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Selective permeability of different connexin channels to the second messenger cyclic AMP

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Received for publication, October 16, 2005, and in revised form, December 15, 2005

Published, JBC Papers in Press, December 22, 2005, DOI 10.1074/jbc.M511235200

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Gap junctions are intercellular conduits that are formed in vertebrates by connexin proteins and allow diffusion exchange of intracellular ions and small molecules. At least 20 different connexin genes in the human and mouse genome are cell-type specifically expressed with overlapping expression patterns. A possible explanation for this diversity could be different permeability of biologically important molecules, such as second messenger molecules. We have recently demonstrated that cyclic nucleotide-gated channels can be used to quantify gap junction-mediated diffusion of cyclic AMP. Using this method we have compared the relative permeability of gap junction channels composed of connexin 26, 32, 36, 43, 45, or 47 proteins toward the second messenger cAMP. Here we show that Cx32 permeates through the investigated connexin channels with up to 30-fold different efficacy. Our results suggest that intercellular cAMP signaling in different cell types can be affected by the connexin expression pattern.

Gap junction channels formed by docking of two hemichannels in the plasma membranes of contacting cells mediate the exchange of small molecules (<1 kDa) and ions between cells. Hemichannels are formed by six protein subunits called connexins (Cx). 3 Gap junction-mediated intercellular communication is thought to play a crucial role in maintenance of homeostasis, morphogenesis, cell differentiation and growth control in multicellular organisms (1).

To date, 20 mouse and 21 human cx genes have been tentatively identified (2). It has been shown that each cx gene is expressed in a distinct spatial and temporal pattern and that most cell types express more than one connexin protein. The diverse expression patterns of the different connexins suggest functional differences, including selective permeability for biologically relevant molecules such as second messengers. Selective permeability of different Cx channels to ions, fluorescent dyes, metabolites, and the second messenger inositol 1,4,5-trisphosphate (IP3) has been described (3–9). For instance, Niessen et al. (8) showed by microinjection of IP3 into monolayers of different HeLa transfectants that Cx32 channels were able to propagate IP3-induced Ca2+ waves 2.5 times better than Cx43 channels and 3–4 times better than Cx26 channels.

Cyclic AMP (cAMP) is a ubiquitous intracellular second messenger that affects cell physiology by directly interacting with effector molecules that include cAMP-dependent protein kinases, cyclic nucleotide-gated ion channels (CNG channels), and hyperpolarization activated channels. In turn, these effectors regulate diverse biological processes such as cardiac inotropy and chronotropy, glycolysis and lipolysis, vascular tone, neurotransmitter and hormone release as well as cell growth and differentiation (for review, see Refs. 10–12). The permeability of gap junction channels for cAMP was already demonstrated more than 30 years ago (13) and confirmed in several subsequent studies (14–18). However, a comparison of the CAMP permeability through different connexin channels has not been published so far.

Recently, we described a method (19) to monitor CAMP diffusion through gap junction channels. Here, we present a quantitative analysis of differential permeability to CAMP of six different gap junction channels composed of murine Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47 proteins. For this purpose, we established six HeLa cell lines, each of them stably expressing one type of Cx protein and mutant CNG ion channels (T537S rCNGα1), which were used as highly sensitive sensors for cAMP concentrations (19). With this method we show here that Cx43 channels are about 3 times more permeable to CAMP than Cx26 channels, 5–6 times more permeable than Cx32 or Cx45 channels, 8–9 times more permeable than Cx47 channels, and more than 30 times more permeable than Cx36 channels.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—For transfection, DNA coding for the T537S-mutated form of the rat olfactory CNG channel α1-subunit (T537S rCNGα1) (20) was inserted into the transfection vector pcDNA3.1/Zeo(+). HeLa wild type cells and the connexin expression cell lines HeLa-Cx26G, -Cx32H, -Cx36K5, -Cx43K7, -Cx45A, and -Cx47K21 (3, 21–24) were stably transfected by lipofection (Tfx®-50 reagent, Promega, Madison, WI) following the protocol provided by the manufacturer. Forty-eight hours after transfection, 100 μg/ml zeocin were added to the medium. Clones were picked after 3–4 weeks and grown under selective conditions. The cells were cultured as previously described (19).

For experiments, cells were plated at low density on glass coverslips placed in a 35-mm plastic Petri dish. Cells were grown for 24–36 h and used for experiments at a confluence level of 10–20%.

For cocultures, HeLa-Cx45/CNG transfectants were plated on a 35-mm plastic dish and grown to confluence. The cells were then incubated with isotonic glucose solution containing 5 μg/ml DiI (Molecular Probes, Eugene, OR) for 15 min at 37°C. After two washes with phosphate-buffered saline (PBS), the DiI-labeled HeLa-Cx/CNG double transfectants and unstained HeLa-Cx45-transfected cells were
trypsinized, centrifuged, and resuspended. Dil-labeled cells were mixed with unlabeled cells at a ratio of 1:1, plated onto glass coverslips, and grown for 48 h.

**Immunofluorescence Analyses**—Cells grown for 24 h on coverslips were fixed in 100% ethanol (−20 °C) for 5 min, blocked with 4% bovine serum albumin (BSA, PAA Laboratories GmbH, Linz, Austria), PBS and incubated with antibodies diluted in 0.4% BSA in PBS overnight at 4 °C. Afterward, cells were washed with 0.4% bovine serum albumin in PBS and incubated with Alexa-conjugated antibodies directed to primary antibodies for 1 h at room temperature. Cells were stained with the following dilutions of primary antibodies: rabbit polyclonal anti-Cx43 (1:2000) (24), polyclonal rabbit anti-Cx26 (1:500; Zytomed, Berlin, Germany), polyclonal rabbit anti-Cx32 (1:250; Zytomed), monoclonal mouse anti-Cx45 (1:100; Chemicon), polyclonal rabbit anti-Cx36 (1:100; Zytomed), and polyclonal guinea pig anti-Cx47 (1:500) (25). Primary antibodies were detected with Alexa488-conjugated goat anti-rabbit immunoglobulin (1:2000; MoBiTech, Goettingen, Germany), Alexa488 goat anti-mouse (1:2000; MoBiTech), and Alexa594 goat anti-guinea pig (1:3000; MoBiTech), respectively. Nuclear staining was performed by 15 min of incubation with 0.2 µg/mL Hoechst 33258 fluorescent dye in PBS (Sigma). Cells were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). Antibody-stained cells were analyzed using the photomicroscope Axiophot (Zeiss, Jena, Germany).

**Fluorometric Measurements and Flash Photolysis**—If not stated otherwise, cells were loaded with 8 µM Fluo-4FF (Molecular Probes), 200 µM cyclic AMP, P1-2-nitrophenyl)ethyl ester (NPE-caged cAMP, Calbiochem), and 0.025% PLURONIC F127 (Molecular Probes) in HEPES-buffered saline (HBS) containing 140 mM NaCl, 5 mM KCl, 3 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, and 10 mM glucose, pH 7.4 (NaOH) for 45 min at 37 °C. After loading, cells were rinsed with HBS without MgCl2 (HBS−), the coverslip was transferred to the experimental chamber, filled with 500 µL of HBS− containing 200 µM caged cAMP, and mounted onto an inverse epifluorescence microscope (Axiostar 100, Zeiss Oberkochen, Germany). Fluorescence measurements were performed using a commercial imaging system (TILL Photonics, Gräfelfing, Germany) consisting of a monochromator (Polychrom 1) and a peltier-cooled 12 bit charged coupled device (CCD) camera connected to a personal computer equipped with the calcium imaging software Fucal (Version 5.12C) (26). The monochromatic light was coupled via an epifluorescence condenser (dual port) to the microscope. All CCD images were background-corrected by subtraction of a dark picture. For photolysis experiments, a high pressure mercury lamp (Osrnam HBO 100W/2) equipped with a shutter (LS6, uniblitz, Rochester, NY) and a short pass filter (SP410) was used. The UV light from the mercury lamp was filtered by a dichroic mirror (Fura) and reflected into the objective by a second dichroic mirror (fluorescein isothiocyanate). For [Ca2+]i, measurements, Fluo-4FF fluorescence was monitored at 470 nm excitation wavelength. In most experiments, 20 frames were recorded at a frequency of 1 Hz, with the first frame preceding the UV flash. The average pixel fluorescence within regions of interest of about 10 × 10 µm was used for further analyses.

To increase the sensitivity of the experiments performed on cocultures, Fluo-4 (Ca2+ binding constant, 345 nM; Molecular Probes) was used instead of the Fluo-4FF (Ca2+ binding constant, 9.7 µM). Additionally, a more sensitive CCD camera (Sensicam QE, PCO Kelheim, Germany) was used during these experiments. The sensitivity was further increased by combining an appropriate operation mode (analog gain, on; low light mode, on) with the 2 × 2 on chip binning option.

**Electrical Recordings**—Pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter × 0.86 mm inner diameter, Harvard Apparatus LTD, Edenbridge, UK) with a horizontal pipette puller (Sutter Instruments Inc., Eugene, OR) and filled with 140 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, 10 mM Hepes, pH 7.2 (KOH). The resistance of patch pipettes was measured to be in the range of 2–4 megaohms. Pipettes were lowered onto the cells by a motor-driven micromanipulator (SM1, Luigs & Neumann, Ratingen, Germany). All experiments were performed at room temperature (−22 °C).

Double whole-cell patch clamp measurements were performed on cell pairs bathed in HBS. Two patch pipettes were connected to two synchronized, single-electrode voltage clamp amplifiers (SEC-05LX, NPI Electronic, Tamm, Germany). These amplifiers switch between voltage measurement and current injection, thereby avoiding artifacts by the series resistance (27). Experiments were performed with a switching frequency of 35 kHz. The test pulses were generated, and currents were recorded on a computer equipped with a 12 bit A/D and D/A converter board by using the program Cell Work (NPI Electronic).

After achievement of the whole-cell patch clamp configuration in both cells of a pair, the cells were clamped to a common holding potential (Vj = Vh = −60 mV). Transjunctional voltages Vj were applied by changing the membrane potential in one cell and keeping the other constant (Vj). The resulting junctional current Ij was observed as a change in current in the unstepped cell. Junctional conductance gj was determined from Ij/Vj. The number of channels per cell pair was calculated by dividing the total conductance between the two investigated cells by the unitary conductance of the corresponding Cx channel (Cj = Cj = 135 pS, Cj = 55 pS, Cj = 15 pS, Cj = 115 pS, Cj = 32 pS, or Cj = 55 pS) (22, 23, 28–30).

Cyclic AMP-induced currents through CNG channels were measured on single cells in the whole-cell configuration. Caged cAMP was diluted to a final concentration of 200 µM in the pipette solution. After achieving whole-cell configuration, the cell interior was equilibrated for at least 5 min with the pipette solution containing the caged compound. Membrane voltage was held at −60 mV. The extracellular solution contained 120 mM NaCl, 3 mM KCl, 10 mM EGTA, 10 mM glucose, 10 mM Hepes, pH 7.4 (NaOH). The caged component was photocleaved by UV light pulses of 4- or 100-ms duration, and the resulting inward currents were recorded.

**RESULTS**

**Characterization of the Double-transfected HeLa Cells**

Immunofluorescence analyses with specific antibodies to the corresponding Cx proteins were used to determine the expression of the connexins in different HeLa transfectants. Fig. 1 shows that the transfected cells expressed Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47 proteins according to the expected punctate immunostaining of gap junction plaques on contacting membranes. To confirm the specificity of the immuno signals, each connexin-transfected HeLa cell clone was also treated with the other Cx antibodies used in this analysis. None of these controls yielded a signal (data not shown).
To confirm the presence of functional gap junction channels between HeLa-Cx cell pairs, we injected the fluorescent dye Lucifer Yellow into one cell of a cell pair. As expected, connexin-transfected HeLa clones showed dye transfer from the injected cell into the neighboring cell. No intercellular diffusion of Lucifer Yellow was detected in HeLa cells not transfected with Cx coding DNA (results not shown).

The expression of CNG channels was shown by whole-cell patch clamp measurements and by application of 8-bromo-cGMP, as previously described (19). The relationship between the number of photolyzed NPE-caged cAMP molecules and the number of applied UV light photons was measured in HeLa-Cx32/CNG double-transfected cells in a previous study (31). In these experiments, CNG channel-mediated inward currents were measured during photorelease of cAMP by 9 successive UV light pulses of equal length (2 ms). The amplitudes of the resulting current steps corresponded to the published dose response curve of the channel (20). This result demonstrates that there is a linear relationship between the number of applied photons and the number of photoreleased CAMP molecules. Moreover it shows directly the relationship between flash duration and CNG channel current.

Quantification of Intercellular cAMP Diffusion

To determine the selective permeability of different gap junctions to cAMP, we used a method that consisted of the following steps. First, cAMP was photoreleased from its NPE-caged analogue in one cell of the investigated cell pair by focusing UV light exclusively onto that cell. Next, the amount of cAMP that had diffused from cell 1 (where cAMP was photoreleased) through the Cx channels into the neighboring cell (cell 2) was quantified. Finally, the number of gap junction channels that connected the investigated cell pair was measured (Fig. 2; see also Ref. 19).

First Step: Photorelease of cAMP from Its Caged Analogue—A prerequisite to measuring cAMP transfer between cells coupled by gap junction channels is the generation of a reproducible cAMP concentration difference between these cells. Uncaging technology is well suited for the generation of a reproducible concentration gradient, because the final concentration of the released compound can be easily controlled by the number of applied UV light photons.

Before investigation, HeLa cells were loaded with 200 μM NPE-caged cAMP as described under "Experimental Procedures." The light of a mercury lamp was focused onto a small spot (30 × 30 μm), limiting photolysis of caged cAMP to a single cell. Complete photolysis of caged cAMP in the irradiated cell was achieved by UV light pulses of 200-ms duration. To avoid unwanted photorelease of cAMP in the adjacent cell, the UV light spot was oriented such that there was no overlap with the contacting cell or the area immediately surrounding it.

Second Step: Quantification of the Amount of cAMP That Diffused to Cell 2—To measure the amount of cAMP that diffused through the gap junction channels into cell 2 after photorelease in cell 1, HeLa cells stably expressing a high sensitivity variant of the CNG channel (T537S rCNGa3 (20)) in addition to the corresponding connexin were used. Gating of this Ca2⁺ conducting channel by cAMP was monitored by Ca2⁺ imaging. The cells were, therefore, incubated with NPE-caged cAMP as well as 8 μM low affinity Ca2⁺ indicator dye Fluo-4FF/AM before the start of the experiment. Fig. 3 shows the changes in the cytoplasmatic Ca2⁺ concentration (|Ca2⁺|) after flash photolysis of caged cAMP in one cell of a coupled HeLa-Cx32/CNG double-transfected cell pair. The UV light induced a strong increase of the |Ca2⁺| in the irradiated cell that propagated into the contacting cell. Typically, this intercellular propagation started after a latency period of 1–10 s at the site of contact. The delay represents the time needed to increase the cAMP concentration in cell 2 to a level where significant CNG channel gating occurs. In some experiments, however, the irradiated and adjacent cells showed simultaneous Ca2⁺ transients. These cell pairs were excluded, since uncaging events in cell 2 due to cytoplasmatic bridges, overlapping processes, diffuse cell boundaries, or light reflections could not be excluded. Such simultaneous transients were also detected in 2 of 100 control experiments performed on HeLa-CNG cell pairs not transfected with Cx channels. In these two experiments not only the starting point but also the magnitude and the shape of the Ca2⁺ transients were similar in the irradiated and neighboring cell. Therefore, the most probable explanation for this result was that the cell pairs were coupled by cytoplasmatic bridges. The occurrence of cytoplasmatic bridges in HeLa cells has been extensively reported (32, 33). However, in 98% of control experiments performed on HeLa-CNG cell pairs, a UV light pulse onto cell 1 resulted in an increase in |Ca2⁺|, in the irradiated cell, but this Ca2⁺ signal remained restricted to the stimulated cell (see Fig. 6; see also Ref. 19). We conclude that the cell-to-cell propagation of the Ca2⁺ signals was dependent on the expression of gap junction channels and that the expression level of endogenous Cx channels in our HeLa cell lines was not significant.

Next we investigated whether the Ca2⁺ transients in cell 2 were actually caused by CAMP-dependent gating of CNG channels and not by intercellular diffusion of Ca2⁺ ions. This question was addressed using cocultures of HeLa-Cx45 transfectants with HeLa-Cx32/CNG double transfectants. The latter were prestained with 5 μM DiI to differentiate between the two cell lines. Before the start of the experiment, DiI fluorescence was used to select HeLa-Cx45/CNG double transfectants that were in contact with Cx45 single transfectants. Selected cells were irradiated for 200 ms with UV light, and the change in intracellular Ca2⁺ concentration was monitored. After a recovery period of 5–10 min, the contacting Cx45 expressing cells were irradiated with the UV light. A typical example of the 12 measurements performed is shown in Fig. 4. The initial cAMP release in the Cx45/CNG-expressing cell induced a considerable Ca2⁺ transient in this cell, but no significant Ca2⁺ increase was detected in the neighboring cell that did not express CNG channels (Fig. 4C). In the second part of the experiment (Fig. 4D) it is shown that both cells were coupled by gap junction channels. The irradiated Cx45 transfectant exhibited no Ca2⁺ transient, whereas the neighboring Cx45/CNG channel-expressing cell showed a significant increase in the intracellular Ca2⁺ concentration. This result strongly indicates that the Ca2⁺ transient in cell 2 of Cx45-coupled cell pairs was based on the intercellular diffusion of cAMP molecules and not of Ca2⁺ ions. It is...
cAMP Permeability of Connexin Channels

Control experiments on single cells expressing CNG channels revealed a considerable variation in both the amplitude of [Ca$^{2+}$] transients and the whole cell CNG channel current after cAMP photorelease (Fig. 5). Most likely this was caused by differences in the expression level of the CNG channels and varying cellular volume. Moreover, we found only a weak correlation between the whole cell current and the maximum amplitude of the respective [Ca$^{2+}$] transient. This indicates that the expression level of the CNG channel was at most weakly correlated with the cellular volume.

To compensate for variations in CNG channel expression in the different cell pairs, the cAMP-induced [Ca$^{2+}$] transients in cell 2 of each cell pair had to be calibrated. As a first step in such a measurement, cAMP was released in cell 1 by a saturating 200-ms UV light pulse (Fig. 2B). Intercellular cAMP diffusion resulted in a [Ca$^{2+}$] transient by CNG channel activation in cell 2. The amplitude of this transient was then compared with the amplitude of transients that were evoked by releasing a defined amount of cAMP in cell 2 by applying UV light pulses of 2.5- or 4.5-ms length (Fig. 2C). A similar amplitude of the Ca$^{2+}$ transient induced by intercellular cAMP diffusion as compared with that induced by one of the short UV light pulses would indicate that the amount of cAMP that diffused from cell 1 to cell 2 was similar to the amount released directly in cell 2. With this calibration procedure one can select cell pairs that transfer similar amounts of cAMP from cell 1 into cell 2. If this is done for cell pairs expressing different types of connexins, one can compare how many gap junction channels of a certain type were necessary to support the above-mentioned standardized increase in cAMP concentration. Due to this equal response strategy, an accurate comparison of the cAMP permeability through different Cx channels is possible even if the CNG channel expression is different from cell to cell. An additional advantage of the normalization procedure is that nonlinearities, such as the Ca$^{2+}$ binding curve of the Ca$^{2+}$ dye or the cytoplasmic...
Ca\textsuperscript{2+} buffering capacity, do not affect the result, since both the diffusion experiment and the calibration generate similar changes in Ca\textsuperscript{2+} concentration. Because CNG channels can only monitor cAMP concentrations, the amount of cAMP that is necessary to produce a certain CNG channel activation depends on the cellular volume. This introduces cell to cell variations and may cause systematic errors if cell lines with different mean cell volumes were used. However, we have no indication that the mean cell volume depends on the type of the Cx protein expressed.

Third Step: Counting Gap Junction Channels—To compare the relative permeability of different connexin channels to cAMP, it was necessary to determine the number of connexin channels in each cell pair that exhibited the standardized increase of cAMP concentration in cell 2. This was done by double whole cell patch clamping (Fig. 2D) of the respective cell pair and division of the transcellular current by the unitary conductivity of the respective Cx channel. Published single channel conductances were used for the calculations, all measured in induced HeLa cell pairs (22, 23, 28–30). We consider it unlikely that the additional expression of CNG channels in the Cx-transfected HeLa cells changes the permeability of the channels for ions.

When conventional patch clamp amplifiers are used for the measurement of gap junction conductance, series resistances can cause serious errors in the measurement, especially when large currents (>5 nS) are recorded. To avoid this problem, we used discontinuous (also termed “switched”) single-electrode voltage clamp amplifiers. These amplifiers have been shown to overcome series resistance problems by injecting current discontinuously and measuring the intracellular potential at a time when no current flows across the pipette. Thus, transjunctional currents up to 100 nS can be measured with a maximal error of 5% (27).

Comparison of the Selective Permeability of Different Connexin Channels to cAMP

Fig. 6 shows the changes in intracellular Ca\textsuperscript{2+} concentration after focal photolysis of caged cAMP in wild type, cx26-, cx32-, cx36-, cx43-, cx45-, or cx47-transfected HeLa cell clones. The UV light pulse onto one cell of a HeLa wild type cell pair resulted in a strong increase of the intracellular Ca\textsuperscript{2+} concentration in the irradiated cell but not in the adjacent cell. Accordingly, no transjunctional current was detected using the double whole-cell patch clamp technique. This is the expected result, since wild type HeLa cells do not express significant amounts of connexin proteins. In contrast, irradiation of Cx-expressing HeLa cells led to propagation of the Ca\textsuperscript{2+} signal into the neighboring cell. In the experiments shown in Fig. 6, the magnitude of the Ca\textsuperscript{2+} signal evoked by
FIGURE 5. Comparison of the electrical and optical signals after flash photolysis of caged cAMP in single cells expressing CNG channels. CNG channels were activated by UV light pulses of 4- and 100-ms duration. Electrical and optical measurements were performed on the same cells. The box plots show averaged data from nine experiments. A maximal fluorescence ($\Delta F/F_0$) induced by the UV light pulses in cells loaded with 200 $\mu$M caged cAMP and 4 $\mu$M Fluo-4FF/AM. B, flash-induced maximal whole-cell currents measured in the same cells. The recording pipette contained 200 $\mu$M caged cAMP. Membrane voltage was held at $-60$ mV. The CNG channel responses induced by the two UV light pulses were highly significant different ($p < 0.0001$, one-tailed paired Student’s t test) in both the calcium imaging and the patch clamp measurements. The current amplitudes and the amplitudes of the Ca$^{2+}$ transients were weakly positively correlated (correlation coefficient $r > 0.66$ and $r > 0.25$ for the 4- and 100-ms UV light pulses, respectively).

FIGURE 6. Measurements of cAMP diffusion through gap junction channels composed of different connexins. HeLa wild type cells and HeLa cells expressing the indicated connexins expressed in addition CNG channels as a cAMP sensor system. Cells were loaded with caged cAMP and Fluo-4FF/AM. Caged cAMP was photolysed by a 200-ms UV light pulse that was focused onto one cell (white squares) of the corresponding cell pair. The time course of Ca$^{2+}$ concentration increase in the cell pairs is shown by three images recorded at the indicated time points. The color bar indicates the intracellular calcium concentration that increases from dark blue to red. White bar, 30 $\mu$m. The amount of cAMP that diffused from the first to the second cell was quantified as described in Fig. 2. Double whole-cell patch clamp measurements performed on the same cell pairs were used to quantify the number of Cx channels. The membrane potential of the two cells was clamped for a short time (200 ms) to two different values ($V_1 = -60$, $V_2 = -20$), and the current evoked by this voltage difference was measured ($I_{ij}$). The measured currents correspond to 270 Cx26 channels, 510 Cx32 channels, 3126 Cx36 channels, 93 Cx43 channels, 450 Cx45 channels, and 651 Cx47 channels. In wild type cells, no transjunctional current was detected.
distinct Cx channel. Previous work has shown that Cx26 and Cx45 form a 13
of molecules are not sufficient to clearly predict their passage through a gap junction channels (9, 37). Therefore, the molecular mass and charge of the tracers but also on other parameters such as the diffusion through gap junction channels depends not only on the size and charge preference (4). Furthermore, coupling studies with fluorescent tracers had indicated that the limiting diameter of Cx channels follows a 2.5-ms UV light pulse (5). Furthermore, Niessen et al. (8) showed that Cx32 channels propagated IP$_3$-induced Ca$^{2+}$ waves 3–4 times better than Cx26 channels. Lucifer Yellow (molecular mass, 443 Da; charge, −2) and IP$_3$ (417 Da; charge, −2 to −4) are like cAMP (329 Da; charge, −1) anionic molecules. In case of a simple size and charge-dependent discrimination, one would expect for the CAMP permeability of Cx32, Cx26, Cx32, and Cx45

gap junctional transfer of CAMP was as high as those generated by a normalizing UV light pulse of 4.5-ms duration for each cell pair of the different HeLa transfectants. Therefore, we concluded that intercellular CAMP diffusion through gap junctions led to identical CAMP concentrations in the neighboring cells. However, the current recordings revealed gap junctional coupling of differing extents between the cell pairs of different Cx transfectants. In the experiments shown in Fig. 6, about 270 Cx26 channels, 510 Cx32 channels, 312 Cx36 channels, 93 Cx43 channels, 450 Cx45 channels, or 651 Cx47 channels were necessary to transfer the same concentration of CAMP from cell 1 to cell 2 of the corresponding cell pair.

We performed two independent subsets of experiments, one in which the intercellular diffusion of CAMP generated a Ca$^{2+}$ signal similar to that generated by a UV light pulse of 2.5-ms duration and a second one in which the signal was similar to that evoked by a light pulse of 4.5-ms duration. The results are summarized in Table 1 and Fig. 7. The mean of both experimental subsets resulted in the following order of CAMP permeability: Cx43 > Cx26 > Cx45 ≡ Cx32 > Cx47 > Cx36.

**DISCUSSION**

We have demonstrated here that gap junction channels composed of Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47 proteins were permeable to CAMP with different efficacies. The differences in permeability observed in this study are highly significant and suggest unique functions of different Cx channels. Moreover, it should be noted that we used cells that overexpressed the Cx proteins so that Cx channels with low permeability for CAMP might act as the barrier under physiological conditions, especially because CAMP molecules have a short lifetime (1–60 s) (34).

The relation between size and charge of molecules and the efficacy by which they were transferred through different gap junction channels was investigated in a large number of studies. Fluorescent tracers of different charges and sizes were used to characterize the specific selectivity of Cx channels (3, 5, 9, 35, 36). These studies showed that the diffusion through gap junction channels depends not only on the size and charge of the tracers but also on other parameters such as the structure. Apparently significant levels of permeant-pore attractions (e.g. van der Waals forces) play an important role for the permeability of gap junction channels (9, 37). Therefore, the molecular mass and charge of molecules are not sufficient to clearly predict their passage through a distinct Cx channel. Previous work has shown that Cx26 and Cx45 form

**TABLE 1**

Summary of results obtained from normalized experiments

| Transfectants | Total conductance$^{b}$ | Single channel conductance$^{a}$ | Number of gap junction channels$^{c}$ | n$^{d}$ |
|---------------|------------------------|-------------------------------|----------------------------------|------|
|               | uS ± S.E.              | pS ± S.E.                     |                                  |      |
| Normalization flash, 2.5 ms |
| HeLa-Cx26/CNG | 21.4 ± 1.4             | 135                           | 159 ± 10.1                       | 8    |
| HeLa-Cx32/CNG | 15.4 ± 2.1             | 55                            | 280 ± 38                         | 4    |
| HeLa-Cx36/CNG | 22.2 ± 2.6             | 15                            | 1480 ± 175                       | 7    |
| HeLa-Cx43/CNG | 4.9 ± 0.5              | 115                           | 43 ± 4.4                         | 7    |
| HeLa-Cx45/CNG | 8.4 ± 0.6              | 32                            | 252 ± 17.1                       | 14   |
| HeLa-Cx47/CNG | 21.3 ± 1.5             | 55                            | 388 ± 27.5                       | 8    |
| Normalization flash, 4.5 ms |
| HeLa-Cx26/CNG | 33.6 ± 1.6             | 135                           | 249 ± 11.9                       | 13   |
| HeLa-Cx32/CNG | 27.1 ± 1.1             | 55                            | 493 ± 20.2                       | 12   |
| HeLa-Cx36/CNG | 45 ± 3                 | 15                            | 3004 ± 198                       | 10   |
| HeLa-Cx43/CNG | 10.4 ± 0.8             | 115                           | 90 ± 6.6                         | 10   |
| HeLa-Cx45/CNG | 14 ± 1.1               | 32                            | 438 ± 35.2                       | 10   |
| HeLa-Cx47/CNG | 39.6 ± 1.1             | 55                            | 720 ± 19.5                       | 13   |

$^{a}$ Total electrical conductance measured between HeLa cell pairs in which the Ca$^{2+}$ signal evoked by diffusion of CAMP in the neighboring cell is similar to that generated by the normalization flash.

$^{b}$ Single channel conductance of the expressed connexin channels measured in HeLa cells (22, 23, 28–30).

$^{c}$ Calculated by dividing the total conductance by the single channel conductance.

$^{d}$ Number of experiments.

**FIGURE 7. Comparison of the CAMP permeability of different gap junction channels.** Bar plot displaying the number of gap junction channels composed of the indicated connexin that allows diffusion of similar amounts of CAMP from cell to cell. The CAMP concentration equals that released from caged CAMP by a 4.5-ms UV light pulse (A) or by a 2.5-ms UV light pulse (B). Error bars ± S.E. The measured difference in the CAMP permeability of Cx32 and Cx45 channels is not significant (p > 0.05; unpaired two-tailed Student’s t test). All other differences are highly significant (p < 0.0001).
channels the order Cx32 > Cx43 > Cx26 > Cx45. Hence, the most surprising finding of this study is the low cAMP permeability of Cx32 channels, which might be explained by obstructive cAMP-pore interactions.

Goldberg et al. (7), using a layered culture system and radioactive labeled metabolites, showed that Cx43 channels are about 8 times more permeable to AMP and ADP and more than 300 times more permeable to ATP than Cx32 channels. However, the layered culture system requires an incubation time of hours for metabolizing the radioactively labeled substances and gap junction formation. Therefore, the results may be affected by diffusion of metabolic intermediates through the gap junction channels. This possible complication can be excluded by the method used in the present study. Nevertheless, our results are in agreement with the findings of Goldberg et al. (7). Because AMP resembles cAMP in molecular mass as well as charge and structure, one could assume that cAMP might display a similar permeability ratio for Cx43 versus Cx32 channels.

It was suggested in several earlier studies that different permeabilities of biologically important molecules could be one of the reasons for the diversity of Cx channels (3–6, 33). Our results support this view. Each cell type may express a specific pattern of Cx channels for rapid intercellular equilibration of second messenger concentrations to assure optimal, coordinated response to external signals. Therefore, certain cell types may express preferentially Cx43 when fast cAMP propagation is essential for normal function or coordinated activity. Cx43 is expressed in many tissues, including working myocardium, alveolar epithelial cells in lung, vascular and intestinal smooth muscles, and endothelial cells (2). In the working myocardium cAMP controls the strength, duration, and frequency of contraction. Hence, an efficient cell-to-cell transfer of cAMP concentrations could be crucial for normal heart beat. The surfactant secretion in alveolar epithelial cells is stimulated by hormones that partially act through a cAMP-mediated pathway. Here the high cAMP permeability of Cx43 channels may be important for rapid and coordinated secretion to prevent lung collapse and to facilitate expansion. In vascular and intestinal smooth muscles, the Cx43-mediated intercellular CAMP diffusion might be important for synchronous relaxation. Fast propagation of cAMP in endothelium could be critical for the barrier functions of this tissue, since it is known that CAMP stabilizes the endothelial barrier (38, 39).

Our studies may also provide important new insights into the role of multiple connexins within the same cell types. For example, Cx26 and Cx32 are coexpressed in hepatocytes (2). It was shown before that Cx32 channels are more permeable to IP3 than Cx26 channels (8). In contrast, our results demonstrate that cAMP is transferred more efficiently through Cx26 channels than through Cx32 channels. Therefore, it is reasonable to assume that in hepatocytes Cx26 channels allow rapid cAMP transfer, whereas Cx32 channels are rather responsible for the IP3 propagation. Recently, it was demonstrated that Cx36 and Cx45 are coexpressed in certain neurons of the mouse inferior olivary (40). Although the major role of the electrical synapses formed by these channels may be the fast transmission of current carrying ions, they also provide a pathway for the exchange of metabolites and second messenger among neuronal cells. Interestingly, the two connexins that are expressed in neurons (41) differ widely in their cAMP permeability. Hence, the metabolic coupling among neuronal cells may be mediated principally by Cx45 channels, whereas Cx36-containing channels may be mainly responsible for the synchronization of electrical activity between neurons.

The modulation of the intercellular signaling by gap junction channels is probably much more complex due to the occurrence of heteromeric and heterotypic channels. Such mixed channels can have unique permeability characteristics distinct from channels composed of only one Cx isofrom. Bevans et al. (16), using a technique called transport specific fractionation, demonstrated that homomeric Cx32 channels conduct GMP and cAMP with similar efficacy. However, heteromeric Cx26/Cx32 channels appeared to be more permeable to cGMP than to cAMP. These results show not only that the isofrom composition of Cx channels can affect the selectivity among second messengers but also the existence of very specific interactions between cyclic nucleotides and gap junction channels.

A further indication for unique functions of different Cx channels is the finding that Cx43 or Cx26 can act as tumor suppressor genes that can reverse the cancer phenotype of mammary carcinoma or glioma cells (42, 43). Because cAMP has been repeatedly reported to play a crucial role in cell growth and differentiation (for reviews, see Refs. 44 and 45), one can assume that the relative high permeability of Cx43 and Cx26 channels to CAMP may contribute to the tumor suppressor function of these channels.

Acknowledgment—We thank Dr. U. B. Kaupp (Jülich) for providing the cDNA encoding the olfactory CNG channel.

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