Identification of a Pore Lining Segment in Gap Junction Hemichannels

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ABSTRACT The ability of certain connexins to form open hemichannels has been exploited to study the pore structure of gap junction (hemi)channels. Cysteine scanning mutagenesis was applied to cx46 and to a chimeric connexin, cx32E$_1$43, which both form patent hemichannels when expressed in *Xenopus* oocytes. The thiol reagent maleimido-butyryl-biocytin was used to probe 12 cysteine replacement mutants in the first transmembrane segment and two in the amino-terminal segment. Maleimido-butyryl-biocytin was found to inhibit channel activity with cysteines in two equivalent positions in both connexins: I33C and M34C in cx32E$_1$43 and I34C and L35C in cx46. These two positions in the first transmembrane segment are thus accessible from the extracellular space and consequently appear to contribute to the pore lining. The data also suggest that the pore structure is complex and may involve more than one transmembrane segment.

INTRODUCTION

Most cells within organized tissues communicate with one another through cell-cell channels. Assemblies of such channels are found in the clustered intramembranous particles of gap junctions (Bennett and Goodenough, 1978; Loewenstein, 1981). Gap junction channels are formed by two hemichannels located in apposing membranes that are bound (docked) to each other in the intercellular gap and are aligned to form an aqueous pathway between cells. Each hemichannel is formed by six subunits, called connexins (Unwin and Zampighi, 1980; Makowski et al., 1977; Caspar et al., 1977). The connexins represent a family of homologous proteins with pronounced tissue specificity and considerable conservation between species (Paul, 1986; Kumar and Gilula, 1986; Nicholson and Zhang, 1988; Willecke et al., 1991; Haefliger et al., 1992).

With respect to membrane topology, connexins are among the best characterized of membrane channel proteins (Revel et al., 1984; Hertzberg et al., 1988; Milks et al., 1988; Goodenough et al., 1988; Zhang and Nicholson, 1994; Rahman and Evans, 1991; Dahl et al., 1994). Data on proteolytic effects on electrophoretic mobility of isolated gap junction proteins, microsequencing, and detailed mapping with peptide-specific antibodies suggest that connexins contain two extracellular loops, four transmembrane segments, and three cytoplasmic domains. A new mapping method, N-glycosylation scanning mutagenesis, allows fine mapping with a resolution of about three amino acids (Dahl et al., 1994; Chang et al., 1994). Data obtained with this procedure are in agreement with the extracellular localization of the E2 sequences, but they also suggest that a considerable fraction of the second extracellular loop is buried, i.e., artificial glycosylation sites are not freely accessible for glycosylation enzymes (Dahl et al., 1994).

Despite the detailed knowledge of the membrane topology, little is known about the functional domains of connexins. In particular, it is not known which sequences constitute the lining of the pore. Since the sequence of the first gap junction protein (connexin 32) was determined (Paul, 1986), it was speculated that the transmembrane segment M3 lines the pore (Unwin, 1989; Bennett et al., 1991). Pore linings were expected to be composed of amphipathic α-helical transmembrane segments. Of the four transmembrane segments predicted by hydrophobicity plots for connexins, only M3 contains a sequence of amino acids that could form an amphipathic α-helix. However, from what became known of other ion channels, a strictly amphipathic α-helical model may not be justified. The crystal structure of porins, for example, has revealed that the pores of these bacterial channel-forming membrane proteins are lined by a 16-stranded antiparallel β-barrel, which contains both hydrophilic and a series of hydrophobic amino acids (Cowan et al., 1992). The pores of potassium channels and probably other voltage-gated and ligand-gated channels also do not appear to be lined by strict amphipathic α-helices in which hydrophobic amino acids would be incompatible with the pore (Durell and Guy, 1992; Akabas et al., 1991; Karlin and Akabas, 1995; Lü and Miller, 1995).

Studies of the channel pore in gap junctions have been complicated because of the lack of specific toxins and the inaccessibility of the pore from the extracellular medium for chemical modifiers. Toxins were instrumental in the assignment of functional domains in other ion channels, including potassium channels (Yellen et al., 1991), and chemical modification in combination with cysteine replacement mutagenesis (a.k.a. cysteine scanning mutagenesis) has proved useful in defining the channel linings of several membrane

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channels (Akabas et al., 1991; Karlin and Akabas, 1995; Lü and Miller, 1995; Xu and Akabas, 1993).

The situation has changed with the discovery of certain connexins, such as cx46 (Paul et al., 1991; Ebihara and Steiner, 1993) and cx32E$_1$43 (Pfahnl et al., 1996), that form open hemichannels when expressed in *Xenopus* oocytes. (Recently, both cx38 and cx43 have also been reported to exhibit hemichannel activity under certain conditions (Ebihara, 1996; Li et al., 1996).) Here we describe the use of these connexins to study the pore lining of gap junction hemichannels with the cysteine scanning mutagenesis approach.

MATERIALS AND METHODS

Generation of cysteine replacement mutants

cx46 cloned into the expression vector SP64T was obtained from Dr. D. L. Paul (Paul et al., 1991). A cassette between the unique *Nhe*I and *Pml*I sites was substituted with corresponding polymerase chain reaction-generated cassettes containing the various mutations. In addition to having the base changes required for cysteine substitution, the primers used for production of the cassettes also contained base changes to create new restriction sites by silent mutagenesis. These sites were used to screen bacterial colonies for the mutant clones.

cx32E$_1$43 is a chimeric connexin and was generated by polymerase chain reaction splicing and overlap extension (Horton et al., 1990) with cx32 (Paul, 1986) in pGEM 3Z (Promega Biotech) (Rabadan-Diehl et al., 1994) serving as backbone and cx43 (Beyer et al., 1987) as donor for the extracellular loop E1 sequences (A. Pfahnl et al., unpublished observations). The cross-over points are amino acids E41 for cx32/cx43 and S72 for cx43/cx32. Cysteine replacement was essentially the same as described for cx46, except that the replacement cassettes defined by the restriction enzymes *Bam*HI and *Bsm*I for S11C, and *Bsm*I and *Pst*I for all other mutants were synthetic oligonucleotides. (The *Pst*I site in the multiple cloning region of pGEM 3Z was eliminated by cutting, followed by mung bean nuclease treatment and blunt end ligation.)

All mutants were verified by sequence analysis (DNA core lab, University of Miami). For in vitro transcription, plasmids were linearized with *Eco*RI (cx46) or *Ssp*I (cx32E$_1$43) and transcribed using the mMessage mMachine kit (Ambion).

Preparation of oocytes

Preparation of oocytes and electrophysiological recording were performed as described previously (Dahl, 1992). Oocytes were injected with ~20 nl of in vitro transcribed connexin mRNA and incubated for 18–24 h in oocyte Ringer's solution (OR2), with elevated Ca^{2+} concentration (5 mM) to keep the hemichannels in the closed state (Ebihara and Steiner, 1993). For electrophysiological recordings oocytes were transferred to regular OR2 (1 mM Ca^{2+}) and voltage clamped at a holding potential of −10 mV. Membrane conductance was determined by using repetitive 10-mV voltage steps of 5-s duration. Before thiol reaction, oocytes were perfused with dithiothreitol (DTT) (1 mM) in OR2 to reduce any existing disulfide bonds. This reduction, however, was not required to observe the effects by maleimido-butyryl-biocytin (MBB) in the relevant cysteine mutants, with the exception of cx32E$_1$43 E146C. MBB was obtained from Calbiochem. MBB was applied in OR2 at the concentrations indicated.

Tracer flux studies

The fluorescent tracer molecules carboxyfluorescein and cascade blue were obtained from Molecular Probes and used at 1 mM concentration in OR2. Oocytes were incubated for 30 min with the tracers. After washing, oocytes were frozen in O.C.T. compound (Miles). Cryosections (20 μm) were taken and viewed in a fluorescence microscope with the filter settings appropriate for carboxyfluorescein and cascade blue.

RESULTS AND DISCUSSION

Identification of reactive cysteines in the M1 segment

In this study two connexins forming open hemichannels were used for cysteine scanning mutagenesis, cx46 and cx32E$_1$43. cx46 is a lens gap junction protein that forms open hemichannels when expressed in *Xenopus* oocytes (Paul et al., 1991). Whether open hemichannels exist in the lens is unclear. However, the cx46 protein appears to be processed differently in oocytes than in lens, as indicated by different electrophoretic mobilities (Paul et al., 1991). Thus it is conceivable that the conductive states of cx46 hemichannels in lens and oocytes differ. cx32E$_1$43 is a chimeric connexin, consisting of connexin32, in which the sequence representing the extracellular loop E1 of cx32 was replaced by the corresponding sequence of cx43. This chimeric connexin was found to induce a membrane conductance in oocytes reminiscent of the cx46 hemichannels (Pfahnl et al., 1996, and unpublished observations). Furthermore, this conductance exhibits gating properties which, with regard to gating by cytoplasmic acidification and voltage, are qualitatively identical to those found for cx32 gap junction channels. Thus cx32E$_1$43 appears to form open hemichannels, whereas neither wild-type cx32 nor cx43 yields a conductance significantly different from that of uninjected oocytes under the same recording conditions.

To study the pore lining of gap junction hemichannels, we exploited the ability of each of two connexins, cx46 and cx32E$_1$43, to form open hemichannels. Individual residues were mutated, one at a time, to cysteines, and their accessibility from the extracellular medium with aqueous thiol reagents was tested. For adaptation of this approach to the peculiar size of gap junction channels, which allow the passage of molecules up to 1 kDa in size (Loewenstein, 1981), maleimido-butyryl-biocytin (MBB), with a molecular weight of 537, was used. This thiol reagent is membrane impermeable (Bayer et al., 1987; Dahl et al., 1991) and thus can interact only with thiol groups that are located extracellularly or are accessible by way of the pore. Effects of MBB on the conductive state of the hemichannels, therefore, are due either to a steric block of the channel or a gating mechanism. Reaction of MBB with thiol groups that are located extracellularly and are not part of the channel mouth could affect conductance only by a gating mechanism. By contrast, reaction with bona fide transmembrane segments should result in a size-dependent steric block. An additional gating effect in such positions, however, is possible.

We mutated, one at a time, two amino acids in the amino-terminal cytoplasmic segment and 12 consecutive amino acids in the first transmembrane segment (M1) of

cx32E$_1$43 to cysteine (Fig. 1). These mutants were expressed in *Xenopus* oocytes by injecting their corresponding in vitro transcribed mRNAs into the cells. With the exception of mutant cx32E$_1$43 I28C, all mutants yielded functional hemichannels. When exposed to MBB, the membrane conductance induced by these mutants remained unaffected, except for cysteines at four positions. Strong inhibition (~30%) was observed for mutants I33C and M34C, and weak inhibition (≤10%) for mutants F31C and R32C.

To test whether the responsiveness of cysteine mutants is unique to the chimeric connexin backbone or whether it is characteristic of connexins in general, the same type of cysteine mutants were generated in cx46. Of the five positions tested, two, I34C and L35C, were found to be MBB sensitive (Fig. 1). These two positions are equivalent to I33 and M34 in cx32E$_1$43, because alignment of the two connexin sequences requires a gap in cx32 (Fig. 2).

MBB caused a small but apparently significant inhibition in the cx32E$_1$43F31C mutant, yet no significant inhibition was observed in the corresponding cx46F32C mutant. Whether this discrepancy reflects true differences between the two channels or whether the inhibition in cx32E$_1$43F31C is a false positive result remains to be determined.

The conductance of hemichannels provided by both wild-type cx46 and the chimeric connexin cx32E$_1$43 in *Xenopus* oocytes remains unaffected by MBB (Figs. 1 and Fig. 4). These connexins have six cysteines located extracellularly, three in each extracellular loop, and at least one disulfide bond connects the two loops (Rahman and Evans, 1991; John and Revel, 1991). Previous studies have shown that subsequent to reduction, MBB inhibits the formation of

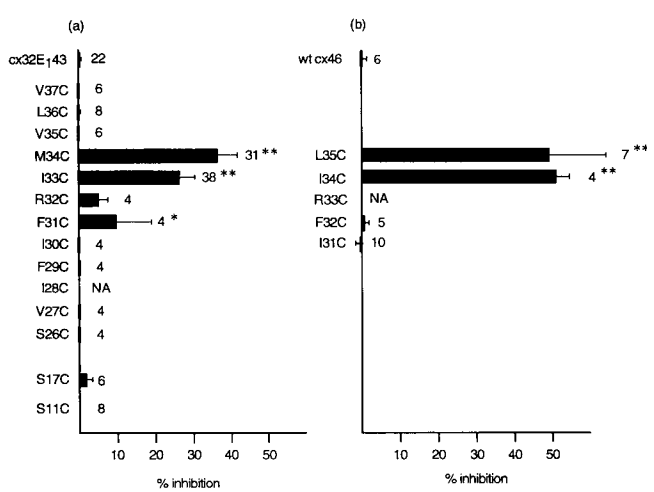

FIGURE 1 Inhibition by MBB (0.1 mM) of membrane conductance of single oocytes expressing cysteine mutants of cx32E$_1$43 (*a*) or cx46 (*b*). The number of oocytes tested is indicated at each bar. In both connexins two positions are found where cysteine replacement renders the channels susceptible to MBB. Mutants cx32E$_1$43I28C and cx46R33C did not form open hemichannels. Values represent means ± SEM of measurements taken 20 min after the application of MBB. *, $p < 0.044$; **, $p < 0.01$ (versus cx32E$_1$43 or cx46 control).

FIGURE 2 Alignment of cx32 and cx46, amino acids 1–42. The first transmembrane segment (M1) is located between positions 24 and 41, approximately. The two positions found in both connexins to be accessible to externally applied thiol reagents are indicated by the bar.

functional gap junction channels, presumably by reaction with the extracellular cysteines. Furthermore, mutation of any one of these cysteines to serine abolishes the ability to form functional channels (Dahl et al., 1991, 1992). The failure of the hemichannels to be affected by MBB thus means that the autochthonous cysteines are not located in positions where reaction with MBB could influence the conductive state of the hemichannels. Hence any effect on membrane conductance seen with cysteine scanning mutagenesis has to be attributed to the cysteines engineered into these connexins. One caveat that applies to any mutagenesis approach has to be considered, however. The cysteine replacement could have remote effects and expose autochthonous cysteines to the extracellular environment. Both cx32E$_1$43 and cx46 have a cysteine in the fourth transmembrane segment that could be affected this way. This, however, would require a major structural rearrangement inconsistent with the observation of formation of regular hemichannels by most of the cysteine mutants and by formation of complete gap junction channels in paired oocytes (data not shown) by at least the cx32E$_1$43 M34C mutant.

Scanning of the M3 segment

Based on the apparent amphipathic property of the third transmembrane segment of connexins, it had been speculated (Unwin, 1989; Bennett et al., 1991) that M3 exclusively provides the lining of the gap junction channel pore. Cysteine scanning mutagenesis was applied to nine positions in the M3 segment of cx32E$_1$43. MBB was found to inhibit ionic conductance in three of these positions, two of which (S138 and E146) are part of the amphipathic segment (Fig. 3). However, the levels of inhibition were considerably smaller than the levels seen with the reactive cysteines in the M1 segment. Although the levels of inhibition by MBB in these mutants (S138C, E146C, M150C) were significantly different from those observed in controls, the biological significance of these findings remains obscure. The contribution of M3 to the pore lining, therefore, must be addressed by another approach.

If the three positions in M3 indeed were lining the pore, two apparently contradictory scenarios could explain the lower level of inhibition as compared to the inhibition seen with the M1 mutants. A hemichannel is probably composed of six subunits and thus contains six potential MBB reaction sites. The size exclusion limit of cx32E$_1$43 hemichannels is at about 900 Da (Pfahnl and Dahl, unpublished observa-

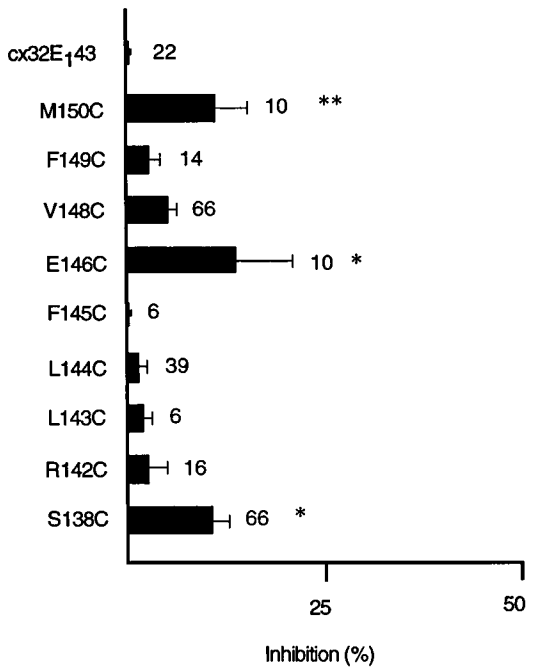

FIGURE 3 Inhibition by MBB (0.1 mM) of membrane conductance of single oocytes expressing cysteine mutants of cx32E$_1$43. Values represent means ± SEM of measurements taken 20 min after application of MBB. *n* is indicated by the number above each bar. *, $p < 0.012$ and 0.013 for S138C and E146C, respectively; **, $p < 0.01$ (versus cx32E$_1$43 control).

tions). It is unlikely that at the part of the channel where size discrimination occurs all six thiol groups within a single hemichannel can be reacted with MBB, considering its molecular weight of 537. If there is a wide region of the channel, up to six MBB molecules could react. Depending on the exact geometry, it is thus possible that MBB binding at a wider part of the pore yields a stronger inhibition than binding at a narrow part. The other scenario, where the residual pore in the vicinity of the MBB site is smallest in the narrow part of the channel, is equally plausible. Thus, without knowledge of the exact molar ratio of maleimide block of conductance for the various positions and the dimensions of the molecules involved, no firm conclusion can be derived about the dimensions of the pore from the inhibition levels.

Characterization of the thiol reaction

Oocytes expressing cx46 or cx32E$_1$43 and stored in regular OR2 medium have a limited lifetime of a few hours. Oocytes were therefore incubated in OR2 with increased Ca^{2+} concentration (5 mM) to keep the hemichannels in the closed state (Ebihara and Steiner, 1993; Pfahnl et al., unpublished observations) and transferred to regular OR2 for the experimental tests. Within 20 min after change of calcium concentration and clamping to a holding potential of −10 mV, the oocytes' membrane conductance after an initial fast rise reached a plateau level or, in some oocytes, continued to rise in a linear fashion. The latter was typically seen in oocytes with high expression levels ($g_m > 15$ μS), and the continued increase of the conductance probably is attributable in part to newly inserted channel protein. This phenomenon has been taken into account for data analysis in the following way: if a linear increase observed before MBB application continued with the same slope, zero inhibition/potentiation by the MBB was assumed (but oocytes with conductance increases of >20% in the 20 min before the MBB application were excluded from analysis). An example is given in Fig. 4, where the conductance of the cx32E$_1$43-expressing oocyte had risen from 17.4 μS to 20 μS in the 20-min period preceding the MBB application. The increase in conductance to 22.6 μS in the 20-min time segment shown, therefore, was probably not caused by MBB.

For testing the inhibition by MBB, the oocytes routinely were first perfused for 5 min with OR2 containing 1 mM DTT to reduce any disulfide bonds, which are known to exist for native extracellular cysteines (Rahman and Evans, 1991; John and Revel, 1991; Dahl et al., 1991). Although this pretreatment did not affect the membrane conductance of the oocytes and was not required for a MBB effect in most of the relevant cysteine mutants, this routine was maintained to provide identical experimental conditions throughout. A notable exception was seen with cx32E$_1$43 E146C, which did not form open hemichannels in the oocyte membrane. However, when oocytes expressing this mutant were treated with 1 mM DTT, a membrane conductance comparable to that obtained with the wt chimera and the other cysteine mutants appeared within 5 min (not shown). This indicates that the engineered cysteine is responsible for a disulfide bond not present in the wt chimeric connexin. The location of this bond is not known; the engineered cysteines of the different subunits could be disulfide bonded to each other, to autochthonous cysteines, or the mutation could result in disulfide bonds between autochthonous cysteines not present in wt connexin. In any case, the bonds keep the hemichannels closed unless they are reduced. Upon washout of DTT, the membrane conductance remained elevated for an extended period of time (>30 min). MBB resulted in an inhibition of the membrane conductance, which was not reversed by DTT.

The effect of MBB on membrane conductance was dose dependent. As shown for cx46 L35C, effects on membrane conductance were observed with micromolar concentrations, and maximal inhibition was seen with 0.1 mM MBB (Fig. 5). The reaction rates of MBB with the various cysteine mutants appeared to be similar. Fig. 4, *b* and *c*, shows the time course of MBB action on mutants M34C and S138C. Despite the different levels of inhibition obtained with these mutants (ranging between 8% and 37%), the reduction of membrane conductance began soon after application of MBB and reached a plateau level within 20–30 min in these and all other reactive mutants. The plateau levels (usually obtained 20 min after the start of MBB reaction) were used to calculate the percentage inhibition shown in Figs. 1 and 3. The differences in inhibition be-

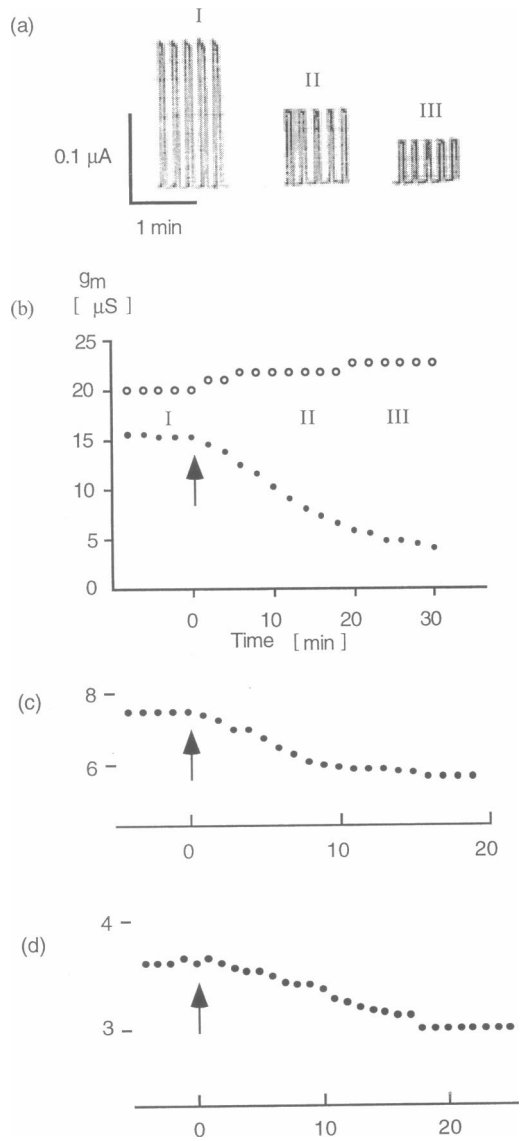

FIGURE 4 Time course of MBB and MPA effects on membrane conductance induced by cysteine mutants of cx32E$_1$43 in *Xenopus* oocytes. The start of bath perfusion with MBB- or MPA-containing medium is indicated by the arrows. The thiol reagents were used at a concentration of 100 μM. Oocytes were voltage clamped at a holding potential of −10 mV, and 10-mV pulses of 5-s duration were applied. A set of resulting membrane currents for the cysteine mutant, cx32E$_1$43,M34C, is shown (*a*). The current samples (I–III) were taken at the time points indicated in *b*. (*b*) Effect of MBB on membrane conductance of oocytes expressing cx32E$_1$43 (○) and cx32E$_1$43,M34C (●). As pointed out in the text, the increase seen with cx32E$_1$43 is a continuation of a conductance increase already present before the time segment shown and therefore probably was not caused by MBB. (*c*) MBB effect on membrane conductance of oocytes expressing cx32E$_1$43 S138C. (*d*) Effect of MPA on membrane conductance of oocytes expressing cx32E$_1$43 M34C.

tween the cysteine mutants, therefore, were unlikely to have been caused by differences in accessibility of MBB to the thiol groups. Instead, they probably reflected the degree of channel block. As pointed out earlier, the increase in membrane conductance seen with cx32E$_1$43 (Fig. 4 *b*) is most likely not attributable to MBB, but is simply the continuation of an increase seen before MBB at the same rate, probably due to insertion of new and/or opening of existing hemichannels.

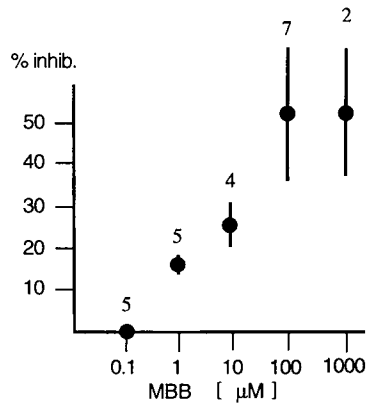

FIGURE 5 Dose-response curve for the effect of MBB on membrane conductance of oocytes expressing cx46L35C. Values represent means ± SEM; *n* is indicated by the number.

To test whether the inhibition of membrane conductance was dependent on the size of the thiol reagent, we used two smaller maleimide-based thiol reagents, *N*-ethyl maleimide (NEM, MW 125) and maleimido-proprionic-acid (MPA, MW 169). NEM was not useful, as oocytes treated with this reagent died rapidly. MPA, on the other hand, was found to inhibit membrane conductance like MBB at the same position (cx32E$_1$43M34C, Fig. 6), albeit to a lesser extent. The time course of inhibition by the two maleimides was similar (Fig. 4 *d*), suggesting that the difference in inhibition is due to the sizes of the maleimides.

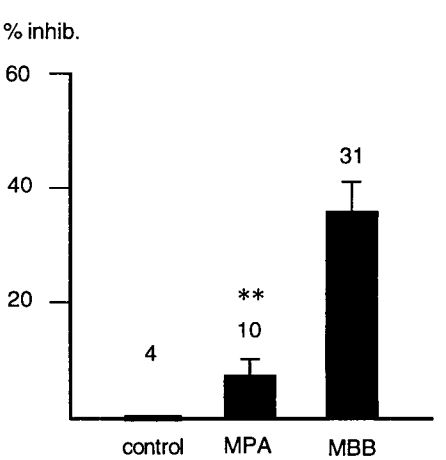

FIGURE 6 Effect of MPA and MBB on membrane conductance of oocytes expressing cx32E$_1$43 M34C. Oocytes expressing cx32E$_1$43 treated with MPA serve as the control. Values represent means ± SEM of measurements taken 20 min after the application of MPA or MBB. *n* is indicated by the number. **, $p < 0.01$ (versus control).

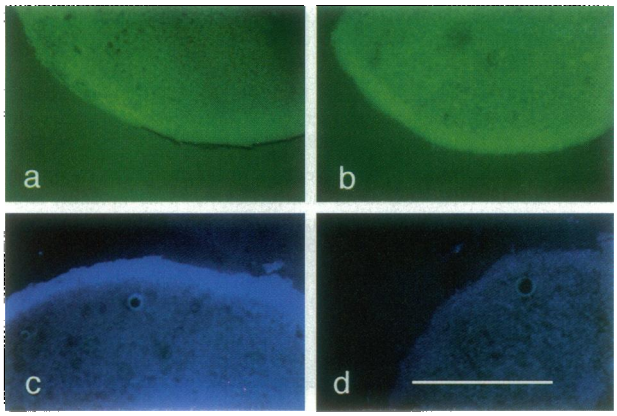

FIGURE 7 Tracer flux into oocytes expressing cx46 L35C without (*a, c*) and after (*b, d*) reaction to MBB for 20 min. Oocytes were incubated for 30 min in medium containing either carboxyfluorescein (1 mM) (*a, b*) or cascade blue (1 mM) (*c, d*). Five oocytes were analyzed for each condition from one pool of cx46L35C-expressing oocytes. Scale bar: 0.5 mm.

Mechanism of inhibition

It is not clear what mechanism MBB uses to reduce the membrane conductance in $cx32E_1 43$ or cx46 hemichannels bearing cysteine replacements in certain positions. The observed block could be achieved by either gating effects on the channel or by a steric block of the channel. The basic interpretation, that the reactive cysteines report pore lining positions in the connexins, is independent of the mechanism of inhibition as long as the sites are located in bona fide transmembrane segments. For example, even if the inhibition is caused by a gating mechanism, the conclusion that position L35 in cx46 is pore lining would stand. This amino acid (L35) is located within a proven transmembrane segment and thus could be reached by the thiol reagent only by way of the pore.

That the inhibition is dependent on the size of the thiol reagent excludes the chemical reaction per se as the cause; it is suggestive, instead, of a steric mechanism but does not rule out gating.

The 40% inhibition could be due to 40% of the channels being shut by an all-or-none gating mechanism or to an equivalent reduction of the open probability. Alternatively, the unit conductance of all channels could be reduced by 40%. These possibilities can be discriminated with the application of tracer molecules to MBB-reacted oocytes. If MBB shuts down 40% of the channels, no change of the cutoff limit should be seen; only the rate of uptake should be reduced. If, on the other hand, each channel's conductance were to be reduced by either MBB-induced gating to a subconductance state or by a steric block, then the cutoff limit for tracer uptake should be lower. However, small tracer molecules should still be taken up. Experiments of this kind with cx46L35C have shown that in response to MBB, the uptake of Cascade Blue (MW 596) is almost completely abolished, whereas carboxyfluorescein (MW 376) uptake appears to be unaltered (Fig. 7). This suggests that in response to MBB the conductance of the channel, γ, is reduced.

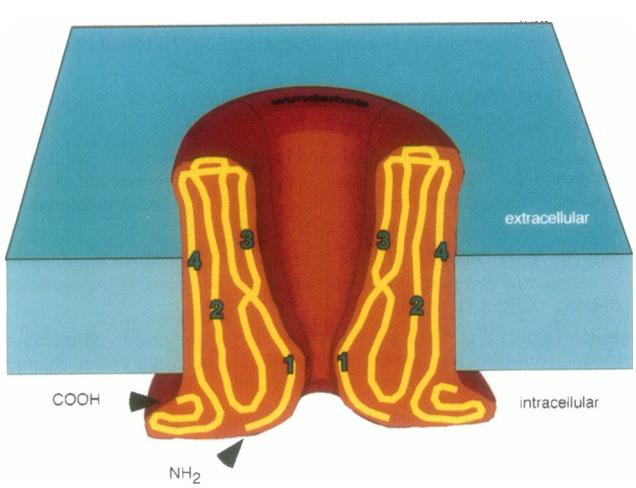

FIGURE 8 Cartoon of a gap junction hemichannel. The hemichannel consists of six subunits, three of which are shown. In each subunit the connexins are folded so that fractions of the first (1) and third (3) transmembrane segments line the channel pore. Whereas the contribution of M1 is supported by the data presented here, the contribution of M3 remains speculative. Furthermore, although the data can be interpreted in terms of differing pore sizes along the channel, the assignment of the narrow part to the M1 segment, however, is arbitrary.

CONCLUSION

Cysteine scanning mutagenesis was used on two connexins that form open hemichannels when expressed in *Xenopus* oocytes. Two positions were identified in the first transmembrane segment (M1) of the chimeric connexin $cx32E_1 43$, where cysteine substitution results in inhibition of ion conductance in response to externally applied thiol reagents. The equivalent positions in another connexin, cx46, were also found to be sensitive to thiol reaction. The data suggest that the M1 segment contributes to the pore lining of hemichannels formed by the connexins.

In both connexins, the reactive positions are adjacent to each other. They represent a segment that is too short to encompass the complete channel lining. One or more other segments of the connexin molecule must, therefore, provide an additional contribution to the pore lining. The M3 segment, which has long been suspected to be the exclusive pore lining sequence, remains a candidate for such a contribution. The data obtained from cysteine scanning mutagenesis of the M3 segment in $cx32E_1 43$, however, have remained inconclusive. The data do not rule out M3 as a pore lining segment, but they do not lend strong support for such a function either. Although inhibition of the $cx32E_1 43$-induced membrane conductance was observed in three positions of the M3 segment, the level of inhibition was very low ($\leq$15%). Either a narrower portion of the pore with access limitation for the thiol reagent or a considerably wider pore with a smaller fractional block could explain

such a low level of inhibition by MBB, if the M3 segment indeed were to be part of the pore lining. A contribution of the M3 segment to the pore lining, therefore, must be confirmed by other approaches. Furthermore, the contribution by other segments remains to be tested.

In consideration of these caveats, Fig. 8 shows a cartoon of a gap junction hemichannel depicting one of the folding schemes of the connexins that could account for the data obtained in this study. Whatever segments may provide the remaining portion of the channel lining, the present data suggest that the gap junction channel pore has a complex structure and appears to follow a different building principle than either voltage-gated ion channels or porins.

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