Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters

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Submitted 9 March 2007; accepted in final form 19 July 2007


Connexin mimetic peptides are widely used to assess the contribution of nonjunctural connexin channels in several processes, including ATP release. These peptides are derived from various connexin sequences and have been shown to attenuate processes downstream of the putative channel activity. Yet so far, no documentation of effects of peptides on connexin channels has been presented. We tested several connexin and pannexin mimetic peptides and observed attenuation of channel currents that is not compatible with sequence specific actions of the peptides. Connexin mimetic peptides inhibited pannexin channel currents but not the currents of the channel formed by connexins from which the sequence was derived. Pannexin mimetic peptides did inhibit pannexin channel currents but also the channels formed by connexin 46. The same pattern of effects was observed for dye transfer, except that the inhibition levels were more pronounced than for the currents. The channel inhibition by peptides shares commonalities with channel effects of polyethylene glycol (PEG), suggesting a steric block as a mechanism. PEG accessibility is in the size range expected for the pore of innexin gap junction channels, consistent with a functional relatedness of innexin and pannexin channels.

GAP JUNCTION MIMETIC PEPTIDES were originally designed to mimic the docking gate of gap junction channels (11, 13). Gap junctions in vertebrates typically are composed of connexins, which form hexameric connexons (22, 41, 45). Under physiological conditions, connexons are closed. Upon docking of two connexons residing in apposed cell membranes, the complete gap junction channel forms and opens. The docking process involves the two extracellular loops of the connexin protein (11, 13, 45). It was expected that peptides with sequences contained in these loops would mimic the homophilic loop-loop interaction and activate the docking gate. However, this expectation was only partially fulfilled. Binding of peptides to connexons did not open them so that a nonjunctional conductance would develop (13). However, the peptides did attenuate gap junction channel formation (11, 13, 44). This inhibition was connexin specific and gave rise to the development of a series of inhibitors of gap junction formation by the various connexins (18, 26).

Connexin mimetic peptides represent the only specific inhibitors for gap junction channel function except for small interfering RNA. However, their use has been limited due to the low efficacy of inhibition (40–50%) and slow onset of action. A faster action has been observed in paired oocytes when peptides are applied to single oocytes before pairing. Application of peptide after pairing had no acute effect on the existing gap junction channels, suggesting that only channel formation, but not channel function, is interfered with by the peptides. This finding explains the slow effect of peptides in tissue cultures, where in the course of turnover of gap junctions, only the new formation of channels can be attenuated.

More recently, a faster action of connexin mimetic peptides has been observed under certain experimental circumstances. For example, “connexin mimetic peptides” inhibited calcium wave propagation but had no effect on dye coupling (5, 8, 14, 15, 17, 19–21, 27, 35). The readout in all these studies was not direct channel activity, but indirect parameters such as measurements of calcium transients and release of ATP. These effects were interpreted as evidence for active “hemichannels”, that is, for connexons to provide patent pathways to the extracellular space. From the attenuation of the measured parameters, it was inferred that the peptides inhibited rather than activated gap junction hemichannels, that is, connexons. While most of the peptides used in these studies contained extracellular loop sequences, recently, it was reported that Gap 24, a peptide with sequence contained in the cytoplasmic loop, was more effective in attenuating the measured parameters (14). The theoretical framework for such an action is not clear.

To date, no evidence for a direct effect of the mimetic peptides on connexon channel function has been provided, although binding is evident (29). Yet mimetic peptides are widely used and the effects on measured parameters like ATP release are interpreted as an involvement of connexons in the measured functions (5, 8, 14, 15, 17, 19–21, 27, 35, 40). A major reason for the lack of documentation of peptide effects on channel function is that direct connexon channel function has not been documented either, except in a few cases. Under physiological conditions, only the lens connexins Cx46 and Cx50 form patent connexons, that is, channels in the nonjunctural plasma membrane connecting cytoplasm and extracellular space (2, 34, 46). Because these channels represent half of a gap junction channel, they are often referred to as “hemichannels.” However, even the observation of functional Cx46 and Cx50 connexons is restricted to the oocyte expression system. This type of channel activity is by and large not seen in other expression systems and might not occur in vivo (10, 42). Connexon activity by other connexins is usually only observed under extreme conditions, like depolarization to more than...
+50 mV, and even then the events of channel openings are extremely rare (7).

Here, we examined the effects of several gap junction protein mimetic peptides on the genuine hemichannel currents mediated by Cx32E143, Cx46, and pannexin1. We report that gap junction mimetic peptides indeed attenuated channel currents. The mechanism of action appears to have been by steric block rather than by sequence-specific interactions, as previously suggested.

MATERIALS AND METHODS

Experimental Procedures

Preparation of oocytes. Preparation of oocytes and electrophysiological recording were performed as described (9, 12). Mouse pannexin1 was kindly provided by Dr. Rolf Dermietzel (University of Bochum), and Cx46 was obtained from Dr. D. L. Paul, (Harvard University). The generation of Cx32E143 was described previously (37).

Synthesis of mRNA. The plasmid containing Cx32E143 (pGEM 3Z; Promega, Madison, WI) was linearized with SspI and transcribed with SP6 polymerase. The plasmid containing Cx46 (rSP64T) was linearized with EcoRI and transcribed with SP6 polymerase. Pannexin1, in pCS2, was linearized with Not I. In vitro transcription was performed with the polymersases T3 or SP6, using the Message Machine kit (Ambion, Austin, TX). mRNAs were quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. In vitro transcribed mRNAs (~20 nl) were injected into Xenopus oocytes.

Electrophysiology. The oocytes were incubated at 18°C for 18–24 h in oocyte Ringer solution (in mM): 82.5 NaCl, 2.5 KCl, 1 MgCl2, 1 CaCl2, 1 Na3HPO4, and 5 HEPES, at pH 7.5.

For determination of junctional conductance, devitellinized oocytes were paired with or without pretreatment with peptides. Each oocyte of a pair was voltage clamped with two intracellular electrodes, and the membrane potential was held at ~50 mV. Five-millivolt voltage steps were applied to one oocyte. The transjunctional current is of the same magnitude but of opposite sign to the current required to keep the potential of the second oocyte constant.

Whole cell membrane current of single oocytes was measured using a two-microelectrode voltage clamp (Genclamp 500B; Axon Instruments, Sunnyvale, CA) and recorded with a chart recorder (Soltec, San Fernando, CA). Both voltage-measuring and current-passing microelectrodes were pulled with a vertical Puller (David Kopf Instruments, Tujunga, CA) and filled with 3M KCl. The recording chamber was perfused continuously with solution. Membrane conductance was determined using voltage pulses. Oocytes expressing Cx32E143 or Cx46 were held at ~20 mV, and depolarizing pulses of 5-s duration and of 5 or 10 mV amplitude were applied. Oocytes expressing pannexin1 were held at ~60 mV, and pulses to +60 mV were applied to transiently open the channels.

Peptides. The use of the peptides 32Gap24, 43Gap26, 43Gap27, and 10Panx1 have been published (14, 18, 36). Their sequences are GHGDPLHLEEVKC (32Gap24), VCYDKSFPISHVR (43Gap26), SRPTEKTIIFH (43Gap27), and WRQAADFVDSY (10Panx1). The additional Pannexin1 peptides used here had the following sequences: E1a, AQEISIGTQIS; E1b, SSFSWRQAFVVD; E1c, SESGNLPLWHLK; E2a, SSSLSEDEFVCSIKS; and E2b, KGSGLRNDSTVPDQ.

Dye uptake. Cx32E143 or pannexin1-expressing oocytes were preincubated with the test substances in Ringer solution for 5 min. The cells were then transferred to a 100 mM potassium gluconate solution containing 10 mM 6-carboxyfluorescein and the test substances for 20 min. After extensive washing in Ringer solution, oocytes were frozen for cryosectioning. Data were analyzed with NIH image software program (http://rsb.info.nih.gov/nih-image/).

Statistics

To determine %inhibition of membrane currents, lines were drawn on enlarged records through peak currents before and after peptide application and during peptide application as shown in Fig. 1. The same was done at the baseline to account for eventual baseline shifts. Percent inhibition was calculated, and t-tests for correlated samples were performed for statistical analysis. In all figures, statistical significance is indicated as **P < 0.01; *P < 0.05; not significant = P > 0.05.

RESULTS

Single connexons are typically closed and may open only under extreme conditions like holding the membrane potential at more than +50 mV or exposing cells to zero extracellular calcium concentration. However, for unknown reasons, the lens connexins Cx46 and Cx50 form patent membrane channels when expressed in Xenopus oocytes (2, 34, 46) but not in other cells. Furthermore, a connexin chimera Cx32E143 provides a nonjunctional membrane conductance when expressed in oocytes (37). Apparently, the mutation, which replaces the first extracellular loop of Cx32 by the equivalent sequence of Cx43, alters the docking gate so that moderate depolarization is sufficient to open the channel. The channel shares gating and permeability properties with the parent Cx32 gap junction channel and thus is a useful model for Cx32 channels (23, 39).

Connexin mimetic peptides used to study nonjunctional effects mainly contain sequences of Cx43 or Cx32. Gap26 and Gap27 are the most commonly used peptides and comprise conserved sequences in the extracellular loops of connexins. 32Gap24 represents part of the intracellular loop domain of Cx32. Cx32E143 is uniquely suitable to test the effect of these peptides because it forms both gap junction channels and a robust nonjunctional membrane conductance. In addition, it contains at least two, if not all, target sequences for the three peptides.

To test the activity of connexin mimetic peptide 43Gap26, Cx32E143-expressing oocytes were devitellinized, treated with 43Gap26 for 15 min, and paired in the continued presence of the peptide. Junctional conductance was determined 3 and 5 h after pairing by dual-voltage clamp. Figure 2A shows that gap junction channel formation is attenuated by the peptide. On the other hand, delayed application of the peptide to oocyte pairs with established gap junction channels did not acutely affect junctional conductance. Only over an extended time span, a moderate attenuation of junctional conductance could be observed (not shown). These findings are in agreement with previous studies that have shown that the peptides interfere with the formation but not the function of gap junction chan-

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Fig. 1. Peak currents induced by depolarizing pulses (remaining current trace is clipped). Upper line connects peaks before and after peptide application. Lower line connects peaks during application of test substance. Distance between lines and full amplitude of current were used to determine %inhibition, which was ~3% in the given example.
nel. 43Gap27 was tested correspondingly, using wild-type Cx43-expressing oocyte pairs (Fig. 2B). The inhibitory effect of both peptides was prominent at the 3-h time point but subsided within 5 h after application of the peptides and cell pairing. Because the peptides were not replenished during this time period, they probably were depleted by various mechanisms, including endocytosis. Another reason for the vanishing peptide effect is the experimental design. Oocytes were allowed to accumulate a large pool from which gap junctions could form rapidly upon cell pairing. This pool of protein can bind the peptides. Subsequently, gap junction formation rate slows as it is then dependent on newly synthesized protein.

As shown previously (23, 24, 37, 39), Cx32E143 induces a nonjunctional membrane conductance, which shares properties with the gap junction channels formed by this connexin chimera. The shared properties include attenuation of channel activity by cytoplasmic acidification. Application of 43Gap26 to voltage-clamped single oocytes did not acutely affect membrane currents carried by Cx32E143 (Fig. 3A). However, an attenuation of the membrane conductance was observed over a time span of several hours (Fig. 3B).

The peptide 43Gap27 similarly had no acute effect on the membrane conductance of Cx32E43 channels. Long treatment with this peptide resulted in conductances that were not significantly smaller than observed in untreated cells (Fig. 3C). 32Gap24, which shares amino acid sequence with cytoplasmic aspects of Cx32, similarly did not acutely affect the membrane conductance provided by Cx32E43 channels (Fig. 4A).

These data apparently are in opposition to those in previous reports, where connexin mimic peptides were used to atten-
uate ATP release and/or calcium wave propagation, and their effect had been interpreted as evidence for the involvement of connexins in these phenomena. However, over the last few years, the pannexin channel has been shown to fulfill the requirements of an ATP release channel better than connexons. Pannexin 1 forms membrane channels that open under physiological conditions. Opening can be achieved by depolarization (6) or at the resting potential by either mechanical stress (1) or by an increase in cytoplasmic calcium concentration (32). We therefore tested the effects of the peptides 43Gap27 and 32Gap24 on pannexon channel activity. Figures 4, B–D show that membrane currents carried by pannexons were rapidly and reversibly inhibited by both peptides. 32Gap24 did not significantly affect the currents carried by Cx46, while a scrambled version of the peptide was similarly effective as the authentic 32Gap24 in attenuating pannexin currents (Fig. 4, B and C).

Because inhibition of pannexin currents by the connexin mimetic peptides was limited, we tested whether peptides with sequences contained in the putative extracellular loops of pannexin1 (“Pannexin mimetic peptides”) would be more specific and have stronger effects on pannexin channel activity. Of the five pannexin1-based peptides covering the extracellular loop sequences of the protein, only two inhibited pannexin currents by more than 10% (Fig. 5). However, the degree of inhibition was not significantly different from that obtained with connexin mimetic peptides on the same channel. Recently, another pannexin1 mimetic peptide (10panx1) was reported to inhibit dye uptake by macrophages without affecting membrane currents of the cells (36). 10panx1 is a three amino acid shorter version of peptide PanxE1b. In oocytes, 10panx1 inhibited pannexin1 currents to a similar extent as PanxE1b (Fig. 5). A scrambled version of 10panx1 was less effective than the authentic peptide.

To test the specificity of the pannexin mimetic peptides, they were applied to oocytes expressing the lens-specific gap junction protein Cx46. Cx46, in addition to forming gap junction channels, also forms nonjunctional membrane channels in oocytes but apparently not in other cells. Even in the lens, membrane currents that could be attributed to Cx46 only were observed when the extracellular calcium concentration was reduced well below normal (16). The depolarization-induced Cx46 currents in oocytes were attenuated by the pannexin mimetic peptide almost to the same extent as the pannexin currents (Fig. 6). In contrast, 32Gap24 and its scrambled version did not significantly inhibit Cx46 connexon currents.

These data suggest, that gap junction mimetic peptides contrary to the widely held belief do not interfere with channel function by sequence-specific peptide–protein interactions. Instead, the data are consistent with a mechanism by which peptides may affect channel function by steric block. Therefore, we tested whether inert molecules like polyethylene glycols (PEGs) interfere with channel currents. As shown in Fig. 4, 32Gap24 affects pannexon currents but not currents carried by Cx32E143. A: oocytes expressing Cx32E143 were held at −20 mV and depolarizing 10-mV pulses were applied. Application of 32Gap24 did not affect acutely the membrane currents, while subsequent cytoplasmic acidification with CO₂ attenuated the currents. B: oocytes expressing mouse pannexin 1 (mPanx1) were held at −60 mV and pulses to −60 mV were applied to activate pannexin channels. 32Gap24 attenuated the currents in a dose-dependent manner. Carbamoyl xanthine also inhibited the currents. C: quantitative analysis of inhibition of membrane currents carried by channels formed by pannexin1, Cx46, and Cx32E143 by 200 μM 32Gap24 or a scrambled version of the peptide (32Gap24scr). Values are means ± SE. The number of oocytes analyzed is indicated above the bars. D: Effect of 43Gap27 on pannexon currents. Oocytes expressing pannexin1 were held at −60 mV and pulses to +60 mV were applied. The current pulses were attenuated acutely by 200 μM 43Gap27 peptide; mean inhibition was 23.2 ± 3.6% (n = 3). *P < 0.05; **P < 0.01.
7, PEGs attenuated pannexin currents in a size-dependent manner, with peak inhibition obtained with PEG1500. The level of inhibition for dye uptake by equimolar peptides was considerably more pronounced than for channel currents.

DISCUSSION

In agreement with previous reports (11, 13, 18, 26, 44), the present data indicate that connexin mimetic peptides inhibit the formation of gap junction channels by binding to their target sequence, the connexins. However, the peptides did not have an acute effect on the nonjunctional membrane channels formed by the connexin chimera used in this study. Only over a time span of hours, a retardation of the typical increase in membrane conductance was observed. This slow effect is inconsistent with a gating mechanism of the channel and is even too slow to account for alteration of channel activity due to peptide-induced secondary modifications of the protein. Instead, the time course is consistent with the life-time of the protein, and it is thus conceivable that the bound peptide tags the protein for degradation.

All three connexin mimetic peptides tested here, however, attenuated the currents carried by membrane channels formed by the unrelated protein pannexin1. A scrambled version of the sequence of one of the connexin mimetic peptides was less effective in inhibiting pannexin1 currents than the peptide with the correct connexin sequence, as if the inhibitory effect was sequence specific. The question thus arises whether pannexin1 exhibits a cryptic binding site for the peptides that is not deciphered by simple sequence alignment between connexin...
and pannexin. If a regulatory binding site existed on pannexin membrane channels, one would expect higher efficacy with authentic pannexin1 mimetic peptides. Pannexin mimetic peptides also inhibited pannexin1 currents, but they were only marginally more effective than connexin-mimetic peptides. Thus a sequence-dependent binding of the peptides seems to be unlikely. On the other hand, for the two peptides, where scrambled versions were tested, the authentic sequences were more effective. Traditionally, such a result is taken as evidence for specific interactions of a peptide with its target sequence. However, the scrambled peptides yielded significant inhibition, albeit low, which moderates the specificity argument. The lesser effect of the scrambled peptides does not rule out a steric effect because, despite the same amino acid content, the scrambled peptides do not need to fold the same way as the authentic sequence. They could be either larger and be (partially) excluded from the channel or they may be smaller and occlude less of the channel. None of the peptides exceeded 30% inhibition of channel currents, indicating that the mechanism of channel closure is inefficient in terms of interference with the permeation of small ions. However, inhibition of dye uptake was much more pronounced. This suggests that the transit of larger molecules, including ATP and dyes, is more severely affected by the peptides because of steric constraints on the channel. This could explain the high levels of inhibition of downstream events reported in other studies (5, 8, 14, 15, 17, 19–21, 27, 35, 40).

The rationale for a specific action of gap junction protein mimetic peptides as inhibitors of nonjunctional membrane channels is murky. If the mechanism of action of the peptides involved a gating process one might expect the contrary, opening of the connexin/pannexin channels, assuming the peptides with extracellular loop sequences were to mimic the action of the docking process in gap junction channel formation. The rationale for the use of 32Gap24, which has the sequence of cytoplasmic aspects of connexin32, is even more mysterious (14). The high peptide concentrations required for significant effects on channel currents as shown here or on downstream events such as ATP release or calcium wave

Fig. 7. Effect of polyethylene glycol (PEG) on pannexin currents. A: oocytes expressing pannexin1 were held at −60 mV and pulses to +60 mV were applied. The current pulses were attenuated acutely by polyethylene glycol (PEG1500) in a dose-dependent fashion. B: size-dependent inhibition of pannexin currents by PEGs. PEGs (1 mM) at the indicated molecular sizes were applied to oocytes expressing pannexin1. C: quantitative analysis of inhibition of pannexin1 currents by 10 panx1, 10panx1scr, and PEG1500 at 200 μM concentration and of PEG 1500 at 1 μM. Values are expressed as means ± SE. The number of oocytes analyzed is indicated above the bars. *P < 0.05; **P < 0.01.

Fig. 8. Dose-response curves of pannexin inhibition by peptides and PEG1500. Peptides and PEG at various concentrations were applied as indicated by the color code. Recording conditions were as described in Fig. 7.
molecules through gap junction channels in invertebrates (33), which are formed by innexins (38).

Connexin mimetic peptides have been widely used to assess the contribution of connexin hemichannels to various physiological functions, particularly their involvement in calcium wave propagation. In these studies, ATP release or downstream events to the release were measured, and the effects of the peptides on these parameters were determined. We are not aware of any published data showing that the connexin mimetic peptides actually interfere with connexin hemichannel activity. Because connexin-mediated hemichannel activity probably does not occur in vivo, it is not surprising that such documentation is lacking. No currents that could be unequivocally attributed to connexin hemichannels have ever been described in vivo. The examples where genuine connexin hemichannel activity has been demonstrated is limited to mutant connexins (28, 37, 43) and extreme experimental conditions for wild-type connexins, like membrane potentials exceeding +50 mV (7). But even under such extreme conditions, channel activity was so low that single channel events could be resolved in whole cell patch recordings. Even the lens connexins, Cx46 and Cx50, which form genuine nonjunctional channels when expressed in Xenopus oocytes, may not do so in the intact lens unless extracellular calcium is removed (16).

The data reported here suggest that the effect of connexin mimetic peptides is indicative of pannexin channel activity rather than channels formed by connexin hemichannels. Therefore, a reinterpretation of reports that describe connexin mimetic peptides as evidence for the involvement of connexin hemichannels in the generation and propagation of intercellular calcium waves (5, 8, 14, 15, 17, 19–21, 27, 35) seems warranted. Pannexons are more likely to be ATP release channels in intercellular calcium waves, because in contrast to connexin hemichannels, the channel properties of pannexons are consistent with such a function. The pannexon channel is highly permeable to ATP, can be opened at the resting potential by mechanical stress, by increase in cytoplasmic calcium, and by extracellular ATP through P2Y or P2X7 receptors (1, 31, 32). Furthermore, pannexin 1 is expressed in the right places, that is, where ATP release takes place. Pannexin 1 is typically found in the nonjunctional membrane and is expressed in cells notable for ATP release and calcium waves, including erythrocytes, endothelial cells, and astrocytes (10, 25, 30, 31).

Recently “pannexin mimetic” peptides have been used to assess the contribution of pannexin 1 in P2X7 signaling (36). It appears that these peptides have the same fate as “connexin mimetic” peptides. Although independent approaches verified the conclusion that the large pore of P2X7 is formed by pannexin 1 (31), the lack of specificity of the “pannexin mimetic” peptides excludes them as evidential tools. The lack of specificity is indicated by the peptide affecting at least one other unrelated channel (Cx46) and by the scrambled version of the peptide sequence also inhibiting pannexin currents. Curiously, the “pannexin mimetic peptide” was reported to affect dye uptake but not currents in response to P2X7 activation in macrophages (36). This observation is consistent with a steric block that partially obliterates the channel, so small ions can flow while dye flux is eliminated.

In summary, neither connexin nor pannexin “mimetic” peptides exhibit the specificity and efficacy required for channel identification. Given that the likely mechanism of action is by
stERIC channel block and that the peptides may have little to do with gap junction protein characteristics, the terms “connexion-” or “pannexin-mimetic peptide” is misleading, and thus should be abandoned.

ACKNOWLEDGMENTS

We thank Dr. K. Muller for valuable discussions and Dr. H. Evans for the generous gift of peptides 43Gap26 and 43Gap27.

GRANTS

This work was supported by National Institutes of Health grant GM48610. S. L. was supported by a predoctoral fellowship from the American Heart Association.

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