional conductance from the coupled cells augmented from zero at 10^{-3} mM IPTG (where no Cx43 channels were observed), to 2.6 nS at 10^{-2} mM IPTG, and to approximately 15 nS at 1 mM IPTG (■). *n* includes coupled and uncoupled cell pairs.

Functional Expression of Cx43: Electrophysiological Experiments

The titration of IPTG for prolonged periods was found to be another way to effectively control the expression of Cx43 and, at a microscopic level, was the best approach for obtaining long recordings with a small number of channels between cells. Using clone 6-30, we determined gj in pairs treated with 0.001, 0.01, 0.1, and 1 mM IPTG for 15–20 h. As shown in Figure 4, at low concentrations of IPTG, gj did not reach values beyond 1 nS. At higher concentrations, gj reached values close to 15 nS. Although the relation between gj and IPTG concentration is not linear, the expression of channels could be controlled from very low to very high levels within a very narrow range of IPTG concentration.

One important issue was to determine that the unitary conductance (γj) and the transjunctional voltage dependence parameters of Cx43 were not affected by either the expression of the LacSwitch plasmid or the application of IPTG. We determined the unitary conductance of Cx43 channels between N2a cells from clone 6-30 treated with 1 mM IPTG for 5 h. As shown in Figure 5, the unitary conductance evaluated using an all-point histogram was 130 pS. The frequency for the total number of events (n = 202) calculated from three different cell pairs resolved conductance levels of 33 ± 8, 112 ± 12, and 133 ± 7 pS. These conductance levels were similar to those obtained with similar recording solutions in other cell types and using uncoupling agents (16). The most important issue here is that it was not necessary to add 2 mM halothane to obtain single-channel recordings, as is normally required while using non-regulatable connexin-expressing cells. With this same clone, we determined the voltage-dependent parameters of multiple Cx43 channels after the application of 1 mM IPTG for 10 h. As shown in Figure 5B, transjunctional currents become inactivated with large voltages in a similar way to other Cx43 currents obtained from other expression systems (17); this is corroborated by comparing their steady-state voltage-dependent parameters (Figure 5C). These parameters were

obtained after fitting the normalized conductance values at steady state (Gss) to a Boltzmann function were Vo = 65 ± 4 mV and Gmin = 0.41 ± 8.

DISCUSSION

In this study, we have taken advantage of a commercially available LacSwitch system to reversibly induce the generation of cell-to-cell communication in N2a cells that are normally devoid of this communication. Regulating the expression of Cx43 protein, which produces gap junction channels by using the two-plasmid LacSwitch system, allowed us to produce these cells.

Our experiments verify through Western blots, dye coupling experiments, junctional conductance measurements, and immunocytochemistry that the LacSwitch system provides a useful tool for controlling Cx43 expression and cell-to-cell coupling. The coexpression of the two different plasmids of the LacSwitch system in a communication-deficient cell line establishes a control system, and the application of IPTG is sufficient to modify dramatically the synthesis of connexin protein and to demonstrate electrophysiologically a parallel establishment of gap junction communication between these cells.

We have shown through Western blots and electrophysiological measurements that the amount of protein expressed in N2a cells immediately after transfection is appropriately regulated by the action of IPTG in 50% of clones, showing the high efficiency of the LacSwitch system. After 1 year, 70% of the initial clones maintained their regulatory capacity.

The LacSwitch system rapidly induced expression of Cx43; the system demonstrated 50% maximal expression in 9.4 h. This fast increase in expression is very similar to the rate of increase of protein expression reported in SKHep1 cells using a tetracycline regulation system (12). In a similar way, the reduction of coupling after IPTG is removed from the media occurred exponentially with a time constant of 4.5 h. The time required to produce this reduction in coupling is in agreement with measurements obtained from other primary cells (18) and in liver after partial hepatectomy (19). Altogether these data suggest that the regulation of protein expression by IPTG in the LacSwitch system may be an essential tool for the study of the processes of protein turnover and other trafficking phenomena in the cell.

We have also advanced the characterization of gap junction channels in this system. Of significance is the fact that expression of proteins was associated with junctional coupling, although this has not always been observed with other regulatory mechanisms like phosphorylation (20–23), which can gate channels already present in the membrane. We have demonstrated through our experiments that expression of Cx43 can be maintained at low enough levels for single channels to be observed even after several hours of induction, with controlled amounts of IPTG. These types of experiments permit the extensive use of cultured cells to determine single-channel properties of channels expressed in low numbers. Substances like octanol and halothane have been used previously to reduce gj to observe the behavior of single channels. It has been found that, in most cases, these drugs do not change the unitary conductance (24,25). They have been also helpful in determining other properties of the channels, such as the correlation of the residual conductance detected at the microscopic level and the steady-state current, determined under macroscopic voltage-dependent protocols (16). Nonetheless, the channel gating behavior of these and non-junctional membrane channels is known to be affected by these drugs (26,27). Experiments with cultured cells that do not require toxic substances will be profoundly useful in

characterizing gap junction channels without the nuisance of changing their channel-gating behavior. Induction with these LacSwitch plasmids also helps to characterize the macroscopic voltage-dependent properties of the channels. The unitary conductance of Cx43, as well as its transjunctional voltage gating parameters, does not appear to be affected by using the LacSwitch system with IPTG application. The single-channel data obtained with the LacSwitch system clearly agree with measurements made with other plasmids in other cell types (28). This unitary conductance corresponds to the maximal conductance recorded for Cx43 channels, which has been associated with the de-phosphorylated form of Cx43 (21). These data agree with our results wherein no intracellular ATP or other phosphorylating agent has been used. The conspicuous residual conductance observed in LacSwitch-regulated cells is a feature of these channels that is usually not observed in transfected Cx43 clones when halothane is used, apparently because halothane induces chemical gating of the channels where changes in conductive states occur only between open and closed states.

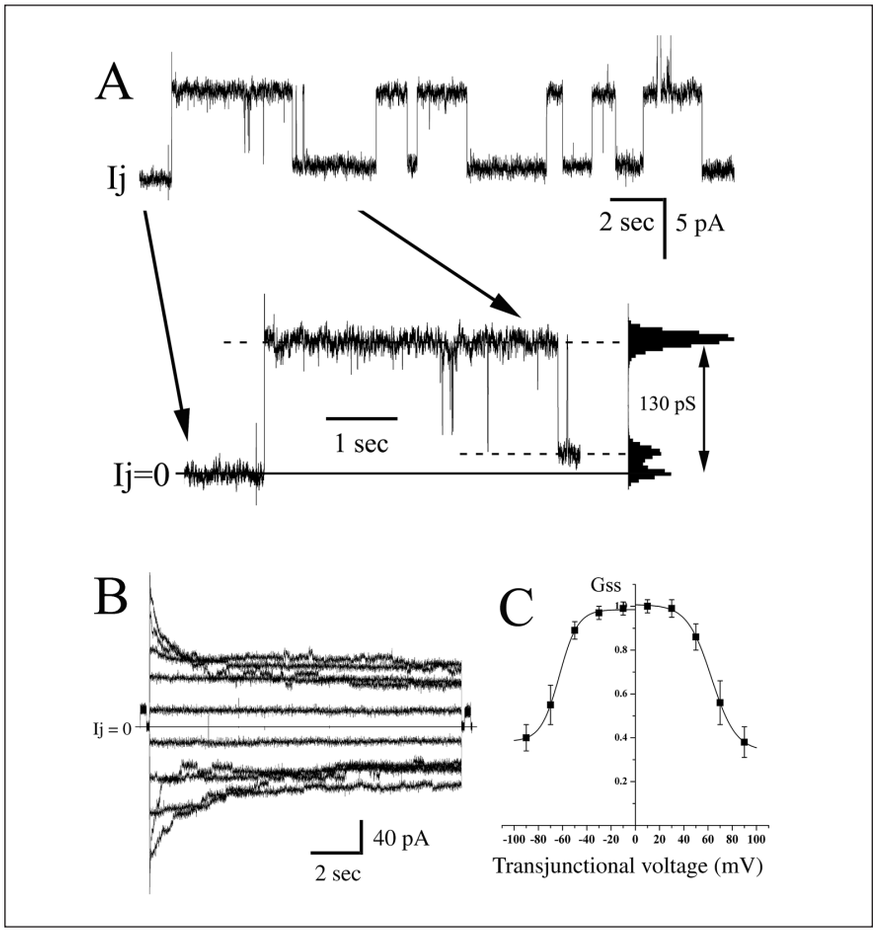

Figure 5. Microscopic and macroscopic analysis of Cx43 gating channels. (A) Cx43 single-channel transitions recorded from cells transfected with the LacSwitch system and treated with 1 mM IPTG for 5 h. The all-points histogram analysis on the right from the time-enhanced region shows the presence of a residual state of approximately 29 pS and a main unitary conductance of approximately 130 pS. The broken lines show the different single-channel transitions. The traces were filtered at 500 Hz and sampled at 2 kHz during a hyperpolarizing voltage pulse of 60 mV. (B) N2a cells transfected with the regulatory system LacSwitch were studied to determine their transjunctional voltage dependence. Junctional currents (Ij) were recorded in one cell of a pair in response to 10-s pulses from -90 mV to 90 mV (in 20-mV increments) that were applied to the other cell. Junctional currents were maximal at the beginning of the pulses and declined to steady-state values in a voltage-dependent manner when the voltage exceeded ± 40 mV. (C) Dependence of Gss (normalized junctional conductance at steady state) on Vj calculated from three different cell pairs. The smooth curve superimposed on the data corresponds to the best fit of a Boltzmann relation to the normalized data.

There is good correlation between the appearance of Cx43 protein and the increase in dye coupling, as indicated by the sigmoid curves fitted to both processes. As soon as IPTG is removed, coupling starts to decrease and, in an exponential form, ceases after 10 h. This fast decrease in expression suggests that IPTG is rapidly metabolized and repression of the Cx43 gene is regained. A prompt decrease in coupling is observed at this time, possibly because of the removal of Cx43 from the membrane.

Using the LacSwitch system with appropriate IPTG concentrations permitted us to consistently obtain cell pairs that had a small number of channels (<10). The tetracycline regulation system was also used for this purpose and resulted in changes of expression, as determined by immunofluorescence and regulation of coupling by dye transfer (12). Unfortunately, our experience with the tetracycline plasmid system indicated it was not as stringent as expected, causing us to consistently obtain "leaky" clones, where the regulated conductance was higher than 1 nS. The high stringency of the LacSwitch system probably occurs because selection of the clones starts with a repressed system, and clones selected must have this characteristic; this property allows finer control of expression of a low number of channels in a leak-proof system. Compared with the tet regulation system, the lac operon has been reported to be too inefficient to express high levels of the gene regulated (11). However, according to our analysis of expression levels through electrophysiological studies (Figure 1), the LacSwitch system expresses Cx43 protein at levels equal to the pCDNA plasmid, which was recommended as a high expresser. Therefore, this system can provide a significant tool for studying expression of connexins in vivo, with the advantage that if inserted in a transgenic genome of an animal, the targeted tissue will not express a specific gene until IPTG is provided (29).

In summary, the data reported here suggest that LacSwitch provides an excellent and easy-to-use genetic tool to

regulate expression of connexins in N2a cells. All molecules involved have been characterized, and probes to detect them are readily available. When used appropriately, LacSwitch provides a fast-response and leak-proof system that allows exploration of the electrophysiological properties of channels at microscopic and macroscopic levels. This regulatory system also shows promise for comparative studies of expression, translocation, and RNA messenger stability for different connexins.

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