Direct High Affinity Modulation of Connexin Channel Activity by Cyclic Nucleotides*

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Connexin channels mediate molecular communication between cells. However, positive identification of biological ligands that directly and nonevocantly modulate their activity has been elusive. This study demonstrates a high affinity inhibition of connexin channels by the purine cyclic monophosphates cAMP and cGMP. Purified homomeric connexin-32 and heteromeric connexin-32/connexin-26 channels were inhibited by exposure to nanomolar levels of the nucleotides prior to incorporation into membranes. Access to the site of action, or affinity for the nucleotides, was greatly reduced following incorporation of the connexin channels into membranes, where inhibition required millimolar concentrations of the nucleotides. The high affinity inhibition did not occur with similar concentrations of AMP, ADP, ATP, cTMP, or cCMP. This is the first report of a direct ligand effect on connexin channel function. The high affinity and specificity of the inhibition suggest a biological role in control of connexin channels and also may lead to the application of affinity reagents to study of connexin channel structure-function.

The gating of connexin channels by ligands is a key uncharacterized element of intercellular signaling. Connexin channels mediate intercellular molecular signaling that is important in developmental and physiological contexts (1–3). However, the biological ligands that directly regulate connexin channel activity have been difficult to positively identify. Direct action on connexin channels is difficult to establish due to the intercellular channel structure and the cytoplasmic location of channel modulatory sites.

Junctional coupling between cells can be reduced by exposure of cells to various compounds (reviewed in Refs. 4–6). In some cases, it is known that the effects on junctional conductance are not due to direct action of the applied compounds on the connexin channels, but to cytoplasmic mediators (e.g. kinases, cf. Ref. 7). However, in most cases the mechanism of action, and whether it is direct or indirect, is not known. The most well documented direct modulators of connexin channels are voltage (cf. Refs. 8–11) and phosphorylation (7, 12, 13), which can each alter substate occupancy of certain connexins. However, voltage is not likely to be a physiologically important regulator except in specific locations in excitable tissues. Cytoplasmic acidification causes most cells to uncouple, but this may not be a direct effect (14–16).

The identification of ligands of connexin channels is important for understanding the physiology of intercellular signaling. It is also important because it can lead to the use of affinity reagents to explore the structure-function of connexins. The biophysical study of connexin channels has been hindered by the absence of specific affinity reagents or toxins. Such agents (e.g. tetrodotoxin and charybdoxin) have been vitally important in elucidating molecular mechanisms of other channels (17, 18).

The studies reported here utilize connexin channels composed of connexin-32 (Cx32) and connexin-26 (Cx26) purified from native tissues by immunoaffinity chromatography. These two connexins have a high degree of sequence identity (62%) and are often found in the same cells and in the same junctional plaques (19–23). Their close association implies a functional and/or structural relationship in situ.

Compounds that alter junctional coupling when applied to cells were tested for efficacy in a reconstituted system. A high affinity, modulatory action of purine cyclic monophosphates, but not other nucleotides, on connexin channel activity was discovered. The chemical specificity and nanomolar affinity suggest a biological role. Preliminary reports of this work have appeared in abstract form (24–27).

EXPERIMENTAL PROCEDURES

Materials—Egg phosphatidylcholine, bovine phosphatidylserine, azolectin (soybean l-phosphatidylcholine), and lissamine rhodamine B-labeled phosphatidylethanolamine were purchased from Avanti Polar Lipids. Tween 20, nitro blue tetrazolium, and diisopropylfluorophosphatase were obtained from Sigma. N-Oethyl-N-glucopiranoside (octylglucoside) was obtained from Calbiochem. Bio-Gel (A-0.5 m; exclusion limit, 500,000 Da) was purchased from Bio-Rad. Alkaline phosphatase-conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indolyl-phosphate were purchased from Boehringer Mannheim Biochemicals. CNBr-activated Sepharose beads were obtained from Amersham Pharmacia Biotech and Immobilon-P transfer membrane from Millipore. Rats and mice were obtained from Taconic. Use and care of animals was according to institutional guidelines.

Immunopurification of Connexin Proteins—Connexin was affinity-purified from an octylglucoside-solubilized crude membrane fraction of rat or mouse liver using a monoclonal antibody against Cx32 as described in Refs. 28 and 29, with the modification that 5 mM EDTA was included in the homogenization and phosphate buffers.

Gel Electrophoresis, Protein Blots, and Immunoblots—Samples of liposomes for gel electrophoresis were prepared by extracting lipids in absolute methanol and washing and concentrating the protein on a 30-kDa cutoff filter cartridge (Ultrafree, Millipore Corp.). Gel electrophoresis, blotting, and staining of blots were carried out as described in Ref. 29.

Antibodies—The monoclonal antibody (M12.13) used in the immunoaffinity purification and for specific staining of connexin-32 on Western

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1 The abbreviations used are: Cx, connexin; CAP, catabolite activator protein; cPMPs, cyclic purine monophosphates cAMP and cGMP; Po, channel open probability; TSF, transport-specific fractionation (of liposomes).
blots is directed against a cytoplasmic domain of Cx32 (30). Reconstitution of Purified Connexin into Unilamellar Phospholipid Liposomes—Liposome formation and protein incorporation followed the protocol of Mimms et al. (31) as modified by Harris et al. (32) and Rhee et al. (28), and summarized in the companion paper (33). To ensure that the potential modulatory agents were available without dilution to the connexin until liposome formation, a 4 mL volume of buffer containing the agents was loaded onto the gel-filtration column before the liposome-forming solution was loaded.

Transport-specific Fractionation (TSF)—The procedure used to fractionate liposomes into two populations based on sucrose permeability is described and fully characterized in Harris et al. (28, 32, 34) and Rhee et al. (28) and summarized in the companion paper (33). Liposomes containing functional channels are separated from liposomes without functional channels by TSF achieved by centrifugation through an isosmotic density gradient formed by urea and sucrose solutions. Equilibration of extraluminal and intraluminal osmolytes is rapid (milliseconds for these 900-Å diameter liposomes). Therefore, even a channel that opens only infrequently for brief times will mediate full exchange of osmolytes and cause liposome movement to the characteristic lower position. The assay will not detect the activity of channels that are not permeable to urea and sucrose. Therefore the channel activity gives a quantitative assessment of the fraction of channels to which ligand is bound at the moment of reconstitution in membranes—it is a "snapshot" of binding. In this way, it is formally analogous to a standard binding assay in which one assesses the average fraction of receptors occupied for a given concentration of ligand. The process is irreversible after reconstitution up to the lifetime of functional channels in the liposomes (~4 weeks).

Rather than looking at the aggregate response of a population of receptors while association-dissociation reactions take place, this assay captures the reactions at a given instant. Thus an irreversible reaction (incorporation into a bilayer) is used to freeze a reversible reaction (ligand binding) under steady-state conditions.

For each preparation of connexin, the percentage of liposomes in the lower band of TSF data was normalized to the maximum value obtained for that preparation. This enabled comparison of modulatory effects across reconstitutions that produced different amounts of channel activity (fractions of liposomes with functional channels). Where several preparations were used, normalized data sets were combined for calculation of means and standard errors.

Previous work with the TSF system suggested that the channels distribute among the liposomes in a manner described by the Poisson distribution (28). For a given protein-lipid ratio (λ) in the liposomes, a Poisson distribution accounts for the fraction of the liposomes that have functional channels. A statistical method was used to correct for the fraction of liposomes that contained more than one channel (described in the companion paper (33)). This calculation compensates for the underestimate of the effect of a test compound introduced by some of the liposomes containing more than one channel, transforming the fraction of permeable liposomes in a population to accurately reflect discrete single channel activities.

RESULTS

Connexin was immunopurified from rodent liver using a monoclonal antibody that recognizes connexin-32. Previous biochemical and functional studies have characterized connexin purified in this way from rat liver as homomeric Cx32 hemichannels, and that from mouse liver as heteromeric Cx32/Cx26 hemichannels (28, 29, 35).

The sensitivities of channels formed by Cx32 and Cx32/Cx26 to several agents were explored and compared by transport-specific fractionation (TSF) of liposomes. TSF has been well characterized (32, 34, 36) and effectively used in studies of channel permeability (28, 29, 37). Purified connexin is incorporated into unilamellar liposomes by gel filtration of a mixture of octylglucoside-solubilized connexin and lipid. TSF fractionates the liposomes on the basis of permeation of osmolytes (urea and sucrose) through the reconstituted channels. Specifically, it employs buoyant density sedimentation to separate into distinct bands liposomes with open channels permeable to urea and sucrose from liposomes that are without such channels. Liposomes that do not contain open connexin channels (i.e., are not permeable to urea and sucrose) migrate to an equilibrium position in the upper part of the gradient. For liposomes that contain open connexin channels, the osmolytes exchange through the channels and the liposomes migrate to a lower position determined only by lipid density. Any significant channel open probability (P_o) results in sufficient osmolyte exchange to change the required change in density. The TSF is therefore an all-or-none assay of per-liposome channel activity. Effects of test compounds on channel activity were assessed by exposing connexin or connexin-containing liposomes to the compounds prior to or during a TSF spin. Effects on channel activity were quantified as changes in the fraction of liposomes in the lower band relative to that for connexin or liposomes not exposed to the test compound.

The fractional change in distribution of liposomes between the two bands is a quantitative measure of the fractional change in activity of the population of the channels. The change in liposome density can result from brief channel openings, so only when P_o changes above or below a low threshold value are changes in channel activity detected by TSF.

Many Compounds That Affect Functional Communication in Cells Do Not Have Effects on Connexin Channels in the TSF System—In initial studies, the effects of test compounds were assessed by including them in the TSF solutions. The results are for both homomeric Cx32 channels and heteromeric Cx32/Cx26 channels, except where otherwise indicated.

Table I lists compounds that can affect junctional ion current or dye transfer when applied to cells but that were without effect in this system. Tables II, III, and IV list additional compounds that were also without detectable effect. Absence of effect indicates that the actions of a compound on connexin channels either (a) are not direct, (b) are not dramatic enough to be detected by TSF, or (c) require the intercellular channel structure (two end-to-end hexameric hemichannels).

However, positive findings in this system indicate dramatic effects on channel activity. When applied at 10 mM, but not 1 mM, to reconstituted hemichannels, cAMP inhibited channel activity (Fig. 1). Activity was not affected by 10 mM adenine or adenosine. By cytoplasmic standards, 10 mM is a high concentration of cAMP, so a sensitivity in this range is not likely to be biological relevant. We speculated that an apparent low affinity for cAMP could arise from restricted accessibility to a high affinity site. This could occur if the binding site were partially obscured by membrane lipids or if the reconstituted channel was only rarely in a conformation that enabled access to the site.

High Affinity Inhibition of Solubilized Connexin Channels by cAMP or cGMP—A high affinity interaction between cyclic nucleotides and connexin was established by studies in which

| Compound          | Concentration |
|-------------------|---------------|
| Glycerrhetinic acid | 1 μM          |
| Halothane         | 1 mM          |
| Heptanol          | 1 mM          |
| Heptanonic acid   | 1 mM          |
| Octanol           | 1 mM          |
| Octanolic acid    | 1 mM          |
| Melatonin         | 10⁻⁸ M        |
| Quinine           | 10⁻⁵ M        |
Table II: Compounds that inhibit other ion channels that were without effect on connexin channels in the TSF system

| Compound                  | Concentration | Known primarily as blocker of          |
|---------------------------|---------------|----------------------------------------|
| Flufenamic acid           | 80 μM         | Chloride channels                       |
|                           | 40 μM         | Ion antiporters                         |
| 5-N,N-Hexamethylene-      |               |                                        |
| amidorilide - HCl         |               |                                        |
| Lidocaine                 | 1 mM          | Sodium and potassium channels           |
| Tetramethylammonium       | 10 mM         | Potassium channels                      |
| Tetraethylammonium        | 10 mM         | Potassium channels                      |
| Tetrapropylammonium       | 10 mM         | Potassium channels                      |
| Tetrapentylammonium       | 10 mM         | Potassium channels                      |

Table III: Divalent ions without effect on connexin channels in the TSF system

| Ion       | Concentration |
|-----------|---------------|
| Ca^{2+}   | 1             |
| Co^{2+}   | 0.2           |
| SO_{4}^{2-}| 5             |

Table IV: Miscellaneous compounds without effect on connexin channels in the TSF system

| Compound            | Concentration | Description                  |
|---------------------|---------------|------------------------------|
| Dimethylpimelimidate| 10–100 mM     | Cross-linking reagent        |
| Dithiothreitol      | 5 mM          | Reducing agent               |
| Sucrose octaacetate | 10 mM         | Acylated analog of sucrose   |

* Other compounds that were tested but that caused aggregation of liposomes or had other effects that made the results difficult to determine were 5% Me_{2}SO or to octylglucoside levels below the critical micelle.

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concentration did not reverse the inhibition. Additionally, attempts to induce the inhibition in reconstituted channels not previously exposed to cyclic nucleotide by exposure of the liposomes to 10 μM cAMP or 8-(4-chlorophenylthio)-cAMP (a hydrophobic analog of cAMP) in 5% Me₂SO or octylglucoside levels below the critical micelle concentration were not successful.

**DISCUSSION**

These experiments demonstrate the existence of a selective, high affinity modulatory site for cyclic purine monophosphates on connexin channels. The site is accessible while the protein is in micelles and/or during its transition from micellar to bilayer environments, and it becomes less accessible following incorporation of the channels into membranes.

The inhibition of channel activity by cPMPs could be achieved by allosteric effects or physical occlusion of the pore. The latter is not likely because cPMPs can permeate reconstituted Cx32 channels (29). The much-reduced sensitivity of reconstituted channels may indicate that after reconstitution the site is accessible only when the protein is in a restricted set of conditions or conformational states. Alternatively, the effects at millimolar levels could be due to a distinct, low affinity site unrelated to the effects at nanomolar levels.

That activity inhibition persists in the absence of free nucleotide after reconstitution suggests that once ligand is bound and the channel is incorporated into a membrane, the ligand is precluded from dissociation. This may occur by conformational changes secondary to binding or to the transition to a membrane environment. Examples of the former include the binding-dependent conformational change that occurs in peptide binding by calmodulin (38, 39) and by the molecular chaperone DnaK (40). Binding at an active site can involve conformational changes that stabilize the bound complex (41, 42) or render it inaccessible to bulk solvent (43). Receptor-ligand complexes can have half-lives of days (44–47). Alternatively, dissociation of ligand may be blocked by membrane lipids, where the bound ligand is shielded from the aqueous phase by the phospholipid bilayer following reconstitution.

Cyclic nucleotides can permeate some junctional channels (48–52), as well as hemichannels (29), so the inhibition at subnanomolar levels may seem paradoxical. However, cytoplasmic concentrations of cyclic nucleotides range between nanomolar and micromolar, well below the millimolar levels required for effects on connexin channels already in membranes. Homomeric Cx32 and heteromeric Cx32/Cx26 have distinguishable but similar affinities for the nucleotides, suggesting that the binding sites for the nucleotides on the two connexins are different, but not dramatically so. The activity of the cyclic nucleotide analogs may be informative regarding the nature of the binding site. Because 1,N⁶-etheno-cAMP and 8-azido-cAMP are derivatized on the purine ring, 2'-[(N-methylanthraniloyl)-cGMP is derivatized on the ribose sugar, and all three are effective at nanomolar levels, it appears that additions to the 2, 8, N⁶ and 2'-hydroxyl groups do not preclude steric fit into the putative binding site.

Cyclic nucleotides directly modulate protein function in several systems. The most well known class is the catabolite activator protein (CAP) family of proteins, which includes the *Escherichia coli* CAP, cyclic-nucleotide dependent protein kinases, and cyclic-nucleotide-gated ion channels (53, 54). However, Cx32 and Cx26 do not contain the amino acid sequences that are strictly conserved at the cyclic nucleotide binding domains of the CAP family. Neither is there obvious sequence similarity with the postulated cGMP binding domain of cGMP-specific phosphodiesterases (55).

Unlike the CAP and phosphodiesterase families of cyclic nucleotide receptors, which have micromolar affinities, the cAR1 and cAR3 chemoattractant receptors of *Dictyostelium* have nanomolar affinities for cyclic nucleotides (56), in the

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**Fig. 3. Dose-response relations for cAMP and cGMP.** Connexin was reconstituted in a range of concentrations of cAMP (triangles) or cGMP (circles). Nucleotide was not present in the TSF gradients. Data were fit using the Hill equation. Bars are S.E. A, homomeric Cx32 channels had essentially identical responses to cAMP and to cGMP. B, heteromeric Cx32/Cx26 channels were more sensitive to cAMP than to cGMP. For both types of connexin channels, binding of nucleotide inhibited subsequent channel activity and appears to involve more than one binding site per channel.
same range that we determined for connexins, and mediate physiological responses at subnanomolar levels (57). Further analogy with the cAR family is provided by our finding that 2'-([N-methylanthraniloyl]-cGMP is just as effective as cGMP, suggesting that substitutions at the ribose 2'-OH position do not interfere with binding to connexin. An unsubstituted ribose 2'-OH group is required for binding of cGMP by the CAP family of receptors (58) but not for cAR1 (59).

The amino acids involved in the cyclic nucleotide binding to cAR1 have not yet been identified. However, a region of 26 amino acids (148–173), thought to control access to the binding pocket (60, 61), shows ~25% identity and ~60% homology with a region of Cx32 and Cx26 that spans the cytoplasmic loop and the third transmembrane domain. The same level of identity and homology extends through the eight positions N-terminal to this sequence before dropping off. This sequence similarity is only suggestive; further analogy with the cAR family must await positive identification of the relevant amino acids.

**SEQUENCE 1. Alignment and homologies were obtained from Align using BLOSUM50. +, identity; +, homology.**

Hill coefficients of between 1 and 2 for the CPM effects suggest that there could be two independent binding sites or more than two cooperative sites per channel. Because all six Cx32 subunits comprising a homomeric hemichannel have identical sequences, there are potentially six binding sites per hemichannel. On the other hand, it is conceivable that the sites of action are within the pore and altered by the micelle-membrane transition. The fact that connexin channels have subnanomolar specific affinities for cAMP and cGMP, and that binding of these cyclic nucleotides closes connexin channels, is likely to be of physiological importance. One possible function is to keep hemichannels closed while they are in Golgi or endoplasmic reticulum membranes. The binding site may be accessible during initial membrane insertion and/or channel assembly, when cytoplasmic CPMs could bind and thus ensure that connexin hemichannels remain closed during trafficking to the plasma membrane. Docking of apposed hemichannels to form the full intercellular channel could cause dissociation of these nucleotides from connexin channels. Connexin-32 is co-translationally inserted into endoplasmic reticulum membrane (62, 63). Intriguingly, it has been shown that during co-translational insertion of polytopic membrane proteins, the transmembrane segments inserted through the endoplasmic reticulum membrane are stabilized in a salt-accessible compartment, apparently not interacting directly with lipid (64, 65). Thus, regions of a folded membrane protein that will be later blocked from aqueous access by membrane lipid appear to be transiently accessible to cytoplasmic components.

In view of this, we favor the view that both the micellar environment in our experiments and the partially unfolded state of the protein as it is inserted into a bilayer renders the CPM site(s) accessible. NMR studies show that nondenaturing detergent such that used in these studies increase molecular motion of and accessibility to protein domains that are exposed to lipid when in bilayers (66, 67). Specifically, there is particularly enhanced accessibility to residues at the membrane-water transition (68, 69). There is evidence that nondenaturing detergents do not fully coat the hydrophobic surfaces of proteins (unlike SDS), leaving a somewhat open structure (70, 71) accessible to small, hydrophilic ligands.

Furthermore, the predicted number of residues for connexin transmembrane helices suggests that the hydrophobic core of the protein is relatively short, composed of five helical turns (28 Å) (72) rather than the six or more typical of channel proteins (73). There may be special lipid requirements for connexin channels in cell membranes to accommodate this. The lipid environment in the present studies is different from that in cell membranes, and may occlude sites that are accessible in them. Differences in the lipid environment may affect accessibility to binding sites, as well as other aspects of channel function (74–78).

This work addressed two long-standing fundamental issues of connexin channel function. One is the identification and mechanisms of biological ligands that control connexin channel activity. The other is the identification of specific affinity reagents with which to probe the structure-function of connexin channels. These new findings, showing that connexin channels can be directly regulated by a cytoplasmic ligand, are likely to lead to structure-function studies of connexin channels and better understanding of the molecular basis for regulation of intercellular signaling.

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