A virus overlay protein binding assay was used to study binding of $^{125}$I-labelled rabies virus to the acetylcholine receptor (ACHR) from Torpedo californica electric organ membranes. After gel electrophoresis of electric organ membranes and transfer of proteins to nitrocellulose, $^{125}$I-labelled $\alpha$-bungarotoxin, a curaremimetic neurotoxin, bound to a 40 kDa band and $^{125}$I-labelled rabies virus bound to 51 kDa and 40 kDa bands. Binding of rabies virus to the 40 kDa band was inhibited by unlabelled $\alpha$-bungarotoxin. In blots of affinity-purified ACHR, labelled virus bound to the 40 kDa $\alpha$ subunit and was competed by $\alpha$-bungarotoxin. Based on binding of rabies virus to the $\alpha$ subunit and the ability of $\alpha$-bungarotoxin to compete for binding, rabies virus appears to bind to the neurotoxin-binding site of the nicotinic AChR $\alpha$ subunit.

Rabies virus is an enveloped, negative-strand, RNA rhabdovirus which, after a possible period of replication in skeletal muscle, primarily infects the mammalian nervous system in vivo (Murphy, 1977). The first step in the viral infectious cycle is attachment of the virus to a component of the host cell surface which acts as a virus receptor (Lentz, 1994). It has been suggested that the nicotinic acetylcholine receptor (ACHR) might serve as a specific host cell receptor for rabies virus (Lentz et al., 1982). Binding of $^{125}$I- and $^{35}$S-labelled rabies virus to affinity-purified ACHR from Torpedo electric organ was demonstrated in dot blots and solid phase assays (Lentz et al., 1986).

The muscle type nicotinic AChR consists of four subunit types, $\alpha$, $\beta$, $\gamma$ or $\epsilon$, and $\delta$, with two $\alpha$ subunits per receptor molecule. The binding site of $\alpha$-bungarotoxin ($\alpha$-Btx), a competitive antagonist of acetylcholine, has been localized to residues 173–204 of the $\alpha$-subunit (Wilson et al., 1985). Because the rabies virus glycoprotein shares a sequence similarity with $\alpha$-Btx (Lentz et al., 1984), and $\alpha$-Btx inhibits rabies virus infection of cultured muscle cells (Lentz et al., 1982; Tsiang et al., 1986), it could be expected that rabies virus also binds to the $\alpha$ subunit of the AChR. In order to test this possibility, protein blots were used to study rabies virus binding to AChR subunits. Protein or Western blots have been used previously to study virus–receptor interactions, the procedure often being referred to as the virus overlay protein binding assay (VOPBA) (e.g., Gershoni et al., 1986; Boyle et al., 1987; Mischak et al., 1988; Dalziel et al., 1991; Jin et al., 1994; Maisner et al., 1994; Broughan & Wunner, 1995). In this assay, proteins from cells or membranes are separated by gel electrophoresis, electrophoretically transferred to a filter, and the filter probed with a radio-labelled virus preparation or with unlabelled virus followed by a labelled antibody against the virus.

Protein blots of Torpedo californica electric organ membrane were prepared and overlaid with $^{125}$I-labelled $\alpha$-Btx (Gershoni et al., 1983) or $^{125}$I-labelled rabies virus challenge virus standard (CVS) strain (Lentz et al., 1985, 1986). Samples (100 µg) of an electric organ membrane preparation were solubilized at room temperature in sample buffer [62.5 mM-Tris–HCl, pH 6.8–10% (v/v) glycerol–5% (v/v) 2-mercaptoethanol–2% (w/v) lithium dodecyl sulphate (LDS) or SDS–0.1% bromophenol blue] and resolved at room temperature on 10% polyacrylamide slab gels. Replicate gels were either stained with Coomassie brilliant blue or were transferred electrophoretically to nitrocellulose filters in 48 mM-Tris–39 mM glycine–0.0375% (w/v) SDS in 20% (v/v) methanol. After transfer, the filters were quenched to reduce nonspecific binding and incubated with $^{125}$I-labelled $\alpha$-Btx (2 x 10$^6$ c.p.m., specific activity 500 c.p.m./fmol) as described by Gershoni et al. (1983) or $^{125}$I-labelled rabies virus. For virus overlay, filters were quenched in 5% (w/v) instant nonfat dry milk in 10 mM-PBS (pH 7.4) for 5–8 h at room temperature. Filters were washed for 10 min in 10 mM-Tris, pH 6.5–5 mM-CaCl$_2$ (binding buffer). Filters were then incubated in incubating buffer [100 ml binding buffer–0.02% (w/v) dry milk–1% (v/v) Triton X-100–0.01% NaN$_3$ containing $^{125}$I-labelled rabies virus (2 x 10$^6$ c.p.m., specific activity 500 c.p.m./fmol) as described by Gershoni et al. (1983) or $^{125}$I-labelled rabies virus. For virus overlay, filters were quenched in 5% (w/v) instant nonfat dry milk in 10 mM-PBS (pH 7.4) for 5–8 h at room temperature. Filters were washed for 10 min in 10 mM-Tris, pH 6.5–5 mM-CaCl$_2$ (binding buffer). Filters were then incubated in incubating buffer [100 ml binding buffer–0.02% (w/v) dry milk–1% (v/v) Triton X-100–0.01% NaN$_3$ containing $^{125}$I-labelled rabies virus (2 x 10$^6$ c.p.m., specific activity

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Fig. 1. 125I-labelled α-Btx and 125I-labelled rabies virus overlays of protein transfers of crude Torpedo electric organ membranes. Membranes (100 μg) were solubilized at room temperature in LDS-containing sample buffer and electrophoresed at room temperature on a 10% polyacrylamide gel. The gels were stained with Coomassie brilliant blue (lane A) or were transferred to nitrocellulose filters. The filters were quenched with nonfat dry milk (5%) and incubated with 125I-labelled α-Btx (2 x 10^6 c.p.m.) (lane B) or 125I-labelled rabies virus (2 x 10^6 c.p.m.) (lane C). Filters were preincubated with unlabelled α-Btx (1.6 x 10^-7 M) and coincubated with α-Btx and 125I-labelled rabies virus (lane D). After washing, the filters were autoradiographed. The positions of AChR subunits are indicated. The molecular masses of the subunits are: δ, 65 kDa; γ, 54 kDa; β, 48 kDa; and α, 40 kDa.

Fig. 2. Overlays of purified AChR. Affinity-purified AChR (20 μg) from Torpedo electric organ membranes was electrophoresed on a 10% polyacrylamide gel and the gels stained with Coomassie brilliant blue (lane A) or transferred to nitrocellulose filters. Filters were incubated with 125I-labelled rabies virus (2 x 10^6 c.p.m.) (lane B) in the absence (lane C) or presence (lane D) of unlabelled α-Btx (1.6 x 10^-7 M). Filters were then washed and autoradiographed.

2 x 10^6 c.p.m./μg protein) at room temperature for 4 h. Filters were washed three times for 10 min in PBS-0.1% Triton X-100 and then autoradiographed at -70 °C for 3–5 days with Kodak XAR-5 film and an intensifying screen (Dupont).

After gel electrophoresis, Coomassie blue staining revealed the membrane preparation to be heterogeneous, containing numerous polypeptides (Fig. 1, lane A). After incubation with labelled α-Btx, a single radioactive band with an apparent molecular mass of 40 kDa was detected (Fig. 1, lane B). After incubation with 125I-labelled rabies virus, two bands were detected, one of 51 kDa and one of 40 kDa (Fig. 1, lane C). When filters were preincubated with unlabelled α-Btx (1.6 x 10^-7 M) and coincubated with unlabelled α-Btx and 125I-labelled rabies virus, binding of rabies virus to the 40 kDa band was markedly reduced while binding to the 51 kDa band was unaffected (Fig. 1, lane D). The nature of the 51 kDa band is unknown and the virus binding observed could represent an alternate receptor or nonspecific binding.

In order to determine whether the 40 kDa band corresponded to the α subunit of the AChR, affinity-purified Torpedo AChR (20 μg) was denatured and electrophoresed on 10% polyacrylamide gels. Coomassie blue staining revealed the four receptor subunits (Fig. 2, lane A). After transfer to nitrocellulose filters and incubation with 125I-labelled α-Btx, only one band of radioactivity, corresponding to the α subunit of the purified AChR, bound toxin (Fig. 2, lane B). Similarly,
after incubation with $^{125}$I-labelled rabies virus, a single band corresponding to the $\alpha$ subunit was detected (Fig. 2, lane C). Preincubation and coincubation with unlabelled $\alpha$-Btx (1.6 $\times$ $10^{-7}$ M) greatly reduced binding of $^{125}$I-labelled rabies virus to the $\alpha$ subunit (Fig. 2, lane D). In order to quantify binding of $^{125}$I-labelled rabies virus, the regions corresponding to the $\alpha$ subunit and an equivalent region of background were excised from the filter, weighed, and radioactivity counted in a gamma counter. After subtraction of background binding, the $\alpha$ subunit region in Fig. 2, lane C, which was incubated with labelled rabies virus had 52 c.p.m./mg filter, while the $\alpha$ subunit region in Fig. 2, lane D, which was incubated with labelled rabies virus and unlabelled $\alpha$-Btx had 17 c.p.m./mg filter.

Previously, it was reported that labelled rabies virus (Lentz et al., 1986) and labelled rabies virus glycoprotein (Bracci et al., 1986) bound to intact AchR purified from Torpedo electric organ. The present study demonstrates that virus interaction with the AchR is mediated by the $\alpha$ subunit. Binding was inhibited by $\alpha$-Btx, a competitive antagonist of acetylcholine. Further evidence for specificity of binding is that the $\alpha$ subunit to which the virus bound is not a major band in the Torpedo membrane gel. The ability of $\alpha$-Btx to compete rabies virus binding indicates the virus binds to the $\alpha$-Btx binding site on the $\alpha$ subunit. The main toxin binding determinant on the $\alpha$ subunit has been localized to residues 173–204 (Wilson et al., 1985), and it has been demonstrated that rabies virus binds to a synthetic peptide comprising this region (Lentz, 1990). These findings support the suggestion that rabies virus binds to the AchR by mimicking the curaremimetic neurotoxins (Lentz et al., 1984).

Although these studies demonstrate the binding of rabies virus to the $\alpha$ subunit of the muscle type nicotinic AchR, it remains unknown whether the AchR is an essential host cell receptor in vivo. Other studies have indicated that other components of the cell surface might act as rabies virus receptors. These constituents include phospholipids or glycolipids (Superti et al., 1984; Wunner et al., 1984), gangliosides (Superti et al., 1986), and sialic acid and other carbohydrates (Conti et al., 1986). In BHK-21 cells, the VOPBA showed specific rabies virus binding to a high molecular mass 'fibronectin-like' protein as well as at least four other proteins migrating between 66 kDa and 200 kDa (Broughan & Wunner, 1995). These findings raise the possibility that rabies virus may utilize more than one type of cellular receptor.

This research was supported by grants NS 21896 and NS 34274 from the National Institutes of Health.

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Received 18 April 1996; Accepted 13 June 1996