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Dominant Negative Mutants of Bacillus thuringiensis Cry1Ab Toxin Function as Anti-Toxins: Demonstration of the Role of Oligomerization in Toxicity

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Abstract

Background: Bacillus thuringiensis Cry toxins, that are used worldwide in insect control, kill insects by a mechanism that depends on their ability to form oligomeric pores that insert into the insect-midgut cells. These toxins are being used worldwide in transgenic plants or spray to control insect pests in agriculture. However, a major concern has been the possible effects of these insecticidal proteins on non-target organisms mainly in ecosystems adjacent to agricultural fields.

Methodology/Principal Findings: We isolated and characterized 11 non-toxic mutants of Cry1AB toxin affected in different steps of the mechanism of action namely binding to receptors, oligomerization and pore-formation. These mutant toxins were analyzed for their capacity to block wild type toxin activity, presenting a dominant negative phenotype. The dominant negative phenotype was analyzed at two levels, in vitro by pore formation activity in black lipd bilayers. We demonstrate that some mutations located in helix α-4 completely block the wild type toxin activity at sub-stoichiometric level confirming a dominant negative phenotype, thereby functioning as potent antitoxins.

Conclusions/Significance: This is the first reported case of a Cry toxin dominant inhibitor. These data demonstrate that oligomerization is a fundamental step in Cry toxin action and represent a potential mechanism to protect special ecosystems from the possible effect of Cry toxins on non-target organisms.

Introduction

Bacillus thuringiensis (Bt) bacteria produce crystal proteins (denominated also Cry toxins) that have insecticidal activity. One of the most successful applications of Cry proteins has been their expression in transgenic crops resulting in their effective protection from insect damage and lowering the use of chemical insecticides [1]. Extensive studies show that Cry toxins used in transgenic crops are safe to the environment and non-toxic to other organisms [2–4]. Nevertheless, there are still concerns related to the possible impact of by products from transgenic Bt crops as Bt-cotton and Bt-corn on non-target organisms in ecosystems adjacent to agricultural fields [5–9].

Pore-forming toxins are important virulent-factors in different diseases induced by several mammalian-pathogenic bacteria [10]. Based on an understanding of their mechanism of action, different strategies have been proposed to neutralize their action [11]. Among these strategies, the use of neutralizing antibodies that recognize toxin regions involved in receptor binding or the use of fragments of toxin-receptors were shown to efficiently protect the cells from intoxication [12,13]. In addition, dominant negative (DN) inhibitors which are inactive mutant-toxins, able to form oligomer structures but affected in their pore formation activity, work as powerful inhibitors since they are able to co-assemble into hetero-oligomers together with the wild type toxin resulting in an effective inactivation of pore formation and toxicity [14–16].

Cry toxins produced by Bt are pore-forming toxins [1]. Their mechanism of action is complex and involves several steps. In the case of Lepidopteran-active Cry1A proteins, the binding to a primary receptor, the cadherin protein, induces the cleavage of an amino-terminal helix α-1 leading to toxin oligomerization [17,18]. Then the Cry oligomer binds to a second receptor. Second receptors such as aminopeptidase N or alkaline phosphatase are anchored to the membrane by a glycosylphosphatidylinositol-anchor, and are localized within lipid rafts [18,19]. The oligomeric toxin inserts into the membrane forming ionic pores causing osmotic lysis of midgut epithelial cells and insect death [1,18].

Although it has been recognized for decades that Cry toxins exert their toxic effect by forming pores into the midgut cells of their target insect, recently an alternative and opposing model was
proposed. The alternative model proposed that after the monomeric Cry toxin binds cadherin, a Mg$^{2+}$-dependent adenyl cyclase/PKA-signaling pathway is activated leading to cell death [20]. In this alternative model, neither oligomerization or pore formation are involved in Cry toxicity.

We hypothesized that mutants of Cry toxins affected in pore formation might work as DN inhibitors. The Domain I of Cry toxins is involved in pore formation [21–25]. In this work we analyzed several mutations in helix α-4, in helix α-3 or in domain II-loop 3. These mutants were affected in pore formation, toxin oligomerization and receptor binding, respectively. We found that DN phenotype is linked to mutations affected in pore formation but that are still able to form oligomeric structures with the wild type toxin resulting in a complete inhibition of its insecticidal activity.

The fact that DN mutations blocked toxicity of wild type Cry toxin, supports the concept that oligomerization is a fundamental step in Cry toxin mode of action in agreement with the pore formation model of Cry toxin action.

**Results**

**Cry1Ab mutant characterization**

We isolated and characterized Cry1Ab mutants affected at different steps of their mode of action, namely receptor binding, oligomerization and pore-formation to determine if any of them showed a DN phenotype. First, we constructed a Cry1Ab mutant G439D located in loop 3 of domain II. We selected this mutation since a similar mutant, previously characterized in another Cry homolog [26], was shown to have reduced toxicity toward *M. sexta*, reduced binding to BBMV and because the loop 3 region is important for binding with cadherin receptor [26–28]. Secondly, we used a previously described Cry1Ab mutant R99E, located in helix α-3 that showed impaired toxin oligomerization [21]. Finally, we constructed several point mutations in helix α-4 of Cry1Ab such as E129K, N135C, D136N, A140K, T142C, T143D, and T143N, that in the context of Cry1Aa toxin showed to be affected in pore formation and toxicity [22,23]. We also constructed two double mutants, the D136N/T143D and E129K/D136N. Binding analysis with *P. xylostella* BBMV, were reported only for E129K and D136N mutants, revealing no effects on binding of these two mutants, and suggesting that loss of binding was not the reason for the loss of toxicity in these Cry1Aa mutants [25]. However, the characterization of these mutants was partial since the binding to *M. sexta* membranes, as well as the oligomerization process was not analyzed.

All of the Cry1Ab mutants analyzed in this work produce bipyramidal crystal inclusions similar to the wild type toxin with exception of mutant T143N that was not further analyzed. With the exception of two mutants, all other mutant toxins showed severe reductions in toxicity when tested against *M. sexta* larvae (Table 1). The two toxins that retain activity corresponds to binding, trypsin activated proteins were labeled with biotin and susceptibility to protease action (data not shown).

We then analyzed the ability of the mutant proteins to oligomerize. In this assay the Cry1Ab mutant-protoxins were proteolytically activated with *M. sexta* midgut proteases in the presence SUV liposomes and the antibody scFv73 that mimics an epitope of the cadherin receptor that interacts with loop 2 of domain II [17,18,21]. The oligomeric structure was observed as a low mobility 250-kDa band in a Western blot assay using a specific anti-Cry1Ab antiserum. As shown in Figure 2, only mutant R99E, located in helix α-3 was affected in oligomerization as previously reported [21]. The oligomeric structure of wild type Cry1Ab toxin was mainly found inserted into the membrane pellet, in contrast with the helix α-4 mutants, that remained in the soluble fraction.

**Table 1. Toxicity of wild type and mutated Cry1Ab toxins against *Manduca sexta* larvae.**

| β-endotoxin | LC$_{50}$ ng/cm$^{2}$ (95% fiducial limits) | Location of mutated residues |
|-------------|------------------------------------------|-----------------------------|
| WI Cry1Ab  | 1.3 (0.9–1.7)                            |                             |
| R99E        | >2000                                    | Helix α-3 of Domain I       |
| E129K       | >2000                                    | Helix α-4 of Domain I       |
| N135C       | 16.4 (10.9–22.7)                         | Helix α-4 of Domain I       |
| D136N       | 2.8 (2.2–3.8)                            | Helix α-4 of Domain I       |
| A140K       | 5.3 (2.8–8.2)                            | Helix α-4 of Domain I       |
| T142C       | 34.9 (28.3–41.7)                         | Helix α-4 of Domain I       |
| T143D       | >2000                                    | Helix α-4 of Domain I       |
| D136N, T143D| >2000                                    | Helix α-4 of Domain I       |
| E129K, D136N| >2000                                    | Helix α-4 of Domain I       |
| G439D       | >2000                                    | Loop 3 of Domain II         |

**Figure 1. Binding competition assays of Cry1Ab mutants to BBMV of *Manduca sexta* larvae.** Binding of biotin labeled toxins was analyzed in the absence (lanes −) or in the presence (lanes +) of 500-fold molar excess of unlabeled toxin. The biotinylated toxins bound to the vesicles, were visualized with streptavidin-HRP conjugate. The Cry1Ab and all mutants located in domain I (helices α-3 or α-4) bound specifically to BBMV only mutant G439D was affected in binding to the *M. sexta* BBMV.

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suggesting that helix α-4 mutants were affected in membrane insertion (Fig. 2A). Finally, the G439D mutant, located in domain II loop 3, also showed an oligomeric structure that was mainly found inserted into the membrane.

Since our oligomeric assay utilizes the scFv73 antibody that mimics the cadherin repeat 11 (CR11) region of the cadherin receptor which recognizes loop 2 in domain II and considering that the G439D mutation is located in a toxin region which interacts with a different region of the cadherin receptor, i.e. the CR12 fragment [28,29], we repeated the oligomerization assay of G439D using a purified CR12 fragment from cadherin receptor, instead of the scFv73 antibody. Under these conditions the oligomerization of the G439D mutant was severely reduced when compared with the wild type toxin (Fig 2B).

In vivo inhibition of toxin insecticidal activity

To compare the potency of the mutants as DN inhibitors, we tested their ability to inhibit the toxicity of Cry1Ab to M. sexta larvae. We fed the larvae with different mixtures of wild type and mutant toxins. We used an equimolar ratio (1:1) as well as a lower ratio (0.25:1 of mutant: wild type). Figure 3A shows that some mutants located in helix α-4 completely blocked toxin action even at sub-stoichiometric ratios. Mutants D136N and A140K did not show DN phenotype because they were not severely affected in toxicity (Table 1), showing an increase in mortality when mixed with the wild type toxin at 1:1 ratio. The higher activity is due to the fact that we used 2 ng/cm² of each toxin, one being wild type

Figure 2. Oligomerization of Cry1Ab proteins. Panel A, Cry1Ab and mutant protoxins were proteolytically activated with M sexta midgut proteases in the presence of SUV liposomes and scFv73 antibody. Membrane pellets were recovered by centrifugation and the toxin detected by Western blot using an anti-Cry1Ab antibody in the supernatant and in the membrane fraction. The oligomeric structure of 250-kDa of the Cry1Ab is observed inserted into the membrane pellet, in contrast with the helix α-4 mutants, that remains in the soluble fraction. The mutant R99E, located in helix α-3 did not form oligomeric structures. Panel B, Oligomerization of Cry1Ab and mutant G439D proteins performed as above but in the presence of the cadherin CR12 fragment instead of scFv73 antibody. Under these conditions the oligomerization of the Cry1Ab wild type is observed inserted into the membrane and oligomerization of G439D mutant was severely reduced. Panel C, Oligomerization of the mixtures of 1:1 Cry1Ab: Mutant proteins performed as in Panel A. The oligomer of double mutants or in the 1:1 mixture of Cry1Ab with the double mutants is observed in the soluble fraction.

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Figure 3. In vivo analysis of the Dominant Negative phenotype of Cry1Ab mutants. Panel A, Toxicity assays against M sexta larvae with Cry1Ab at 2 ng/cm² of diet (black bar) or with a mixture of the same concentration of Cry1Ab wild type with the mutant proteins at two different ratios, 0.25:1 mutant:Cry1Ab (white bars) or 1:1 (grey bars). Some mutants of helix α-4 show a clear DN phenotype. Panel B, Toxicity assays against M sexta larvae as panel A but at 10:1 mutant:Cry1Ab ratio (dashed bars). R99E reduce toxicity of wild type under this condition in contrast mutant G439D did not affect toxicity of the wild type toxin.

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and the other being either the D136N or A140K mutant that showed reduced toxicity but remain active (Table 1). This mixture represents, therefore, the additive mortality of the two toxin proteins. In contrast, helix α-3 R99A and domain II-loop 3 G439D mutants did not show a DN phenotype. The R99A mutant, showed a competition phenotype since only a high ratio of 10:1 reduced the toxicity of Cry1Ab. In contrast, the G439D mutant showed no effect on Cry1Ab toxicity even at a 10:1 ratio (Fig. 3B).

**Discussion**

The helix α-4 mutations analyzed in this study do not impair toxin assembly in a pre-pore structure, but rather block an essential conformational transition of the assembled complex necessary for membrane insertion and pore formation. The helix α-4 mutations that resulted in loss of toxin action act as DN antitoxins blocking toxicity and pore formation of wild type toxin. These data strongly indicate that oligomerization and pore formation are necessary steps in the mode of action of Cry toxins. In contrast, the helix α-3 R99A mutant that is affected in the process of oligomerization but retain binding capacities to membrane receptors, displayed competitive binding for the receptor at 10:1 ratio (mutant: wild type). Finally a mutant in domain II, G439D, with altered binding interaction with the BBMV and the cadherin receptor, did not compete with Cry1Ab for binding and neither showed a DN phenotype.

These data are similar to some reported mutants of the anthrax toxin; a mutant affected in its activation by furin, was unable to undergo oligomerization, yet still bound to, and competed receptor binding causing a competitive inhibition of toxin action only at high at 10:1 ratios [30,31]. In another report an anthrax mutant affected in toxin oligomerization did not show a DN phenotype since it was unable to form hetero-oligomers with the wild type toxin [32]. Finally, an anthrax mutant with altered receptor binding did not compete for receptor binding and neither affected wild type activity [32].

The molecular mechanism observed in DN phenotype involves toxin oligomerization between different Cry toxin-monomers forming hetero-oligomeric structures between mutant and wild type monomers. The hetero-oligomer that is formed with the double mutants and the wild type Cry1Ab toxin was severely affected in membrane insertion and pore formation activity suggesting a problem in the transition from pre-pore to pore as was previously proposed for anthrax DN mutants [16].

If the assembly of the Cry toxin oligomeric structure is an stochastic procedure, then at a 1:1 ratio the probability to have at least one subunit of the DN mutant in the resulting oligomeric-complex is high.

In vitro inhibition of toxin pore formation activity

To determine if pore formation inhibition by the DN mutants depends on the ability to form hybrid complexes with wild type toxin, we produced homo- and hetero-oligomers and measured their ability to form conductive ion channels in black lipid bilayers. Wild type Cry1Ab or the D136N/T143D and E129K/D136N double mutants were activated in the presence of SUV liposomes and scFv73 antibody as described above to produce oligomeric structures. The hetero-oligomers were prepared by mixing the DN mutants with the wild type in a 1:1 ratio during activation under similar conditions described above. We analyzed oligomer formation in the supernatant and pellet fractions, after centrifugation of the activation reaction to separate toxin inserted into liposomes from soluble proteins. Figure 2C shows that the 250-kDa oligomer was observed mainly in the pellet in the case of Cry1Ab. Nevertheless, in the case of the D136N/T143D and E129K/D136N double mutants or in the 1:1 mixture of Cry1Ab with the double mutants, the 250-kDa oligomers were observed in the soluble fraction (Fig 2C). The soluble and membrane pellet fractions of activation reactions were used to assay pore formation activity in black lipid bilayer system as described previously [21].

The results indicated that oligomers produced by the mutant toxins were severely affected in their pore formation activity when compared with wild type toxin. The hetero-oligomers formed by a mixture of wild type and mutant proteins were also inactive in pore formation. Figure 4A shows representative traces of the activity of Cry1Ab, the mutant E129K/D136N and the mixture of these two proteins in lipid bilayers. Similar data were obtained with the mutant D136N/T143D (data not shown). Current-voltage curves are presented in figure 4B, showing that only wild type Cry1Ab toxin has pore formation activity. These results are consistent with the notion that DN mutants inactivate the wild type toxic action in vivo by forming inactive hetero-oligomers unable to insert into the membrane.

![Figure 4](https://example.com/figure4.png)
If one mutant monomer is enough to completely block the wild type toxin activity, then at 1:1 ratio an effective blockage of toxin action is expected. The fact that we found inhibition of wild type toxin activity at 0.25:1 ratio strongly indicates that a single mutant subunit is sufficient to inactivate the oligomer activity and that oligomerization is an important step in toxin action.

The data presented here provides unequivocal evidence that oligomerization is a key step in the mode of action of Cry1Ab and further supports that pore formation is an important event triggering insect cell death. These data support the pore-forming model of the mode of action of Cry toxins and contradict the model of cell death induced by the interaction with cadherin receptor and subsequent induction of signal transduction pathway.

Recent reports raised the concern that the Cry1A toxins may affect non-target organisms [5–9]. Nevertheless, Cry1A toxins used in transgenic plants have been extensively shown to be specific against target insects and safe to non-target organisms [2–4]. In any case the antitoxins of Cry1A described here could be used to inhibit toxicity of Cry toxins in special conditions like, for example, for attenuation of an accidental effect or a release of unregulated Cry toxin, since they offer an efficient alternative to neutralize and counter the Bt toxin action that would help protect potentially endangered organisms in a particular ecosystem.

Materials and Methods

Construction of Cry1Ab mutants

Mutants were produced by site-directed mutagenesis (Quick-Change, Stratagene, La Jolla, CA) using the pHTr315Ab harboring cry1Ab gene. Appropriate oligonucleotides were synthesized for each mutant. Automated DNA sequencing at UNAM’s facilities verified the single point mutations. Acrystalliferous Bt strain 407 harboring a point mutation D136N was transformed with recombinant plasmids and selected in Luria broth at 30°C supplemented with 10 μg ml⁻¹ erythromycin. For construction of double mutants we used pHTr315Ab-D136N harboring a point mutation D136N as template to introduce additional point mutations as E129K or T143D.

Cry1Ab toxin purification

Bt transformant strains were grown at 30°C in nutrient broth sporulation medium with erythromycin until complete sporulation. Crystals were observed under phase contrast microscopy and purified by sucrose gradients [33]. Crystals were solubilized in 50 mM Na₂CO₃, 0.2% β-mercaptoethanol, pH 10.5. The monomeric toxins were obtained by trypsin activation in a mass ratio of 1:20 (1 h, 37°C). Phenylmethylsulfonyl-fluoride (1 mM final concentration) was added to stop proteolysis. The oligomeric Cry1Ab structure was produced as described [17,21] by incubation with svFv73 antibody (1:4 toxin:antibody ratio) purified as described [17,21] by incubation with svFv73 antibody (1:4 toxin:antibody ratio). Appropriate oligonucleotides were synthesized for each mutant. Automated DNA sequencing at UNAM’s facilities verified the single point mutations. Acrystalliferous Bt strain 407 harboring a point mutation D136N was transformed with recombinant plasmids and selected in Luria broth at 30°C supplemented with 10 μg ml⁻¹ erythromycin. For construction of double mutants we used pHTr315Ab-D136N harboring a point mutation D136N as template to introduce additional point mutations as E129K or T143D.

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References

1. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, et al. (1998) Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 775–806.
2. Mendelson M, Kough J, Vaituzis Z, Matthews K (2003) Are Bt crops safe? Nature Biotechnol 21: 1003–1009.
3. Chen M, Zhao JZ, Collins HL, Earle ED, Cao J, et al. (2008) A critical assessment of the effects of Bt transgenic pants on parasitoids. PLoS ONE 3: e2284.
4. Romeis J, Meissle M, Bigler F (2006) Transgenic crops expressing Bacillus thuringiensis toxins and biological control. Nature Biotechnol 24: 63–71.
5. Rosi-Marshall E, Tank JF, Royer TV, Whiles MR, Evans-White M, et al. (2007) Toxins in transgenic crop products may affect headwater stream ecosystems. Proc Natl Acad Sci USA 104: 16204–16208.

6. Kramarz PE, de Vaufleury A, Zygmunt PM, Verdun C (2007) Increase response to cadmium and Bacillus thuringiensis maize toxicity in the snail Helix aspersa infected by the nematode Phasmarhabditis hermaphrodit a. Environ Toxicol Chem 26: 71–79.

7. Zwahlen C, Hilbeck A, Howald R, Nentwig W (2003) Effects of transgenic Bt corn litter on the earthworm Lumbricus terrestris. Mol Ecol 12: 1077–1086.

8. Bohn T, Primicerio R, Hessen DO, Tzaav T (2008) Reduced fitness of Daphnia magna fed a Bt-transgenic maize variety. Arch Environ Contam Toxicol 55: 584–592.

9. Douville M, Gagné F, André C, Blaise C (2009) Occurrence of the transgenic corn crylAb gene in freshwater mussels (Elliptio complanata) near corn fields: Evidence of exposure by bacterial contamination. Ecotoxicol Environ Saf 72: 17–23.

10. Parker MW, Feil SC (2005) Pore-forming protein toxins: from structure to function. Progress Biophys Mol Biol 88: 91–124.

11. Rainey GJ, Young JA (2004) Antitoxins: novel strategies to target agents of bioterrorism. Nature Rev Microbiol 2: 721–729.

12. Maynard JA, Maassen CB, Lappla SH, Brasky K, Patterson JL, et al. (2002) Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. Nature Biotechnol 20: 597–601.

13. Scobie HM, Rainey GJ, Bradley KA, Young JA (2003) Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci USA 100: 5170–5174.

14. Wai SN, Westermark M, Oscarsson J, Jass J, Maier E, et al. (2003) Antitoxin binding facilitates proteolytic cleavage of helix a of domain II loop 3 of Bacillus thuringiensis delta-endotoxin involved in toxicity and specificity. J Biol Chem 278: 2311–2317.

15. Masson I, Tahashnik BE, Liu YB, Bousseau R, Schwartz JL, et al. (1999) Helix 4 of the Bacillus thuringiensis Cry1Aa toxin lines the lumen of the ion channel. J Biol Chem 274: 31996–32000.

16. Smalley DP, Ellar DJ (1996) Mutagenesis of three surface loops of Bacillus thuringiensis insecticidal toxin reveals sites important for toxicity receptor recognition and possibly membrane insertion. Microbiology 142: 1617–1624.

17. Rajamohan F, Hussain SA, Cottrill JA, Gould F, Dean DH (1996) Mutations at domain II loop 3 of Bacillus thuringiensis Cry1Aa and Cry1Ab a-endotoxins suggest loop 3 is involved in initial binding to lepidopteran midgut. J Biol Chem 271: 25220–25226.

18. Xie R, Zhuang M, Ross LS, Gómez I, Ohlman DI, et al. (2005) Single amino acid mutations in the cadherin receptor from Heliothis virescens affect its toxin binding ability to Cry1A toxins. J Biol Chem 280: 8416–8425.

19. Pacheco S, Gómez I, Gill SS, Bravo A, Soberón M (2009) Enhancement of insecticidal activity of Bacillus thuringiensis Cry1A toxins by fragments of a toxin-binding cadherin correlates with oligomer formation. Peptides 30: 583–588.

20. Beauregard KE, Collier RJ, Swanson JA (2000) Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization. Cell Microbiol 2: 251–258.

21. Singh Y, Chaudhary VK, Leppla SH (1989) A deleted variant of Bacillus anthracis protective antigen is non-toxic and blocks anthrax toxin action in vivo. J Biol Chem 264: 19103–19107.

22. Yan M, Collier RJ (2003) Characterization of dominant negative forms of anthrax protective antigen. Mol Med 9: 46–51.

23. Thomas WE, Ellar DJ (1983) Bacillus thuringiensis var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vivo and in vitro. J Cell Sci 66: 181–197.

24. Soberón M, Pardo-López J, López I, Gómez I, Tabashnik B, et al. (2007) Engineering modified Bt toxins to counter insect resistance. Science 318: 1640–1642.

25. Wolfenden M, Luethy P, Maurer A, Parenti P, Sacchi FV, et al. (1985) Preparation and partial characterization of amino acid amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (Pieris brassicae). Comp Biochem Physiol 86A: 301–308.

26. Mueller P, Rudin DO, Tien HT, Westcott WC (1962) Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. Nature 194: 979–980.