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THE M2δ TRANSMEMBRANE DOMAIN OF THE NICOTINIC CHOLINERGIC RECEPTOR FORMS ION CHANNELS IN HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY. A synthetic peptide with the sequence of the M2δ segment of the nicotinic acetylcholine receptor from *Torpedo californica* forms pores in human erythrocyte membranes as determined by hemoglobin and potassium release. This peptide forms a permeability pathway with an apparent cross-sectional diameter of 7-9 Å. The M2δ pore is oligomeric and a pentamer is the species that accounts for the properties of the permeation path. Peptides that mimic other identifiable segments of the *Torpedo* acetylcholine receptor, M1δ and MIR, do not form channels in erythrocytes under the same conditions.

INTRODUCTION. The nicotinic acetylcholine receptor (AcChoR) of *Torpedo californica* is composed of four glycoprotein subunits (α, β, γ, δ) with stoichiometry $\alpha_2\beta\gamma\delta$ (1, 2). A high degree of amino acid sequence homology exists among the four subunits, and all exhibit four putative transmembrane regions designated as M1, M2, M3 and M4 (3). *In vivo*, the AcChoR pentamer acts as a ligand activated cation channel (4) with an effective pore diameter of ~ 7 Å (5). The specific assignment of subunits involved in channel lining has been a subject of intense investigation. Evidence suggests that M2 is the segment which lines the pore (4, 6-8). Significantly, a synthetic 23-mer peptide with the sequence of M2δ forms ion channels in lipid bilayers with single channel properties that emulate those of authentic AcChoR ion channels (8). Here we provide evidence of channel formation by the AcChoR M2δ peptide in biological membranes.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by solid phase methods on an Applied Biosystems model 430 peptide synthesizer, purified by HPLC and sequenced, essentially as previously described (8, 9). The amino acid sequences of the peptides studied are: M2δ - EKMSTAISVLLAQAVFLLLTSQR (8), M1δ - LFYVINFITPCVLISFLASLAFY (8), MIR (a segment of the main immunogenic region) - VNQIVETNVR (10), and a peptide composed of 23 serine residues (poly S).

Hemolysis Assay. Hemolysis was assayed according to Tosteson et al. (11). Briefly, recently outdated blood from the Veteran's Administration Medical Center Blood Bank (La Jolla, CA) was washed three times with 0.3 M sucrose buffer (0.3 M sucrose, 0.01 M Tris-Hepes, 0.001 M EGTA,

The abbreviations used are: AcChoR, acetylcholine receptor, MIR, main immunogenic region, TFE, trifluoroethanol, Hb, hemoglobin.

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pH 7.4). An erythrocyte suspension was made 1% (v/v), and 2.5 ml samples were used for all assays. Synthetic peptides were dissolved in trifluoroethanol (TFE; Aldrich, Milwaukee, WI) and added to erythrocyte suspensions with immediate vortexing. Suspensions were incubated at 22 ± 2°C for desired time periods, whereupon two 1 ml aliquots were withdrawn and layered over 0.1 ml dibutylphthalate (Aldrich, Milwaukee, WI). Samples were immediately centrifuged for 5 minutes in an Eppendorf Model 5414 centrifuge, and the absorbance of the supernatants at 540 nm was recorded. The effect of equivalent amounts of pure TFE (5-30 µl) without peptide were subtracted from the lysis produced by peptides. Total lysis was obtained by solubilizing erythrocytes with Triton X-100 at a final concentration of 0.5% (v/v). K^+ was measured on a Perkin-Elmer Atomic Absorption Spectrophotometer Model 5000 (λ = 766.5 nm). Supernatants were combined, and 1 ml aliquots diluted to a final volume of 5 ml with distilled water.

RESULTS AND DISCUSSION

Synthetic M2δ peptide has hemolytic activity. Erythrocytes suspended in sucrose buffer are lysed by the synthetic M2δ peptide. Figure 1 illustrates the time course of hemoglobin (Hb) release produced by three different concentrations of M2δ. Initially, Hb release increases linearly with time, leveling off at a steady state value. K^+ release from erythrocytes is also a measure of cell lysis as illustrated in Figure 2B. The time courses of Hb and K^+ release are similar, although the initial rate of K^+ release is faster than that for Hb.

M2δ forms channels in lipid bilayers (8). Therefore, a likely mechanism of lysis is that M2δ creates a pore through which the high levels of intracellular K^+ exit the cell. Accordingly, K^+ efflux generates an osmotic imbalance leading to cell lysis. This model implies that external sucrose is too large to pass through the M2δ pore. Therefore, sucrose in the buffer was replaced by Tris (at 0.15 M), and no lysis was obtained (Figs. 1 and 2A). Tris was selected for this assay because it is known to permeate through both the authentic AcChoR (5) and the M2δ pore (8). Presumably, $Tris^+$ equilibrates with intracellular K^+, and no osmotic imbalance is created. The dimensions of $Tris^+$ (8Å x 7Å x 6Å) (12) and sucrose (11Å x 9Å x 8Å) (13) predict an effective cross-sectional diameter of the permeability path formed by the M2δ pore of 7-9 Å, in agreement with the apparent cut-off size of the synthetic M2δ (8) and authentic AcChoR (5) channels.

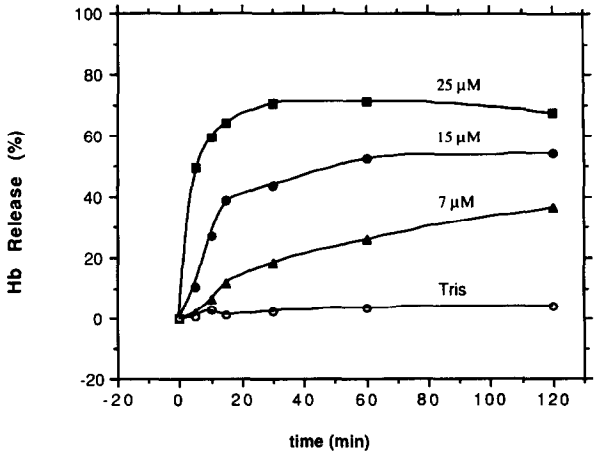

Fig. 1. Hb release from erythrocytes suspended in sucrose buffer supplemented with M2δ at 25 µM (■), 15 µM (●), and 7 µM (▲). Open circles (○) designate Hb release from erythrocytes suspended in 0.15 M Tris buffer containing M2δ (15 µM).

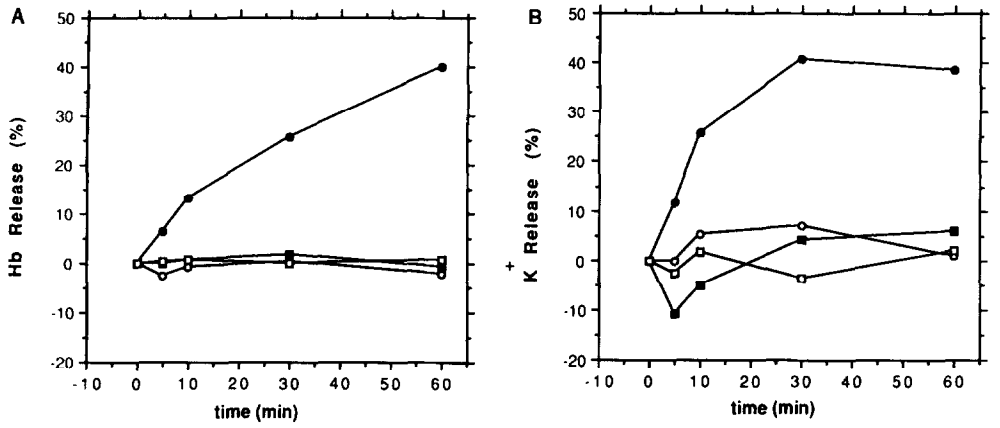

Fig. 2. Hb release (A) and K^+ release (B) from erythrocytes suspended in sucrose buffer (●, ■) or in Tris buffer (○, □) and exposed to 15 μM M2δ (●, ○) or 15 μM polyserine, n = 23 (■, □).

The M2δ pore is oligomeric. A plausible structural model for the ion-conductive pore formed by M2δ postulates a pentameric array of 5 amphipathic α-helices arranged such that the polar residues line a central hydrophilic pathway, and the apolar residues interact with the apolar core of the bilayer (8). Information about the size of oligomeric channels is obtainable from membrane conductance measurements in planar lipid bilayers (14). Double logarithmic plots of conductance vs. concentration of channel forming peptide have a slope equal to the number of peptides per channel. Since the rate of hemolysis is dependent on membrane conductance, a double logarithmic plot of the initial rate of hemolysis vs. concentration of M2δ will have a slope equal to the size of M2δ oligomers involved in the rate limiting step of the hemolysis pathway. Accordingly, Figure 3 shows that the assembly of a trimer is the rate limiting step in the formation of a functional ion channel. However, based on Tris permeability (5, 8), it is likely that pentamers are the dominant species responsible for the M2δ conductive pore.

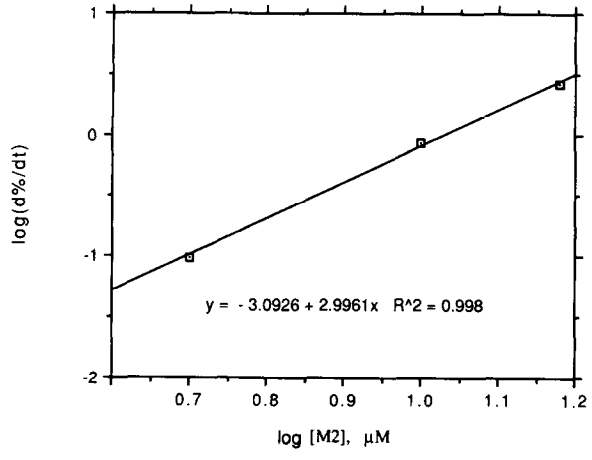

Fig. 3. Plot of log (initial rate of hemolysis) vs. log [M2δ]. Initial rates are initial slopes from a plot of % Hb release vs. time. Initial rates for 5, 10 and 15 μM M2δ were determined in duplicate, and the mean rates were plotted vs. [M2δ]. Slope of 3.0 was calculated by linear regression.

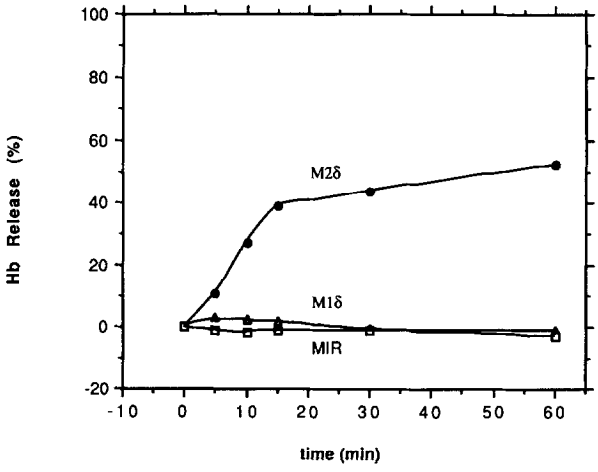

Fig. 4. Hb release from erythrocytes suspended in sucrose buffer containing 15 μM M2δ (●), M1δ (□), or MIR (Δ).

Specificity of the assay. To test the specificity of M2δ hemolytic activity, synthetic peptides with sequences derived from other AcChoR structural domains were studied. M1δ (3, 8) is a putative transmembrane segment (15), whereas the extramembranous synaptic domain of the α subunits contains a hydrophilic segment- the main immunogenic region (MIR) (10). Neither M1δ nor MIR (α) peptides would be expected to be cytolytic, and indeed they show no activity when tested under the same conditions as M2δ (Fig. 4). Figure 2A,B also show that a polyserine 23-mer has no cytolytic activity. This is significant because serines are postulated to line the polar face of M2δ (4, 8) and lack of cytolysis by polyserine supports the notion that amphiphilicity is a hallmark of channel forming α-helices (7, 8).

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