J. Membrane Biol. 148, 243–253 (1995)

The Journal of
Membrane
Biology

Cyclic AMP Induces Rapid Increases in Gap Junction Permeability and Changes in the Cellular Distribution of Connexin43

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Received: 9 February 1995/Revised: 22 August 1995

Abstract. The rapid effects of cAMP on gap junction-mediated intercellular communication were examined in several cell types which express different levels of the gap junction protein, connexin43 (Cx43), including immortalized rat hepatocyte and granulosa cells, bovine coronary venular endothelial cells, primary rat myometrial and equine uterine epithelial cells. Functional analysis of changes in junctional communication induced by 8-bromo-cAMP was monitored by a fluorescence recovery after photobleaching assay in subconfluent cultures in the presence or absence of 1.0 mM 1-octanol (an agent which uncouples cells by closing gap junction channels). Communicating cells treated with 1.0 mM 8-bromo-cAMP alone exhibited significant increases in the percent of fluorescence recovery which were detected within 1–3 min depending on cell type, and junctional communication remained significantly elevated for up to 24 hr. Addition of 1.0 mM 8-bromo-cAMP to cultured cells, which were uncoupled with 1.0 mM octanol for 1 min, exhibited partial restoration of gap junctional permeability beginning within 3–5 min. Identical treatments were performed on cultures that were subsequently processed for indirect immunofluorescence to monitor Cx43 distribution. The changes in junctional permeability of cells correlated with changes in the distribution of immunoreactive Cx43. Cells treated for 2 hr with 10 μM monensin exhibited a reduced communication rate which was accompanied by increased vesicular cytoplasmic Cx43 staining and reduced punctate surface staining of junctional plaques. Addition of 1.0 mM 8-bromo-cAMP to these cultures had no effect on the rate of communication or the distribution of Cx43 compared to cultures treated with monensin alone. These data suggest that an effect of cyclic AMP on Cx43 gap junctions is to promote increases in gap junctional permeability by increasing trafficking and/or assembly of Cx43 to plasma membrane gap junctional plaques.

Key words: Gap junction—Connexin43 (Cx43)—Cyclic AMP—Fluorescence recovery after photobleaching—Junctional communication

Introduction

The gap junction protein connexin43 (Cx43) is the most abundant and widespread member of an evolutionarily conserved multigene family of channel-forming proteins found in a variety of cell types including excitable and nonexcitable cells (Willecke et al., 1991). Cx43 and other members of the gap junction protein family assemble as hexameric hemichannels (connexons) in cell membranes and form an aqueous channel of about 1.5 nm diameter upon alignment of connexons in adjacent cells (Hall, Zampighi & Davis, 1993).

A number of studies indicate that Cx43 is a phosphoprotein which exists in multiple phosphorylation states. Cx43 phosphorylation may be required for assembly and/or activation of gap junctions (Musil & Goodenough, 1993) and the unitary conductances of Cx43 may be regulated by phosphorylation (Moreno et al., 1994). There is evidence that activation of several protein kinases can downregulate gap junction permeability. For example, sustained activity of one or more isoforms of protein kinase C and the tyrosine protein kinases, $pp60^{v\text{-}src}$ and $p130^{gag\text{-}fps}$, uncouples cells and leads to, respectively, serine or tyrosine and serine phosphorylation of Cx43 (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990; Brissette et al., 1991; Goldberg & Lau, 1993; Godwin et al., 1993; Kanemitsu & Lau, 1993; Kurata & Lau, 1994). The binding of epidermal growth factor (EGF) or platelet-derived growth fac-

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tor (PDGF) to their respective receptors (receptor tyrosine kinases) leads to serine phosphorylation of Cx43 and disruption of junctional communication (Maldonado, Rose & Loewenstein, 1988; Kanemitsu & Lau, 1993; Pelletier & Boynton, 1994). In the case of rat liver epithelial cells, uncoupling of cells caused by lysophosphatidic acid treatment or EGF binding may be mediated in part by mitogen-activated protein kinase (Kanemitsu & Lau, 1993; Hii et al., 1994).

To date, the only kinase which has been reported to cause an increase in junctional communication in some cell types is the cyclic AMP-dependent protein kinase (*reviewed,* Stagg & Fletcher, 1990). Cx43 may be subject to multiple levels of regulation by this kinase because cAMP analogues can increase gap junction permeability in a number of cell types over time courses ranging from seconds to hours (Stagg & Fletcher, 1990; Mehta, Yamamoto & Rose, 1992). In myometrial cells, cyclic AMP agonists may reduce communication under certain conditions (Cole & Garfield, 1986; Sakai, Blennerhassett & Garfield, 1992) and increase it under others (Godwin et al., 1993; Dookwah et al., 1992; Nnamani et al., 1994).

In the present study, several cell types which express different levels of junctional communication and Cx43 were used to determine the rapid effects of cAMP on cell-cell communication. Direct effects were evaluated by treating cells with a membrane permeable cAMP analogue (8-bromo-cAMP) and monitoring rapid changes in gap junction permeability by means of a dye transfer method (Wade, Trosko & Schindler, 1986) adapted to calculate rate constants for dye diffusion between coupled cells (Barhoumi et al., 1993). Additional analyses of the effects of cAMP on junctional communication and Cx43 distribution in subconfluent cultures were also performed by adding 8-bromo-cAMP in the presence of 1.0 mM 1-octanol (an agent that uncouples cells by reducing the open probability of gap junction channels to near zero (Burt, 1991)) or 10 μM monensin (an ionophore which inhibits translocation of Cx43 from the Golgi (Purnam et al., 1993)). Upregulation of cell-cell communication induced by 8-bromo-cAMP was correlated with increased trafficking and/or assembly of Cx43 to plasma membrane gap junctional plaques.

Materials and Methods

Materials

Culture media, Dulbecco's phosphate buffered saline (PBS), serum, 1-octanol, and all general chemical reagents were purchased from Sigma Chemical (St. Louis, MO). Tissue culture flasks and dishes were obtained from Corning (Oneonta, NY) and 4-well LabTek slides were purchased from Nunc (Naperville, IL). 5-carboxyfluorescein diacetate (CFDA), was purchased from Molecular Probes (Eugene, OR). Stock solution of CFDA was prepared in DMSO (2.0 mg/ml) and diluted to 10 μg/ml in serum-free medium without phenol red. Octanol was prepared as a 2.0 mM stock in serum- and phenol red-free culture medium for dilution to 1.0 mM during experiments. The cAMP analogue, 8-bromo-cyclic adenosine-3′:5′-monophosphoric acid (8-bromo-cAMP), was purchased from Boehringer Mannheim (Indianapolis, IN). Stock solution of 8-bromo-cAMP was prepared in medium without serum or phenol red (10 mM) and diluted to 1.0 mM in the same medium for experiments described below. Monensin was purchased from Calbiochem (La Jolla, CA) and prepared by dissolution in ethanol before addition to medium at a final concentration of 10 μM (ethanol < 0.01%). Monoclonal mouse anti-connexin43 developed against a synthetic peptide corresponding to 19 residues (251–270) of rat Cx43 (Clone: Z039) was purchased from Zymed Laboratories (San Francisco, CA). Biotinylated secondary antibody and fluorescein-streptavidin were purchased from Amersham Corporation (Arlington Heights, IL).

Cell Cultures

Several clonal cell lines and primary cultures exhibiting immunoreactive Cx43 were used in these studies. Clonal cell lines included Clone 9 (ATCC, CRL 1439, passage 17), a cell line derived from normal rat hepatocytes, bovine coronary venular endothelial cells (CVEC, (Schelling et al., 1988)), and a spontaneously immortalized granulosa cell line (SIGC) derived from a BD-IV strain of rats (Stein et al., 1991). With the exception of SIGC, cell lines were used within 10 passages of arrival in the laboratory. Passage numbers 24 and 321 of SIGC were selected for analysis (SIGC has been maintained in the laboratory in continuous culture for >380 passages). Primary cultures of rat myometrial cells were isolated as described (Dookwah et al., 1992) and equine uterine endometrial epithelial (UEE) cells were isolated from transcervical endometrial biopsies as described (Brady et al., 1993). Clone 9 cells were grown in Ham's Nutrient Mixture F-12 with 10% fetal bovine serum. SIGC were grown in Dulbecco's Modified Eagle's Medium with F-12 salts (DME-F12) containing 5% fetal bovine serum. Primary cultures and CVEC were grown in the same medium containing 10% fetal bovine serum.

Functional Analysis of Gap Junctions

Junctional communication was monitored with a Meridian ACAS 570 workstation (Meridian Instruments, Okemos, MI) using a fluorescence recovery after photobleaching technique (Wade et al., 1986). Cells were plated into 35 mm culture dishes for 48 hr. Cultures were stained with 10 μg/ml CFDA in serum-free medium without phenol red for 15 min at 37°C, followed by three rinses and maintenance in serum-free medium without phenol red. CFDA, a nonfluorescent membrane-permeable probe, is converted by intracellular esterases to a polar fluorescent product (CF) that is retained by cells with intact plasma membranes.

A microscopic field containing aggregates of at least 20 cells was selected for analysis, and single cells, when present within fields, served as photobleached negative controls (which should not recover fluorescence). Single cells or small isolated groups of nonphotobleached cells were used as positive controls to monitor background photobleaching due to image scans and to monitor any increase in fluorescence that would indicate the presence of uncleaved dye. The epifluorescence microscope objective focused the laser beam for photobleaching. Following collection of the first image scan, a series of point bleaches reduced the dye photochemically in 1–3 selected cells to a level sufficient to observe fluorescence recovery without causing visible cell damage at the light microscopic level. After bleaching,

redistribution of fluorescence was measured from sequential scans beginning immediately postbleach, and then at 1-min intervals. Data from the fluorescence recovery experiments were collected from at least 30 cells tested from 3 culture dishes per treatment group.

Estimation of a rate constant (k) for fluorescence recovery was performed by fitting the percent fluorescence intensity at a given time, $F(t)$, to the following equation:

$$F(t) = F_{eq}(1 - e^{-kt}) + F(0)$$

where F_{eq} represents the percent fluorescence recovery of the bleached cell at equilibrium and $F(0)$ is the percent fluorescence intensity immediately following photobleaching. A curve fitting regression analysis allowed extrapolation over time to determine the rate constant, k and F_{eq} (Barhoumi et al., 1993). While the value F_{eq} is a function of the number of contacting cells and the initial level of photobleaching, k is characteristic of the cell line studied (Burghardt et al., 1994). Variability in F_{eq} was limited by selecting groups of at least 20 cells for analysis.

Experiments designed to examine the direct effects of 8-bromo-cAMP on junctional communication employed two approaches. One approach involved two photobleaching and fluorescence recovery experiments performed on the same cells to determine the rate constant before and directly after addition of 8-bromo-cAMP. When examining the effect of 8-bromo-cAMP in the presence of octanol, cells were uncoupled with 1.0 mM octanol for 1 min and the second experiment was then performed directly after addition of 1.0 mM 8-bromo-cAMP to monitor the dynamics of restoration of junctional communication. Alternatively, fluorescence recovery experiments were performed on different control, 8-bromo-cAMP, and octanol plus 8-bromo-cAMP treated cells which gave identical results.

Similar experiments were performed on cells treated for 2 hr with 10 μM monensin prior to 8-bromo-cAMP treatment. Two fluorescence recovery experiments were performed on the same cells to determine the rate constant before and directly after addition of 8-bromo-cAMP, or on different monensin, and monensin plus 8-bromo-cAMP treated cells.

Indirect Immunofluorescence and Image Analysis

Clone 9 and SIGC (passage 24 and 321) were selected for Cx43 immunolocalization and image analysis. Experimental treatments of cultures in LabTek slides were conducted including (in mM): control (no treatment); 1.0 8-bromo-cAMP for 5 min; 1.0 8-bromo-cAMP for 15 min; 1.0 octanol for 1 min; 1.0 octanol for 1 min followed by 1.0 8-bromo-cAMP for 5 min; 1.0 octanol for 1 min followed by 1.0 8-bromo-cAMP for 15 min. Additional immunofluorescence analyses in Clone 9 and SIGC were performed following incubation of cells with 1.0 mM 8-bromo-cAMP or 10 μM monensin for intervals up to 24 hr. Cells treated with monensin for 2 hr were also exposed to 1.0 mM 8-bromo-cAMP for 5 or 15 min. Immediately following treatments, LabTek slides were processed for indirect immunofluorescence analysis of Cx43. Slides were briefly washed in 0.02 M PBS and cells were then fixed with –20°C methanol for 10 min. After fixation, slides were air dried and subsequently rehydrated with 0.02 M PBS with 0.3% Tween 20 (PBS/Tween). Anti-connexin43 mouse monoclonal antibody (1 mg/ml stock in 50% glycerol, 20 mM sodium phosphate, 150 mM NaCl, 3 mM NaN_3, pH 7.5) was added to each treatment at a dilution of 1:200 and allowed to incubate overnight at 4°C. Following two 10-min washes in PBS/Tween, a biotinylated secondary antibody (sheep anti-mouse IgG, diluted 1:200) was added for 1 hr at 37°C. Slides were then washed in PBS/Tween and incubated for 1 hr at room temperature with fluorescein-streptavidin. Following additional washes, slides were subsequently mounted in glycerol containing p-phenylenediamine. Immunofluorescence controls consisted of the secondary and fluorochrome conjugates without primary antibody.

Slides were scanned with the ACAS 570 after selecting fields of contacting cells to collect average fluorescence intensity/area as well as quantify the number of immunoreactive punctate cell surface fluorescent "plaques"/area using 180 × 180 μm areas occupied by cell aggregates. Background fluorescence adjustment was the same for all treatments. Fluorescent plaques at the cell surface (identified by obtaining phase contrast images prior to digital image acquisition) were quantified. Cx43 plaques were identified if they were less than 10 μm^2 in area. Images were collected from 4 areas/wells and from 4 different wells/treatment. Conventional light microscopy was used to photograph phase contrast and fluorescent images of cells from each treatment group with a Zeiss Photomicroscope III (Zeiss, Thornwood, NY). Results from immunofluorescence experiments were verified from four separate experiments performed on the Clone 9 and SIGC (passage 24 and 321).

Table 1. Dynamic changes in cell-cell communication rate (k) detected during the 4 min of 1.0-mM 8-bromo-cAMP treatment

Cell Type	k (min^{-1})[a]	
	Control	8-bromo-cAMP
Clone 9	0.84 ± 0.04	0.95 ± 0.10
SIGC (passage 24)	0.31 ± 0.02	0.36 ± 0.06
myometrial	0.19 ± 0.01	0.33 ± 0.07[b]
UEE	0.18 ± 0.02	0.31 ± 0.01[b]
SIGC (passage 321)	0.10 ± 0.01	0.21 ± 0.01[b]
CVEC	0.07 ± 0.01	0.28 ± 0.04[b]

[a] Values are mean k ± SEM determined from at least 30 cells from 3 different culture dishes.
[b] Significantly different at $P < 0.05$.

Statistical Analysis

Data were analyzed using the General Linear Models ANOVA procedure of SAS/STAT (1985) to determine the significance of differences ($P < 0.05$) in fluorescence recovery, rate constants, or number of Cx43 immunoreactive plaques. Duncan's New Multiple Range test was utilized for between-treatment group comparisons.

Results

Physiological Assessment of Cell-Cell Communication

Direct Effects of Cyclic AMP

The effects of 8-bromo-cAMP on gap junction-mediated intercellular communication were analyzed in several cell types which were found to express Cx43. Determination of the rate constant (k) for fluorescence recovery following photobleaching in each cell type revealed a considerable range in communication rate among the different cell types (Table 1, Control column). The kinetics of fluorescence recovery in Clone 9 and a high passage number (p321) SIGC were compared (Fig. 1).

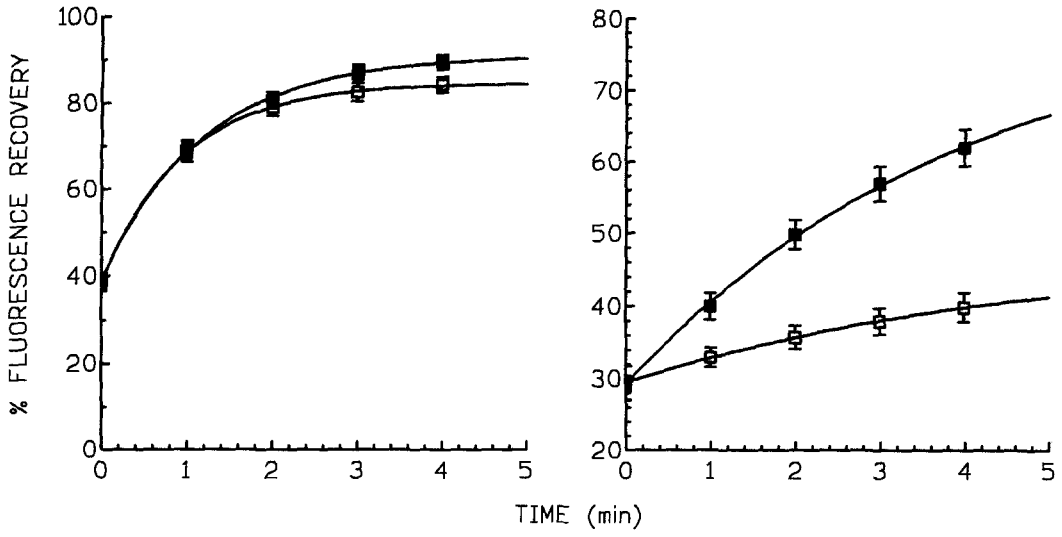

Fig. 1. Analysis of the pattern of fluorescence recovery following photobleaching in the same cell before (open squares) and immediately after addition of 1.0 mM 8-bromo-cAMP (solid squares) (n = 30). Even though Clone 9 cells (left panel) normally exhibit high levels of junctional communication, a slight increase in fluorescence recovery was detected within 2 min of addition of 8-bromo-cAMP and reached significance at 3 and 4 min postbleaching ($P < 0.05$). SIGC, passage 321 (right panel) which are characterized by uniformly low levels of junctional communication nevertheless show significant increases in fluorescence recovery within 1 min after addition of 8-bromo-cAMP.

The control Clone 9 and SIGC passage 321 are characterized, respectively, by uniform high and low levels of cell-cell communication. Also shown is the fluorescence recovery in the same individual cells immediately after addition of 1.0 mM 8-bromo-cAMP. Although the kinetics of fluorescence recovery shown are from the same cells prior to, and immediately after addition of 8-bromo-cAMP, identical results were obtained when photobleaching and fluorescence recovery analysis was performed on separate control and treated cells. Moreover, control experiments in which five sequential photobleaching and fluorescence recovery analyses were performed on the same cells revealed no significant change in rate constant as previously reported (Stein, Boonstra & Burghardt, 1992; Dookwah et al., 1992).

Although Clone 9 cells exhibit very high levels of junctional communication, a small but significant increase in fluorescence recovery was detected 2 min after addition of 8-bromo-cAMP (Fig. 1). SIGC passage 321 exhibit low levels of communication compared to Clone 9 cells and significant increases in fluorescence recovery in these cells were detected within 1 min of 8-bromo-cAMP treatment. Dynamic changes in communication rate (k) in control and 8-bromo-cAMP-treated cell lines are summarized in Table 1. A rapid 8-bromo-cAMP-induced increase in junctional communication relative to controls was more obvious in cells which exhibit lower initial levels of communication (e.g., myometrial, UEE, SIGC passage 321, or CVEC), i.e., increases in cell-cell communication were proportionately greater in cells with low initial levels of communication. In the case of cells with high initial levels of communication, changes in rate constants were more difficult to detect over the short 4-min time course while small but significant changes in fluorescence recovery could be detected within 2–3 min of 8-bromo-cAMP treatment. Significant increases in rate constants were also observed in cells maintained in 1.0 mM 8-bromo-cAMP for up to 24 hr (*data not shown*).

Effects of Cyclic AMP after Uncoupling by Octanol

In cell types where direct effects of 8-bromo-cAMP were small within the 4-min timeframe of the fluorescence recovery experiments (e.g., Clone 9 and SIGC, passage 24), analysis of the effect of 8-bromo-cAMP was performed after suppression of junctional communication with 1.0 mM octanol. The procedure, designed to reset the level of cell-cell communication to a zero base, required an understanding of the kinetics of octanol-induced elimination of communication. Experiments performed in each cell line to determine minimum intervals in which changes in junctional communication may be detected by analysis of fluorescence recovery indicated that image scans of individual cells could be obtained every 12 sec. Selected scans from a typical single kinetic analysis performed at 12-sec intervals in an untreated Clone 9 cell are shown in Fig. 2. The kinetics of fluorescence recovery in that cell is shown in Fig. 3*a*. A second photobleaching and fluorescence recovery experiment was then performed on the same cell directly after addition of 1.0 mM octanol (Fig. 3*a*). In all cell lines, elimination of junctional communication was abrupt and complete within 1 min. Cells remained un-

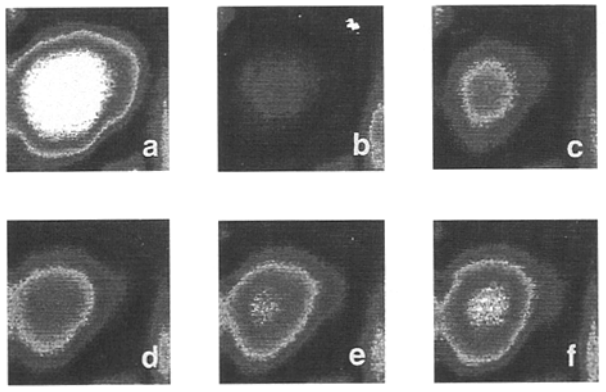

Fig. 2. One of numerous fluorescence recovery following photobleaching experiments performed in each of the cell lines to determine how quickly changes in junctional communication may be monitored by this technique. Image scans were recorded each 12 sec. Shown here are the prebleach scan (*a*), the immediate postbleach scan (*b*), and every other image scan (i.e., 24-sec intervals) of a Clone 9 cell following photobleaching (*c–f*). The rapid fluorescence recovery in Clone 9 cells is illustrated in panel *c*, recorded 24 sec after the first postbleach scan. Images were recorded on black and white film from pseudocolor intensity images. Width of each field is 35 μm.

coupled during 30 min of octanol exposure. Directly after removal of octanol, communication was partially restored within minutes (Fig. 3*b*). By 6 min after octanol washout, the rate constant was restored to control values (*data not shown*).

Because elimination of junctional communication could be induced in less than 1 min, two sequential photobleaching and fluorescence recovery experiments on the same cells were performed in each cell line to examine the effects of 8-bromo-cAMP after 1 min of octanol treatment. Figure 4 illustrates one of many fluorescence recovery analyses performed on an untreated Clone 9 cell. The control cell showed complete fluorescence recovery within approximately 5 min. Addition of 8-bromo-cAMP after uncoupling with octanol caused a return of fluorescence into the photobleached cell within 2–5 minutes and reaching equilibrium within 20–30 min. These data indicated that addition of 8-bromo-cAMP to octanol-uncoupled cells transiently restored junctional communication ($k = 0.1$ min^{-1}) to approximately 12% of the control rate constant ($k = 0.82$ min^{-1}). However, when fluorescence recovery experiments were performed 30 min later, the cells were again uncoupled (*data not shown*).

Effects of Cyclic AMP on Monensin-treated Cells

The effect of monensin (10 μM for 2 hr), which inhibits translocation of Cx43 (Purnam et al., 1993), was examined in fluorescent recovery experiments prior to and following exposure of Clone 9 and passage 24 SIGC to 8-bromo-cAMP (Table 2). Rate constants in monensin-treated cells were significantly lower than control and remained unchanged following treatment with 1.0 mM 8-bromo-cAMP.

Analysis of Immunoreactive Cx43 in Clone 9 and SIGC Cells

Initial indirect immunofluorescence screening of Cx43 in cells treated with 8-bromo-cAMP suggested that there was a perceptible increase in the amount of cell surface label by 15 min. Clone 9 and SIGC were selected for further quantitative analysis of patterns of Cx43 immunostaining because the cells exhibit a uniform and flattened morphology which facilitated rapid digital image acquisition and analysis. Figure 5 shows composite phase contrast and conventional fluorescence images of Clone 9 and SIGC (passage 321) cultures illustrating the difference in Cx43 distribution in both cell types. Digital images of these cells immunostained with Cx43 antibody were recorded following treatments with octanol and/or 8-bromo-cAMP for different intervals. Results obtained with Clone 9 cells are shown in Fig. 6 although similar results were obtained with SIGC.

Quantitative analysis of these Cx43 fluorescence patterns and intensities revealed significant differences in the number of punctate immunoreactive plaques among treatment groups (Fig. 7). When direct effects of 8-bromo-cAMP on Cx43 in Clone 9 cells were analyzed (Fig. 7*a*) an increase in the number of cell surface plaques was detected within 5 min following treatment and reached significance at 15 min. This increase was maintained for up to 24 hr of treatment with 8-bromo-cAMP. In addition, all of the plaques were located at regions of cell-cell contacts. In cells uncoupled with octanol for 1 min, the number of plaques was not significantly different from controls (Fig. 7*b*), although there appeared to be more cytoplasmic staining near the plasma membrane. Addition of 8-bromo-cAMP to these uncoupled cells caused a significant increase in cell surface Cx43 staining starting at 5 min. Similar results were obtained with SIGC (passages 24 and 321).

Immunostaining of Cx43 in Clone 9 and SIGC in monensin-treated cells revealed the replacement of the typical light perinuclear staining profile by a more intense vesicular pattern (Fig. 8). This was accompanied by detectable reduction of surface plaques by 2 hr. After 2 hr of monensin treatment, addition of 8-bromo-cAMP resulted in no change in the number of gap junctions at the cell surface by 15 min (*data not shown*).

Discussion

The objective of this study was to examine the rapid effects of cyclic AMP on gap junctional permeability in

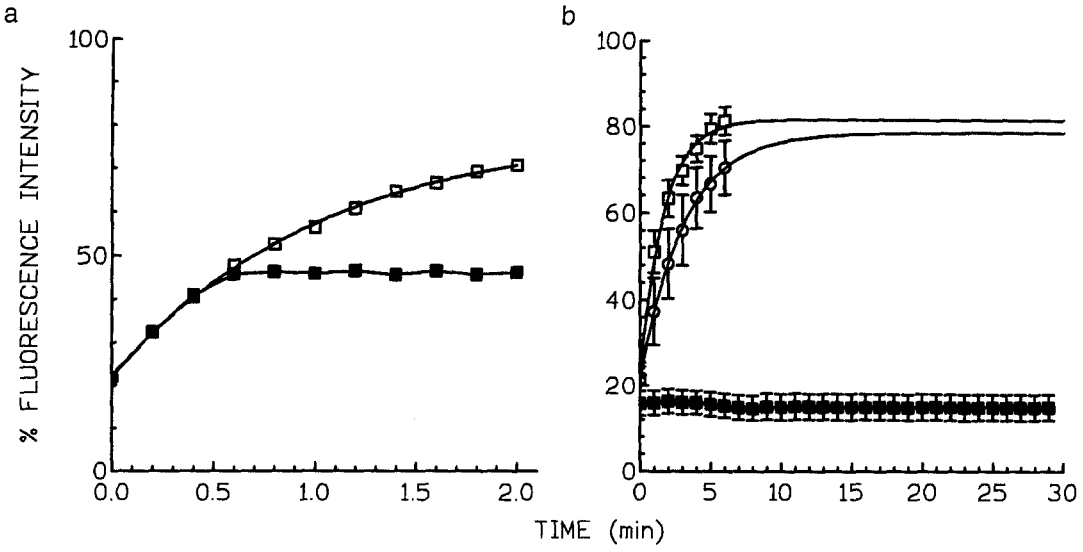

Fig. 3. Analysis of junctional communication in control and octanol-treated cells. (*a*) Left panel. Evaluation of the kinetics of octanol-induced elimination of cell-cell communication: A typical single cell fluorescence recovery analysis which was performed at 12-sec intervals in an untreated Clone 9 culture (open squares) and in the same cell directly after addition of 1.0 mM octanol, i.e., $t = 0$ (solid squares). Suppression of cell-cell communication was abrupt and always complete in less than 1 min; some cells were uncoupled within 12 secs. (*b*) Right panel. Evaluation of the duration of octanol-induced uncoupling and restoration of junctional conductance upon octanol washout: Sequential fluorescence recovery analyses performed on Clone 9 cells ($n = 6$). The rate constant for cell-cell communication in control cells (open squares) was 0.6 ± 0.06 min^{-1}. Uncoupling of cells with 1.0 mM octanol (solid squares) for 1 min prior to fluorescence recovery analysis resulted in uniform suppression of fluorescence recovery over 30 min. Following replacement of octanol with fresh culture medium, another experiment was performed (open circles) and the rate constant was restored to 0.4 ± 0.02 min^{-1}. Complete restoration of the control rate constant was obtained following a fourth photobleaching and fluorescence recovery experiment on the same cells, i.e., within 6 min of octanol washout (*data not shown*).

a variety of cells which express Cx43 (including rat myometrial and equine UEE primary cultures, and immortalized rat granulosa cells [SIGC], bovine coronary venular endothelial cells [CVEC] and rat liver Clone 9 cells). Rate constants for junctional communication obtained from fluorescence recovery after photobleaching experiments have been shown to be a useful parameter for evaluating changes in cell-cell communication in cultured cells (e.g., Dookwah et al., 1992; Stein et al., 1992; Barhoumi et al., 1993; Stein et al., 1993; Pluciennik, Joffre & Délèze, 1994). An added utility of the fluorescence recovery analysis is the ability to perform sequential experiments on the same cells. By analyzing dynamic changes in rate constants, (i.e., a change in the rate of fluorescent probe diffusion during an experimental treatment), it was found that junctional communication can be rapidly enhanced by administration of 8-bromo-cAMP in several cell types which express Cx43. In some cell types, dynamic changes in cell-cell communication rates were underestimated (e.g., Clone 9 and SIGC, passage 24) because significant increases in fluorescence recovery were not detected until 2–3 min after addition of 8-bromo-cAMP in contrast to other cells (myometrial, UEE, SIGC, passage 321, and CVEC) where increases were detected within 1 min. This may be the basis for the recent observation (Godwin et al., 1993) that injection of active cAMP-dependent protein kinase into well coupled ovarian granulosa cells had no effect unless cells were first injected with alkaline phosphatase.

The increase in junctional communication caused by 8-bromo-cAMP was not transient and was detected in all cell types examined for up to 24 hr. Quantitative analysis of Cx43 indicated that enhanced communication is correlated with an increase in the immunoreactive Cx43 at the cell surface. With the possible exception of the CVEC line, Cx43 is the predominant connexin in each of these cell types. Clone 9 and myometrial cells have little immunoreactive Cx26 (Saez et al., 1993; Winterhager et al., 1991) and equine UEE has no detectable Cx26, however, the contribution of this connexin (which is not a phosphoprotein) to the fluorescence recovery rate constant in these cells is thought to be minor. Coexpression and colocalization of Cx43 and Cx40 (a phosphoprotein) has recently been observed in endothelium of resistance vessels in rat (Little, Beyer & Duling, 1995) and we cannot exclude the possibility that effects of 8-bromo-cAMP on this cell type involve both connexins.

Another method for evaluating the effect of 8-bromo-cAMP on cell-cell communication was to suppress communication by an uncoupling agent before treatment with cAMP. The effects of octanol (an agent frequently used to close gap junction channels) are rapid and reversible (Burt, 1991). The strategy employed was

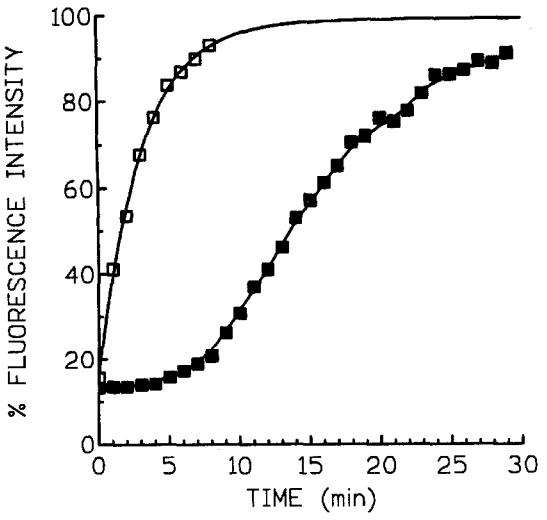

Fig. 4. An example of a sequential fluorescence recovery after photobleaching experiment performed on the same Clone 9 cell. The first analysis was performed on an untreated control Clone 9 cell (open squares) in an aggregate of at least 20 cells. Cells were then uncoupled with 1.0 mM octanol for 1 min and a second experiment was initiated immediately upon addition of 1.0 mM 8-bromo-cAMP. In this particular experiment, return of fluorescence into photobleached control cells (open squares) was complete by 10 min with a rate constant (k) of 0.82 min^{-1}. A slower fluorescence recovery began 5 min after addition of 8-bromo-cAMP in the uncoupled cell (solid squares) and by 10 min, the recovery of fluorescence reaches equilibrium but with a slower communication rate, k, of 0.10 min^{-1}.

Table 2. Effect of 10 μM monensin on cell-cell communication rate (k) before and after 1.0-mM 8-bromo-cAMP treatment

	k (min^{-1})[a]		
Cell type	Control	Monensin	8-bromo-cAMP
Clone 9	0.78 ± 0.06^b	0.37 ± 0.04^c	0.33 ± 0.07^c
SIGC (passage 24)	0.33 ± 0.02^b	0.19 ± 0.02^c	0.21 ± 0.02^c

[a] Values are mean $k \pm$ SEM determined from at least 30 cells from 3 different culture dishes.

[b,c] For each cell line, values identified by the same letter are not significantly different ($P < 0.05$).

to reset junctional communication to a zero base which facilitated detection of any increase in communication. All cells included in this study were uncoupled within 1 min of octanol exposure and cells remained uncoupled during at least 30 min of octanol exposure. However, rapid partial restoration of communication was achieved in cells treated with 8-bromo-cAMP following octanol exposure for 1 min, even though octanol remained in the medium during the cAMP treatment.

Neither the mechanism by which octanol uncouples cell junctions nor the mechanism by which 8-bromo-cAMP transiently restores junctional communication in uncoupled cells are yet understood. However, this fluorescence recovery after photobleaching strategy is particularly useful for monitoring changes in intercellular communication while they are happening. Octanol is thought to interact with lipids surrounding channel proteins and hydrogen bond with the connexins resulting in changes in membrane fluidity and perturbation of tertiary structure (Burt, 1991) without changing $[Ca^{2+}]_i$, pH, cAMP, or nonjunctional membrane conductance (Chanson et al., 1989; Meda et al., 1991). Restoration of intercellular communication by 8-bromo-cAMP following octanol uncoupling does not appear to be mediated by the restoration of membrane fluidity to original values (Burghardt, Barhoumi & Dookwah, 1993). Effects of cAMP on octanol-treated cells has been previously described where suppression of spontaneous uncoupling of cells was observed in cells exposed to 0.1 mM dibutyryl cAMP and 5 mM ATP (Somogyi & Kolb, 1988). However, a significant cAMP-mediated increase in junctional conductance was not detected using the double whole cell patch clamp technique.

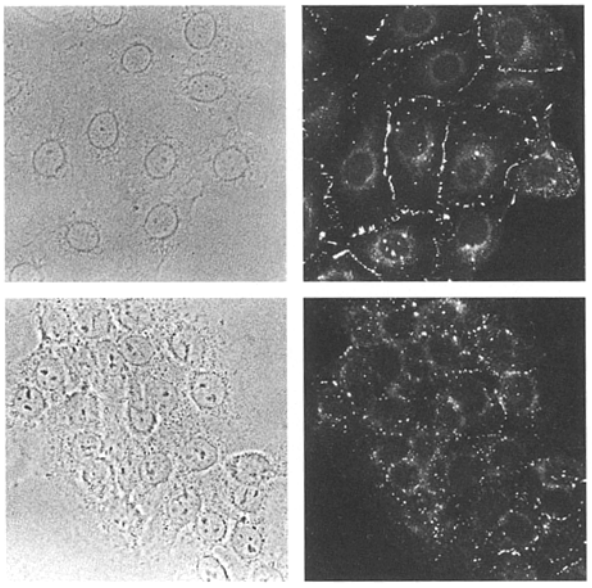

Fig. 5. Phase contrast and conventional fluorescence microscopy of Clone 9 (upper panel) and SIGC, passage 321 (lower panel) showing Cx43 immunostaining along cell-cell contacts and light staining in perinuclear regions of the cell. Note that in cells located at the periphery of aggregates, greater cytoplasmic and cell surface immunoreactivity can be seen. Width of each field is 200 μm.

In the present study, octanol-uncoupled cells retained immunoreactive Cx43 at or near the cell surface and exhibited increased surface Cx43 upon addition of 8-bromo-cAMP. This correlated with the partial restoration of junctional conductance suggesting that Cx43 trafficking and/or assembly may add new functional gap junctions to the cell surface. Pretreatment of cells with octanol seemed to accelerate the transfer of immunoreactive Cx43 to the cell surface because the number of

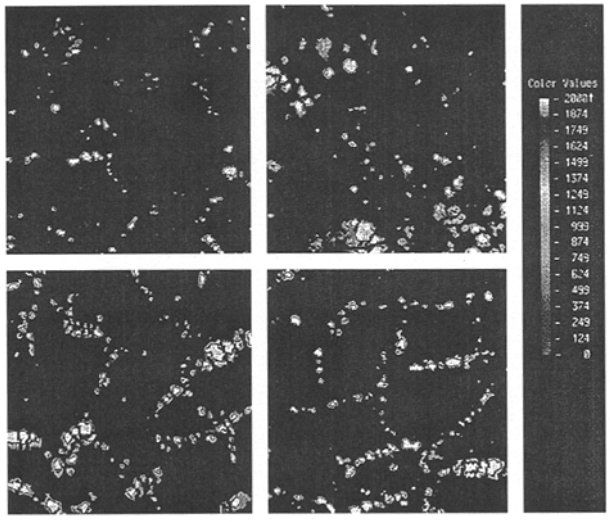

Fig. 6. An illustration of digital fluorescence images of Cx43 immunostaining in Clone 9 cells obtained with the ACAS 570. Treatment groups shown are control (upper left), 1-min octanol (upper right), 1-min octanol followed by 5-min 8-bromo-cAMP (lower left), and 15-min 8-bromo-cAMP (lower right). Cells treated for 15 min with 8-bromo-cAMP were similar to the 1-min octanol, 5-min 8-bromo-cAMP treatment; both were significantly different from control. Only cells treated for 1 min with octanol exhibited less uniform staining with some aggregates located below the plasma membrane. Pseudocolor intensity images were reproduced in black and white. Width of each field is 100 μm.

plaques seen within 5 min of 8-bromo-cAMP following octanol uncoupling of cells was comparable to that seen after 15 min of 8-bromo-cAMP alone. However, as noted above, the rate constant was about 12% of the control rate constant and the cells were again uncoupled after 30 min. These data suggest that as new channels are assembled at the cell surface, they soon become uncoupled by the octanol.

Purnam et al. (1993) have shown that the metabolic inhibitor, monensin, blocks the processing of Cx43 resulting in connexin accumulation in the Golgi apparatus. The failure of monensin-treated Clone 9 and SIGC to exhibit 8-bromo-cAMP-induced increase in junctional communication or an increase in Cx43 immunoreactive surface plaques is also consistent with the interpretation that 8-bromo-cAMP can accelerate Cx43 trafficking and or assembly when the connexin processing pathway is intact.

Early studies by Flagg-Newton, Dahl and Loewenstein (1981) suggested the involvement of cAMP in the increased cell-cell communication and increased gap junctional plaques recognized by freeze fracture analysis. They speculated that upregulation of cell-to-cell channels was due to cAMP-dependent phosphorylation (*reviewed,* Loewenstein, 1985). Given that there are at least 12 members of the gap junction multigene family (Willecke et al., 1991) and that not all connexin family members are phosphoproteins (Saez et al., 1990), the involvement of cAMP-dependent protein kinase in the modulation of gap junction permeability among gap junction family members is not yet understood. Cx43 (Pressler & Hathaway, 1987) and Cx32 (Saez et al., 1990) have been reported to be substrates for cAMP-dependent protein kinase. It appears that cell-cell communication in cells which express Cx43 may be controlled at multiple levels by components of the cAMP-dependent protein kinase regulatory cascade (Stagg & Fletcher, 1990). The development of communication involves a complex sequence of events including connexin synthesis (transcription and translation), trafficking to the plasma membrane, connexon assembly, aggregation of hemichannels in apposed membranes, and gating. Each element of this sequence, which could potentially affect the degree to which cells share small regulatory and informational molecules, may be regulated by phosphorylation events involving the cAMP-mediated cascades.

A number of agents including estrogens (Petrocelli & Lye, 1993; Yu, Dahl & Werner, 1994), retinoic acid (Rogers et al., 1990), and cAMP (Mehta et al., 1992; Schiller et al., 1992) can lead to elevated mRNA levels, rates of transcription, and functional expression of Cx43 cell-cell channels. These effects require several hours and it is unlikely that the responses observed in the present studies involve altered transcriptional or translational events.

Assembly of connexins into functional structures may be regulated by phosphorylation (Musil & Goodenough, 1993). The time scale involved in the assembly of functional channels from precursors is not known although this is probably a rapid process. Rapid changes in junctional permeability observed in the present study are consistent with the possibility that activation of cAMP-dependent protein kinase and subsequent phosphorylation of either gap junction protein or accessory factors may lead to an increase in communication. Rapid modulation of gap junctions by cAMP has previously been observed in cardiac cells which express Cx43 and there is experimental evidence for the role of cAMP-dependent protein kinase in this process in vivo (*reviewed,* De Mello, 1994). A relationship between rapid upregulation of Cx43-mediated junctional communication and changes in the phosphorylation state of Cx43 has not yet been established. Berthoud et al. (1992) found no evidence of altered electrophoretic mobility of Cx43 in MDCK cells treated with 8-bromo-cAMP whereas Granot and Dekel (1994) reported that forskolin (a potent activator of adenylyl cyclase) caused an increase in the relative intensity of phosphorylated Cx43 species in rat granulosa cells which was maximal at 30 min. Studies in progress employing the cell types used in this investigation are designed to evaluate differences in phosphorylation of Cx43 species caused by activation of cAMP-dependent protein kinase.

These studies add to a growing list of investigations

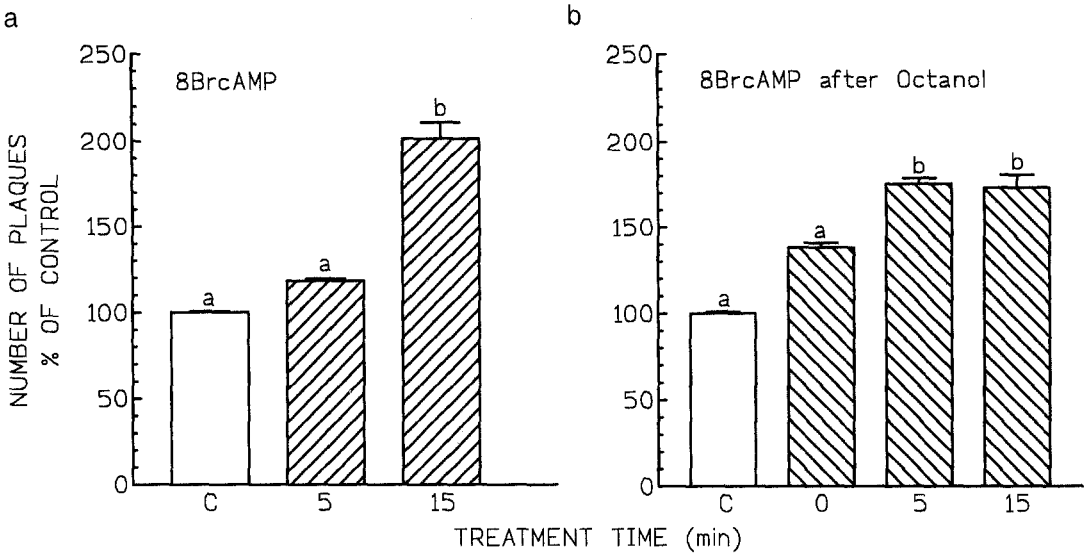

Fig. 7. A summary of the quantitative analysis of Cx43 plaques obtained from digital images of Clone 9 cells following treatments. Fluorescent plaques measuring up to 10 μm^2 in area were counted from regions of contact between cells. Significant differences in the number of punctate immunoreactive plaques among treatment groups were observed. (*a*) Left panel. Results obtained in untreated control (C) and 8-bromo-cAMP treatment for 5 or 15 min. An increase in the number of plaques was detected within 5 min following 8-bromo-cAMP treatment. By 15 min there was approximately twice the number of plaques seen in untreated cells. (*b*) Results obtained in untreated cells (C) and in octanol-uncoupled cells treated with 8-bromo-cAMP for 0, 5, or 15 min. In cells uncoupled with octanol for 1 min, the 5 and 15 min 8-bromo-cAMP treatments significantly increased the number of plaques relative to untreated (C) and 1 min-octanol (0). Values shown were obtained from the mean number of plaques counted in 16 areas (4 areas/dish, 4 dishes per treatment). Values identified by the same letter are not significantly different ($P < 0.05$).

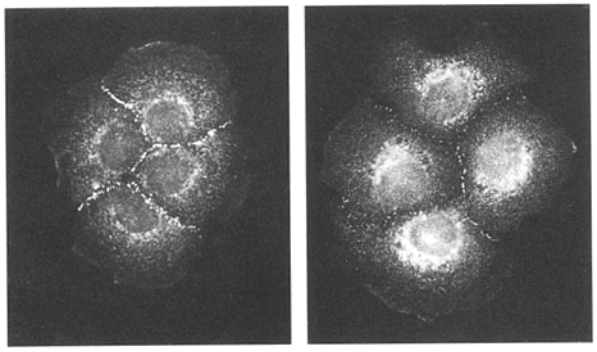

Fig. 8. Fluorescence microscopy of Clone 9 cells treated for 1 and 2 hr with 10-μM monensin. Compared to control cells (Fig. 5) monensin treatment for 1 hr (left panel) and 2 hr (right panel) caused increased vesicular Cx43 staining in the cytoplasm and loss of punctate surface staining. The reduced punctate membrane Cx43 staining correlated with a reduction in rate of fluorescence recovery (Table 2).

in which rapid upregulation of junctional communication can be induced by cAMP, activators of cAMP-dependent protein kinase, and catalytic subunits of this kinase in a variety of cell types (Godwin et al., 1993; Nnamani et al., 1994). This effect can be observed even in cells in which gap junction channels are uncoupled prior to cyclic AMP treatment. These data also indicate that an action of 8-bromo-cAMP was to cause increased trafficking and/or assembly of Cx43 into cell surface plaques.

We acknowledge the technical assistance of Richard Lewis and Meghan Abella. We thank Dr. Hugh Dookwah for contributions to the myometrial cell isolation protocol and Drs. Stephen H. Safe, Timothy D. Phillips, and Evelyn Tiffany-Castiglioni for helpful discussions. This work was funded by NIH (HD-26182, P42-ES04917, ES05871-01A1), the March of Dimes Birth Defects Foundation Basic Research grant #1-0796, and USDA 92-37203-7952.

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