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Design and characterization of the first peptidomimetic molecule that prevents acidification-induced closure of cardiac gap junctions

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Abstract

Background—Gap junctions are potential targets for pharmacological intervention. We have previously developed a series of peptide sequences that prevent closure of Cx43 channels, bind to cardiac Cx43 and prevent acidification-induced uncoupling of cardiac gap junctions.

Objective—We aimed to identify and validate the minimum core active structure in peptides containing an RR-N/Q-Y motif. Based on that information, we sought to generate a peptidomimetic molecule that acts on the chemical regulation of Cx43 channels.

Methods—Experiments were based on a combination of biochemical, spectroscopic and electrophysiological techniques, as well as molecular modeling of active pharmacophores with Cx43 activity.

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Results—Molecular modeling analysis indicated that the functional elements of the side chains in the motif RRY form a triangular structure. Experimental data revealed that compounds containing such a structure bind to Cx43 and prevent Cx43 chemical gating. These results provided us with the first platform for drug design targeted to the carboxyl terminal of Cx43. Using that platform, we designed and validated a peptidomimetic compound (ZP2519; molecular weight 619 Da) that prevented octanol-induced uncoupling of Cx43 channels, and pH gating of cardiac gap junctions.

Conclusion—Structure-based drug design can be applied to the development of pharmacophores that act directly on Cx43. Small molecules containing these pharmacophores can serve as tools to determine the role of gap junction regulation in the control of cardiac rhythm. Future studies will determine whether these compounds can function as pharmacological agents for the treatment of a selected subset of cardiac arrhythmias.

Keywords

Connexin 43; Arrhythmias; Gap Junctions

INTRODUCTION

Gap junctions are intercellular channels that allow for direct passage of ions and small molecules between cells. In the heart, gap junctions provide a low-resistive pathway for propagation of excitatory currents, a step necessary for the synchronous activation of the cardiac muscle. Gap junctions are formed by oligomerization of an integral membrane protein called connexin. The major cardiac connexin isoform is the 43 kDa protein Connexin43 (Cx43). Closure of Cx43 channels and the consequent loss of electrical coupling in cardiac disease have been invoked as potential arrhythmogenic events. Furthermore, gap junctions are also present in non-myocyte cells of the heart,¹ playing an important role in the control of heart development² and, likely, heart function.³ Given their importance in cardiac homeostasis, gap junctions are considered valuable targets for pharmacological intervention.^{4,5} Encouraging results in experimental models of heart disease have been obtained with two molecules shown to preserve gap junction communication: rotigaptide⁶ and GAP-134.⁷ However, current evidence suggests that the effect of these molecules on gap junctions is indirect, mediated by intermediary steps, and likely involving protein kinases with catalytic activity and thus, functional effects on multiple substrates.^{8,9} As an alternative, we hypothesized that current knowledge on structure-function relation of Cx43 can be used to design molecules that modify gap junction function.^{6,10–12} Our approach was based on the observation that the carboxyl terminal (CT) domain of Cx43 is necessary for chemical gating of the channel. We thus utilized high throughput screening methods to identify peptides that can a) bind the Cx43CT domain, b) interact with cardiac gap junctions and c) prevent chemically-induced gap junction closure. Those experiments led to the identification of a series of peptides that share a consensus Cx43-binding sequence “RXP” (R is arginine, P is proline and X is any amino acid). Of that series, a 34 amino acid peptide, dubbed “RXP-E,” was found to preserve intercellular communication and action potential propagation between cardiac cells.¹³ Additional experiments led to a first approximation of the core active sequence of “RXP” peptides. As a result of those efforts, we demonstrated that activity was preserved in a linear octapeptide of sequence RRNYRRNY.¹⁰ Our data led to the hypothesis that the core structure in this and other cyclized analogs was provided by the guanidino groups of the arginine side chains and the aromatic ring of tyrosine.¹⁰ Yet, a more precise assignment of the active components of these peptides still remained necessary, to address the question of whether a three-amino acid model can serve as a platform for small molecule design with potential application in living organisms. Here, we have used a combination of biochemical, spectroscopic and electrophysiological techniques to demonstrate that the primary sequence RR(X)Y (“X” being a linker amino acid) is a Cx43 binding motif. *In silico* analysis, validated experimentally, further showed that the triangular

secondary structure formed by the two arginines and the tyrosine side chains (a “pharmacophore triangle”) serves as platform for the design of synthetic molecules that target cardiac gap junction channels. Using this platform, we generated the first peptidomimetic compound capable of preventing chemical gating of Cx43 (compound “ZP2519”). Our studies demonstrate that rational, structure-based drug design can be applied to cardiac gap junction pharmacology. These molecules can then serve as tools to determine the role of gap junction regulation in cardiac arrhythmogenesis and, potentially, serve as pharmacological agents for treatment of a selected subset of cardiac arrhythmias.

METHODS AND MATERIALS

Transfection of N2a cells with Connexin 43 and patch clamp methodology

Experiments were conducted in transiently transfected N2a cells. In all cases, the dual-whole-cell voltage clamp technique was used to record gap junction currents as described previously (see ref¹⁰ and those within for details). Synthetic peptides were diluted in the pipette solution to a final concentration of 100 μ M. Traces of junctional currents were acquired and analyzed using Clampex software (pClamp version 10.0, Axon Instruments, Union City, CA). Octanol superfusion was initiated 5 minutes after patch break and continued for 10 minutes. Octanol concentration was 1.5mM in all experiments. Junctional Conductance (Gj) was measured in control cells and those having peptide in the intracellular pipette solution. Experiments were conducted in murine neuroblastoma cells (N2a) obtained from American Type Culture Collection (Manassas, VA). Details on methods for cell culture and transient transfection, and characteristics of the vector, can be found in ref¹⁰ and others within.

Production of GST fusion proteins

GST-RXPE and RXPE mutant fusion proteins were produced as previously described.¹³ Briefly, synthetic oligonucleotides from RXP-E's and full-length sequences were inserted into pGEX-6P-2 (Amersham) and expressed in BL-21-competent bacterial cells. The resultant GST-fusion proteins were purified using a glutathione column. Protein concentration was measured using the Bio-Rad DC Protein Assay. Protein purity was assessed by SDS-PAGE.

Cell dissociation and culture of rat neonatal ventricular myocytes (NRVMs)

All experiments involving animals conformed to the protocols in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, Revised 1996). Primary cultures of NRVMs for patch clamping were obtained using established procedures^{10,14}.

Preparation of heart lysates

Fresh rat heart lysates were prepared by homogenizing tissue in heart lysis buffer, as described in detail in¹³ and others within. Total protein content of heart lysate was determined by DC protein assay (Bio-Rad) with bovine serum albumin as a standard.

GST-Pulldown assay and immunochemical detection of Cx43

Bound GST fusion proteins were incubated with approximately 15 mg of pre-cleared rat or mouse heart lysate in 1ml of lysis buffer for 90 minutes, rocking at 4°C. A separate sample was incubated with lysis buffer only as a control. The final pellet was resuspended in Laemmli sample buffer and probed by western blotting. Western blots for Cx43 were conducted using methods previously described.¹³

Surface Plasmon resonance (SPR)

SPR is a spectroscopic method to determine binding amplitude and kinetics in real time.¹⁵ Experimental details were as described previously.¹¹ Briefly, recombinant Cx43CT was

covalently bound to a carboxymethyl dextran matrix. Dissociation constants (K_D) were calculated from the time course of binding and unbinding of the ligand, using a 1:1 (Langmuir) association and dissociation kinetic model (*Biacore software package*).

Statistical analysis

Data were statistically compared by Student's t test. Comparisons were made utilizing the average asymptotic G_j value of either control, or peptide-treated cells. Only one comparison (against control) was allowed for each data set. Differences yielding p values <0.05 were regarded as significant. Average data are presented as mean ± standard error of the mean.

Molecular modeling

The HyperChem (version 7.5, Hyper Cub. Inc.) program was used for visualization of spatial structures, overlays and conformations. Force-field analysis was conducted using Amber97, and for optimization the Polak-Ribiere algorithm (conjugate gradient) was applied.

RESULTS

Peptide “RRNY” failed to prevent octanol-induced uncoupling

Our previous studies demonstrated that sequence RRNYRRNY and similar cyclized analogs prevented octanol-induced uncoupling of Cx43 channels.¹⁰ From those and other results, we speculated that the tetrapeptide “RRXY” contained the core active sequence. However, initial studies showed that this tetrapeptide (X=N) was unable to preserve coupling in Cx43-expressing N2a cell pairs exposed to 1.5mM octanol. Figure 1 shows the results. For each experiment, junctional conductance (G_j) during octanol superfusion was measured relative to control (i.e., G_j recorded prior to initiation of superfusion with the uncoupler). Average values were calculated for each time point after the onset of octanol superfusion. Black symbols correspond to data obtained in the absence of peptide, and show the rapid development of uncoupling caused by octanol superfusion. Red symbols depict data obtained when peptide RRNY was present in the internal pipette solution. The G_j rapidly progressed to zero in the presence of peptide RRNY, in a manner similar to that observed in the control (fig 1). We speculated that the lack of activity in peptide RRNY was consequent to the loss of a stable structure of the core active component, or its rapid degradation in the intracellular space (see¹⁰; see also “Discussion”). However, an alternative hypothesis was that the active element in the RRNYRRNY peptide¹⁰ is a different combination of amino acids within that sequence (e.g., RNYR, NYRR, RNYRR, etc). As an approach to assess the latter possibility, we generated peptides where the RRNY elements were preserved, but separated by a “linker” sequence that did not affect the Cx43-related function of the peptide. The choice of a sequence for such a linker was based on results obtained with our first-generation compound RXP-E¹¹, as described below.

Linker sequence GGGSAPFYSHS as an inactive component of RXP-E

RXP-E is a 34-amino acid peptide that prevents chemical gating of Cx43 and of cardiac gap junctions.¹⁰ The molecule is composed of two RXP-containing domains, separated by a linker of sequence GGGSAPFYSHS (one-letter amino acid code; see¹¹). To assess the relevance of the linker in RXP-E function, a peptide was generated where the linker sequence was deleted (Figure 2, top). As shown in Figure 2A, Cx43 was pulled down from adult rat heart lysate using glutathione beads coated with a GST-RXP-E protein. Cx43 was also precipitated by the linker-less RXP-E peptide (RXPE-LX), but not by a protein where residues 5, 7, 31 and 33 were substituted with alanine, thus eliminating two of the three RXP motifs in the sequence. Cumulative data are shown in Figure 2B. To determine whether these results correlated with the activity of RXP-E on functional gap junctions, we tested the time course and extent of

octanol-induced uncoupling in a peptide where either the linker domain alone, or in addition to the RXP-containing domains, was disrupted. As shown in Figure 2C, RXPE remained active even if the linker sequence was deleted. Indeed, although a decrease in average G_j was observed, all Cx43-expressing N2a cell pairs tested remained coupled after 10 minutes of octanol superfusion. Yet, as shown in panel 2D, substitution of residues 5, 7, 31 and 33 for alanines abolished the ability of the peptide to prevent octanol-induced uncoupling, indicating the importance of specific RXP motifs to retain activity and emphasizing that, as demonstrated in our previous publication,¹¹ the RXP sequence is not, per se, capable of preventing octanol-induced uncoupling (see, e.g., Figure 3A of¹¹). These results were consistent with the ability of the peptide to biochemically interact with the Cx43 protein (2A and 2B) and show that the linker sequence does not, per se, confer activity to RXP-E. We therefore used that sequence to assess whether peptide RRNYRRNY retains function when the two RRNY elements, purportedly the unitary active cores, were physically separated and as such, less likely to conform an active pharmacophore that is not contained within the RRNY structure.

RRNY domains separated in primary sequence retained pharmacophore activity

Figure 3 shows dual patch clamp results obtained when RRNY repeats were separated by linker sequences. “L” refers to the linker sequence in RXP-E (see also Figure 2, top). For the experiments in panels A and B, the sequence RRNY-L was repeated three and two times, respectively. There was a tendency toward improved efficacy when more RRNY elements were included in the same primary sequence. More importantly, the three peptides tested prevented complete uncoupling in all experiments, and the minimum G_j value at the end of the 10 minutes of octanol superfusion was significantly different from that recorded from control cells ($P=0.003$, 0.004 and 0.003 respectively). Finally, surface plasmon resonance studies showed that the ability of peptides to bind to the carboxyl terminal domain of Cx43 was unaffected by the physical separation of the two RRNY elements (Figure 4). Panel A shows the binding of peptide RRNYGGSAVPFYSHSRNY to the CT domain of Cx43, covalently bound to a carboxylmethyl dextran matrix (see¹⁵ for details). Upward arrow points to the onset of superfusion of the peptide and downward arrow indicates the beginning of washout. The rates of association and dissociation were too fast to estimate binding kinetics but the peptide showed a clear interaction with the bound ligand. Panel B shows the relation between binding amplitude (in resonance units; ordinates) and the molecular weight of the peptides tested. Two RRNY domains were separated by various linkers. The sequences of the peptides are shown on the right and the molecular weights are shown in parentheses. The data show a linear association between binding amplitude and molecular weight of the analyte. A linear association is expected when the binding amplitude is unaffected by the specific changes in the sequence, and only a function of the difference in mass (see¹⁵). Overall, our data showed that two or more RRNY elements physically separated by various sequences retained their ability to bind Cx43CT and prevented octanol-induced uncoupling of Cx43 channels. We therefore sought to modify the design of the basic RRXY sequence to enhance its activity when isolated from other flanking residues.

Molecular design of peptide ZP2519

In our previous studies, we proposed that the pharmacophore activity of RRXY peptides was related to the distance between the guanidino groups of the two arginine side chains, and the aromatic ring of tyrosine. We therefore carried out computer simulations to identify a synthetic molecule that retained this pharmacophore triangle structure but utilized non-natural building blocks. Indeed, we speculate that the RRNY tetrapeptide may be subject to degradation; accordingly, substitution with peptidomimetic structures would make the compound less likely to act as a substrate for digestion by intracellular proteases.¹⁶ As shown in Figure 5A, the successful strategy involved: a) replacing the linker amino acid asparagine (N) with lysine; b) covalently linking the aromatic ring of the tyrosine side chain to the ϵ -amino group of the lysine

and c) deleting the C-terminal tyrosine. This compound (RRK-(4-hydroxyl-benzoyl); molecular weight, 619Da; peptide dubbed “ZP2519”) presented the same triangular structure as RR-Y in the original parent molecule (Figure 5B; see also ¹⁰) and effectively prevented octanol-induced uncoupling of Cx43 channels in Cx43-expressing N2a cells (Figure 5C).

Effect of ZP2519 on pH gating of cardiac gap junctions

Cx43 uncoupling can be induced by a variety of modifications in the microenvironment of the channel. Of particular relevance to pathophysiology, an increase in intracellular proton concentration (a decrease in intracellular pH) leads to closure of connexin channels.¹⁷ We therefore assessed whether peptide ZP2519 can prevent acidification-induced uncoupling of cardiac gap junctions. Results are shown in Figure 6A. Neonatal rat ventricular myocytes were recorded in the dual patch clamp configuration. The pH of the internal pipette solution was decreased to 6.2, and peptide ZP2519 was dissolved in this solution, as in our previous studies.^{10,11,13} Red and black symbols indicate, respectively, the average normalized G_j value recorded as a function of time after patch break in cell pairs where ZP2519 was present, or absent, in the internal pipette solution. ZP2519 prevented acidification-induced uncoupling; indeed, in the presence of the peptide, G_j decreased progressively, but reached an asymptotic value that was significantly higher than the one recorded in control conditions ($p < 0.001$). These results show that pH gating of cardiac gap junctions can be prevented by a small peptidomimetic molecule derived from the “RXP” family of Cx43-binding compounds. Similar results were obtained in Cx43-expressing N2a cells, thus confirming the interaction between ZP2519 and Cx43, regardless of the cellular environment (figure 6B).

ZP2519 activity requires the presence of the CT domain of Cx43

Given the molecular weight of ZP2519, binding experiments were not feasible, since its mass is lower than the level of detection of conventional surface plasmon resonance. However, to assess (even if indirectly) the hypothesis that ZP2519 interacts with the carboxyl terminal domain of Cx43, experiments were conducted in N2a cells transfected with a Cx43 mutant construct lacking most of the carboxyl terminal domain (mutant M257; position K258 replaced with a stop¹⁸). As shown in Figure 6C, octanol-induced uncoupling of M257 channels progressed with a similar time course regardless of whether or not the peptide was present in the patch pipette. Moreover, ZP2519 was ineffective at preventing octanol-induced uncoupling of Cx40 channels (Figure 6D), thus arguing that this molecule requires of specific domains of the Cx43CT domains for its action.

DISCUSSION

In a previous study, we reported that short peptides, six to eight amino acids long, prevented chemically-induced uncoupling of Cx43 channels.¹⁰ Molecular modeling suggested that the activity of those peptides was conferred by the triangular structure formed by the guanidino groups of two arginines, and the aromatic ring of a neighboring tyrosine residue. That prediction, however, awaited experimental confirmation using a molecule minimized to its three key structural elements. The data presented here provide strong experimental support to the *in silico* results. The two Arginines and the phenol group were assembled forming the correct triangular structure using Lysine as a chemical scaffold. In this manner, we have generated the first RXP-derived peptidomimetic molecule with preserved activity as a gap junction opener (ZP2519). More importantly, we have identified this simple triangular pharmacophore model as a valid platform for the design of synthetic compounds that prevent chemical gating of Cx43. The latter represents a major step toward generation of future compounds of minimum size and preserved activity that can be modified for use in tissue and organ preparations.

The data presented here result from our continuous effort to develop a pharmacology for gap junctions.^{10,11,13} This project focuses on Cx43 regulation as our target. Yet, we have utilized drug design strategies previously applied to the generation of other modifiers of gap junction channels.⁶ From that perspective, our studies represent the combination of two previously-independent lines of investigation: one, to characterize the molecular mechanisms of Cx43 pH gating¹⁹ and the other one, to generate pharmacologically active compounds based on peptidic sequences with biological action.¹¹ In this process, we have reduced the active compound from the original 34 amino-acid polypeptide (RXP-E¹¹) to a ~600 Da peptidomimetic with preserved function. We have also demonstrated that the activity of RXP-E resides in the RXP-containing domains and not in the linker region. Further minimization of RXP-E may lead to additional structural information relevant to the design of Cx43-binding molecules.

Our previous studies, and those presented here, show that the activity of these pharmacophores on gap junction channels requires the preservation of the CT domain of Cx43. At first glance, it seems paradoxical that these peptides prevent octanol-induced uncoupling, since the effect of n-alkanols is not dependent on the integrity of the CT domain (see, e.g., Figure 6 and also¹¹). On the other hand, our previous data indicate that the interaction of RXP-derived peptides with the CT domain stabilizes the channel in the open configuration (see Figure 4 of¹¹). We therefore speculate that RXP-derived peptides bind to the CT domain of Cx43 and that this interaction, directly or indirectly, “locks” the channel in the open configuration, thus preventing closure even when non-selective uncouplers are used.

Our data indicate that the RR-Y structure is a novel Cx43CT binding motif, and its secondary structure predicts a corresponding high order region in the Cx43CT molecule. Cx43 is a highly promiscuous molecule, capable of interacting with a variety of kinases, scaffolding and adapter proteins.²⁰ Yet, structural information for Cx43 binding domains is available only for a limited number of Cx43 partners, such as the SH3 domain of src²¹ and the second PDZ domain of ZO1. In both cases, the binding pockets provide information as to the structure of the interacting region of Cx43CT once in contact with its partner molecule. Identification of the Cx43CT region that binds the RR-Y structure, and characterization of the Cx43CT structure when in the presence of the pharmacophores, will give new insight into the structure-function relation of the regulatory domain of Cx43 and further enhance our ability to design more effective pharmacophores. Taken altogether, our studies validate the notion that the carboxyl terminal domain of Cx43 can be an important target for pharmacological intervention (see also^{10,11,13}).

At present, pharmacological intervention of cardiac arrhythmias relies, largely, on the use of molecules that impair the function of ion channel proteins. Moreover, while a number of drugs are available to affect sodium, calcium or potassium currents, gap junctions have eluded the action of exogenous compounds. Whether cardiac gap junctions can be successfully targeted in antiarrhythmic therapy remains unclear. One of the reasons why this question remains unanswered is the lack of compounds that can, selectively and effectively, interfere with gap junction function. In this project, we set out to develop small molecules that can restore or preserve the function of the major cardiac gap junction channel, connexin43. In the course of our studies, we have progressed, from a 34 amino acid peptide¹¹ to the first structure-based small peptidomimetic molecule with Cx43 action. Moreover, we have identified a structural platform that can be used in the design of improved Cx43 regulators. The latter represents an important new advance in the progression toward rational drug design for gap junction pharmacology.

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Abbreviations

Cx43   Connexin 43

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Figure 1.

Peptide RRNY failed to prevent octanol-induced uncoupling of Cx43 channels exogenously expressed in N2a cells. Peptide was diluted in the internal pipette solution at a concentration of 100 μ M. Red and black symbols represent, respectively, data obtained from cell pairs in the presence or absence of the peptide (N=5 for both groups). These results contrast with those previously obtained from peptide RRNYRRNY.¹⁰

Figure 2.

Linker sequence in RXPE is not necessary for activity. **A.** Western blot for Cx43 precipitated from rat heart lysate by using a GST-RXPE recombinant protein. Symbols “-” and “+” correspond, respectively, to experiments done in the absence or presence of heart lysate. Construct “GST” acts as a negative control. “RXPE-LX” refers to an RXPE construct lacking the linker domain (amino acids in black on the top of panel; one letter code). Notice Cx43 still precipitates with RXPE in the absence of the linker sequence. However, substitution of amino acids 5R, 7P, 31R and 33P for alanines (thus eliminating active “RXP” motifs; construct RXPE 5,7,31,33a) failed to associate with the gap junction protein. Cumulative data from densitometry relative to native RXPE binding is shown in **B.** Panels **C** and **D:** Dual patch clamp experiments in Cx43-expressing N2a cells showing junctional conductance values (Gj; relative to value prior to onset of octanol superfusion in each experiment) during exposure to 1.5mM octanol). Sequence of the peptide tested (100μM for both **C** and **D**) is shown on top. Red and black symbols correspond, respectively, to data obtained in the presence and in the absence of the peptide. Consistent with the pulldown data in panels **A** and **B,** loss of active “RXP” consensus sites prevented the effect of the peptide; however, the peptide was still active even if the linker sequence was deleted.

Figure 3.

Peptides with single RRNY domains separated by inactive linkers still prevented octanol-induced uncoupling in Cx43-expressing N2a cells. “L” refers to the linker sequence in RXP-E. The number in subscript refers to the number of RRNY-L repeats in the peptide. Red and black symbols correspond, respectively, to data obtained in the presence or absence of the peptide diluted in the patch pipette to a final concentration of 100 μ M. Abscissae reflect the time elapsed after onset of octanol superfusion; for panels A, B and C, N values for the peptide series are 6, 5 and 8, respectively. N = 6 for control groups.

Figure 4.

Surface plasmon resonance analysis of peptide binding to recombinant carboxyl terminal domain of Cx43 (Cx43CT). Panel A: Trace obtained from exposure of Cx43CT, bound to a carboxymethyl dextran matrix, to peptide of sequence shown at the top of the panel. Upward and downward arrows correspond, respectively, to the onset and end of peptide perfusion. Panel B: Collective data of binding amplitude (in resonance units) elicited by the peptides shown on the right, as a function of the molecular weight (m.w.) of the analyte. The data indicate that linker sequences did not interfere with the ability of domain RRNY to bind to Cx43CT.

Figure 5.

Modification of peptide RRNY to AcRRK(4-HO-Benzoyl)-NH₂ (dubbed “ZP2519”). Panel A: Strategy for size reduction and generation of the peptidomimetic. Panel B: In silico data showing preservation of the pharmacophore triangle in peptide ZP2519 when compared to the parent RXP-4 molecule (see¹⁰). Panel C: Dual patch clamp data show that ZP2519 (100μM) prevents octanol-induced uncoupling of Cx43-expressing N2a cells (red symbols, N=7; black symbols, N= 6).

Figure 6.

Panels A and B: ZP2519 prevented, respectively, acidification-induced uncoupling of neonatal rat ventricular myocytes and of Cx43-expressing N2a cells. ZP2519 (100 μ M) was diluted in an internal pipette solution buffered to pH 6.2 (red symbols; N=6; black symbols: data collected in the absence of peptide; N= 6). Panels C and D: ZP2519 (100 μ M) did not prevent octanol-induced uncoupling in N2a cells expressing a mutant of Cx43 lacking its carboxyl terminal domain (N=5 for peptide and controls) or an alternative connexin isotype, Cx40 (panel D, N=5 for both groups).