Stoichiometry for α-bungarotoxin block of α7 acetylcholine receptors

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α-Bungarotoxin (α-Btx) binds to the five agonist binding sites on the homopentameric α7-acetylcholine receptor, yet the number of bound α-Btx molecules required to prevent agonist-induced channel opening remains unknown. To determine the stoichiometry for α-Btx blockade, we generate receptors comprised of wild-type and α-Btx-resistant subunits, tag one of the subunit types with conductance mutations to report subunit stoichiometry, and following incubation with α-Btx, monitor opening of individual receptor channels with defined subunit stoichiometry. We find that a single α-Btx-sensitive subunit confers nearly maximal suppression of channel opening, despite four binding sites remaining unoccupied by α-Btx and accessible to the agonist. Given structural evidence that α-Btx locks the agonist binding site in an inactive conformation, we conclude that the dominant mechanism of antagonism is non-competitive, originating from conformational arrest of the binding sites, and that the five α7 subunits are interdependent and maintain conformational symmetry in the open channel state.

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urotoxins from the venom of snakes in the family Elapidae are notorious for producing long-lived neuromuscular blockade\(^1\). They are basic peptides comprised of 60–70 amino acids that, through intra-molecular disulfide bonds, form three fingers that extend from a globular base\(^2\). Binding of α-neurotoxin to the acetylcholine receptor (AChR) at the motor synapse is mutually exclusive toward binding of small molecule agonists and antagonists\(^3\), and X-ray crystallographic studies show that α-neurotoxins, agonists and antagonists contact overlapping regions at the ligand-binding site\(^4\)\(^-\)\(^9\). The overall physiological, pharmacological and structural studies suggest that α-neurotoxin blockade operates through a competitive mechanism.

Members of the nicotinic AChR family contain from two to five agonist binding sites. Studies of the muscle AChR, which contains two agonist binding sites, showed that α-neurotoxin occupancy of one site rendered the agonist-induced response undetectable\(^10\)\(^-\)\(^11\). Those observations harmonized with subsequent single channel measurements showing that AChRs occupied by one agonist opened the channel with much lower efficiency than those occupied by two agonists\(^12\)\(^-\)\(^2\). Thus consistent with a competitive mechanism, occupancy of one site by α-neurotoxin prevented binding of a second agonist required for efficient channel opening. However, given subsequent structural data\(^2\)\(^-\)\(^4\), an alternative mechanism is equally plausible: α-neurotoxin occupancy locks the binding site in an inactive conformation and conformational arrest of that site prevents channel opening.

To distinguish between competitive and conformational arrest mechanisms, we devise a strategy to assess α-neurotoxin occupancy of the α7 AChR simultaneously with agonist-induced channel opening. The α7 AChR contains five identical subunits and binds the α-neurotoxin, α-Bungarotoxin (α-Btx), at each of its five agonist binding sites (Fig. 1)\(^13\)\(^-\)\(^15\), thus offering the maximum number of sites to study the occupancy-channel opening relationship. Taking advantage of a mutant subunit that confers α-Btx resistance, we generate receptors comprised of wild-type and α-Btx-resistant subunits, and tag one of the subunit types with conductance mutations to report subunit stoichiometry\(^17\)\(^-\)\(^18\); following incubation with α-Btx; measure the current amplitude of each channel opening event to determine the subunit stoichiometry\(^17\)\(^-\)\(^18\); following incubation with α-Btx, infer the number of bound α-Btx molecules from the subunit stoichiometry.

A functional α-Btx-resistant mutant. To generate functional, α-Btx-resistant α7 receptors, we considered sequence differences among naturally occurring nicotinic AChR α-subunits, such as the α1–subunits from the snake and mongoose\(^19\)\(^-\)\(^2\) and the α2–α4 subunits from heteromeric neuronal AChRs\(^2\)\(^-\)\(^2\). All of these subunits harbour subtle changes in a structure known as loop C, a β-hairpin at the entrance of the AChR ligand binding site, and a major site for interaction with α-Btx (Fig. 1). We chose to mimic sequence differences in loop C from the neuronal α3 subunit, a strategy that was successful in generating a functional, α-Btx-resistant α7/5-HT3A receptor\(^2\)\(^-\)\(^2\)\(^3\), and mutated the pair of residues flanking a canonical tyrosine residue within loop C to their α3 counterparts (Fig. 1).

To confirm that receptors comprised of α-Btx-resistant subunits fulfilled the dual requirements of α-Btx resistance and agonist responsiveness, we transfected cDNAs encoding either wild-type or α-Btx-resistant subunits into HEK cells, and incubated the cells with either buffer alone or buffer containing 50 nM α-Btx. We then formed a cell-attached patch with the potentiator PNU-120596 (PNU) in the pipette solution to prolong the inherently brief α7-channel openings on subsequent addition of agonist\(^2\)\(^-\)\(^2\). After recording a baseline free of channel openings, we added the membrane-permeable agonist nicotine to the external solution. For cells expressing either wild-type or α-Btx-resistant receptors, addition of nicotine elicits single channel

![Figure 1](image-url)
Figure 2 | A functional α-Btx-resistant α7 mutant. Difference in toxin sensitivity for homopentameric receptors formed from (a) wild-type (WT, top two traces) and (b) mutant α-Btx-resistant subunits mutant (MU, bottom two traces). After recording a stable baseline free of single channel openings (that is, no agonist present), nicotine (100 μM final concentration) was added to the bath solution surrounding each patch (arrow). For the lower traces, in both ‘a’ and ‘b’, cells were incubated with 50 nM α-Btx for 20 min before forming a cell-attached patch. A concentration of 10 μM PNU was included in all patch pipettes to prolong agonist-activated openings and facilitate their visualization (see Methods). Note that each recording was obtained from a different membrane patch, on a different cell, so the magnitude of the evoked currents is not comparable between recordings. Traces are filtered at 100 Hz, each increment of the vertical axes (right) indicates 10 pA, and scale bar, 5 s, 25 pA.

currents that sum in a staircase manner, indicating robust receptor activation by agonist (Fig. 2). Following incubation with α-Btx, channel opening of wild-type α7 receptors is completely blocked, whereas channel opening of α-Btx-resistant receptors still occurs. Thus, receptors comprised of α-Btx-resistant subunits respond to agonist but are not blocked by α-Btx.

Another requirement is that for receptors containing both α-Btx-resistant and wild-type subunits, α-Btx binds normally to sites formed from wild-type subunits. Thus, we transfected HEK cells with cDNAs encoding the wild-type α7 subunit, the α-Btx-resistant subunit or a mixture of the two. We then incubated intact cells with increasing concentrations of radio-labelled α-Btx, and measured the amount of bound radio-ligand under steady state conditions. For wild-type α7, the total binding of α-Btx increases abruptly at low concentrations, but as the concentration is increased, binding increases more gradually and linearly (Fig. 3). To determine non-specific binding, we measured binding of α-Btx to cells transfected with the human-muscle AChR β1-subunit, which does not form α-Btx-binding sites, and observed only the gradual and linear increase in binding over the range of α-Btx concentrations. The difference between total and non-specific binding reveals that binding of α-Btx to cells expressing wild-type α7 receptors is saturable and the dissociation constant is ~3 nM. By contrast, for cells expressing α-Btx-resistant receptors, binding of α-Btx is very similar to that for cells transfected with the AChR β1-subunit (Fig. 3). Thus, specific

Figure 3 | Steady state α-Btx binding measurements. Radiolabeled [125I]-α-Btx binding to cells expressing (a) wild-type, low-conductance (WTLC) subunits, (b) mutant subunits (MU), or (c) mixtures of MU and WTLC subunits (1:4, MU:WTLC). In each case, total binding (closed circles, solid line), non-specific binding (open circles and dashed line), as well as specific binding (grey circles and line) are shown. Non-specific binding was determined from cells transfected with the cDNA encoding the human muscle AChR β1-subunit, which does not form α-Btx binding sites. The apparent Kds for cells expressing WTLC subunits, and mixtures of MU and WTLC subunits are 2.7 nM (2.26–3.14 nM) and 2.4 nM (1.90–2.84 nM), respectively, (95% confidence limits), where n = 3 and the error bars represent one s.d. of the mean. (d) Time course of [125I]-α-Btx dissociation from cells expressing WTLC (closed circles and solid line), or mixtures of MU and WTLC subunits (1:4, MU:WTLC), open circles and dashed line). The dissociation half-life for cells expressing WTLC subunits and mixtures of MU and WTLC subunits are 322.6 min (239.4–494.3 min) and 316.7 min (258.0–410.0 min), respectively, (95% confidence limits), where n = 2 and error bars are one s.d. of the mean.
binding of α-Btx to receptors containing α-Btx-resistant subunits is negligible over the concentration range tested. For cells transfected with both wild-type and α-Btx-resistant subunits, both the concentration dependence of α-Btx binding and the rate at which bound α-Btx dissociates mimic those observed for cells transfected with wild-type α7 subunits alone (Fig. 3). Thus, for receptors containing both α-Btx-resistant and wild-type subunits, α-Btx binds normally to sites formed from wild-type subunits.

**Electrical fingerprinting.** To determine the number of bound α-Btx molecules required to block agonist-induced single channel currents, we employed our previously described electrical fingerprinting method15–18. The method relies on co-assembly of wild-type and mutant subunits, and in one of the subunit types, substitution of arginine for polar or anionic residues in cytoplasmic portals to alter the unitary current amplitude26. Previously, following co-assembly of normal and low conductance α7 subunits, patch clamp recordings revealed five distinct current amplitudes, corresponding to pentameric receptors with zero to four low conductance subunits; receptors with five low conductance subunits were not observed, presumably owing to their low current amplitude17,18. However, in the course of testing different ionic conditions, we found that removal of calcium unmasked small but detectable unitary currents from receptors comprised solely of low conductance subunits. Thus in the following experiments, we used a calcium-free pipette solution to allow identification of all six possible subunit combinations based on current amplitude.

For cells transfected with cDNA encoding the α-Btx-resistant, normal conductance subunit, patch clamp recordings reveal long-lived single channel openings with large and uniform current amplitudes (Fig. 4). The pipette solution contained ACh.

**Figure 4 | Electrical fingerprinting to determine subunit composition from single channel amplitudes.** (a) Mutant α-Btx-resistant subunits produce channel openings with a uniform large current amplitude (MU, top), while wild-type, low conductance subunits (WTLC) produce openings with a uniform small current amplitude (middle trace). Co-expressing MU subunits with WTLC subunits (1:4; mutant:WTLC) gives rise to receptors with different subunit stoichiometry and channel openings with a distribution of amplitudes (bottom trace). For each trace, the detected amplitude for individual clusters of openings, corresponding to a single channel, is overlaid (see Methods for detection criteria; open is up; scale bar, 10 s, 10 pA). (b) Event-based amplitude histograms for pooled clusters of openings from mutant subunits (top; 7 recordings and 89 clusters), WTLC subunits (middle; 7 recordings and 62 clusters), and mixtures of mutant and WTLC subunits (bottom; 21 recordings and 225 clusters, pooled from 1:4 and 1:3 cDNA ratios). Amplitude scatter plots corresponding to the openings are also shown above each histogram. Histograms have been fit with a sum of Gaussian components, corresponding to the amplitude classes used to determine (c) the relationship between single-channel amplitude and the stoichiometry of MU and WTLC subunits. For each histogram in ‘b’, the Gaussian components are labelled ‘0-5’ according to the presumed number of incorporated mutant subunits. The mean amplitude and s.d. (α) of each component was used to determine the amplitudes and associated error bars (1σ) used for the plot in ‘c’. The amplitudes for the MU and WTLC classes (yellow and green points) were determined from the corresponding MU and WTLC histograms, whereas for the channels with mixed stoichiometry (blue points) the amplitudes were from the corresponding components in the MU + WTLC histogram.
and the potentiator PNU, which prolongs channel openings to allow accurate determination of current amplitude, a requirement for electrical fingerprinting. PNU does not affect the ability of α-Btx to bind to α7 receptors or block channel opening (Fig. 2), likely because it binds within an inter-helical transmembrane cavity. Furthermore, rather than promoting channel opening, PNU dramatically slows the onset of desensitization, which develops from the open channel state. Owing to the submaximal concentration of PNU (1 μM), brief un-potentiated channel openings, resolved only partially at the chosen filter bandwidth, also occur throughout the recordings. For cells transfected with cDNAs encoding the wild-type α7, low conductance subunit, patch clamp recordings again reveal long-lived single channel openings, but their current amplitude, though uniform, is much smaller (Fig. 4). The two diverging current amplitudes represent the maximum and minimum baselines with which to compare receptors formed from combinations of the two types of subunits.

We then co-transfected HEK cells with cDNAs encoding both the α-Btx resistant, normal conductance subunit and the wild-type, low conductance subunit and made patch clamp recordings. The recordings again reveal long-lived single channel openings, but the openings exhibit several different current amplitudes (Fig. 4). As expected from a pentameric assembly of the two types of subunits, the distribution of current amplitudes contains six Gaussian components, corresponding to all possible subunit combinations. Furthermore, the decrease in amplitude between successive components is regular, suggesting that the single-channel amplitude is determined by the number of incorporated low conductance subunits (Fig. 4). Thus, for each channel opening episode, the current amplitude registers the stoichiometry of high- and low-conductance subunits.

Although the two types of subunits co-assembled to form functional receptors, we found that the partitioning of the different current amplitudes did not follow a simple binomial distribution based on the ratio of transfected cDNAs. For example, a 1:1 transfection ratio yielded mainly channel openings with the largest current amplitude, corresponding to receptors with five α-Btx-resistant, normal conductance subunits. The major cause appeared to be a difference in both the time course and level of expression of the two types of receptor subunits. Thus, to obtain the range of current amplitudes in Fig. 4, we transfected the cells with a three- to four-fold molar excess of cDNA encoding the wild-type, low conductance subunit.

**Stoichiometry of α-Btx block.** To determine the stoichiometry for α-Btx block of α7 channel opening, we co-transfected cells

![Figure 5](https://example.com/figure5.png)
with the subunit cDNAs in ratios of 1:4 and 1:3 (α-Btx-resistant, normal conductance to wild-type and low conductance) and recorded agonist-induced channel openings without and with pre-incubation with α-Btx; a concentration of 50 nM α-Btx was chosen to maximize occupancy of the wild-type binding sites and minimize occupancy of the α-Btx-resistant sites. We first describe results from the 1:4 transfection ratio, which gave the greatest diversity of current amplitudes, and thus the broadest range of subunit stoichiometry. In the absence of α-Btx, patch clamp recordings revealed frequent channel openings, which often superimposed, and the unitary current amplitudes spanned a wide range (Fig. 5). However, after incubation with α-Btx, the frequency of channel opening was greatly suppressed and many patches (13 of 21) exhibited no openings at all (Fig. 5). Equally important, only openings with large current amplitude were present. Thus, in addition to blocking the vast majority of channel openings, α-Btx narrows the distribution of current amplitudes, indicating block depends on the subunit stoichiometry.

Figure 6 | Stoichiometry of α-Btx inhibition of α7. Amplitude histograms, where the number of events in each bin has been scaled to their relative frequency (see Methods), for pooled clusters of openings from cells co-expressing mixtures of mutant (MU) and wild-type, low conductance (WTLC) subunits at cDNA ratios of (a) 1:4; MU:WTLC and (b) 1:3; mutant:WTLC. In each case, the top histogram represents the distribution and frequency of clusters and the total recording time for each condition (see Methods). For the 1:4 transfection ratio, in the absence of α-Btx, the amplitude histogram contains five of the six possible amplitude classes; openings from the largest amplitude class, corresponding to receptors with five α-Btx-resistant, normal conductance subunits, were not evident as a distinct component (Fig. 6). After incubation with α-Btx, the frequency of clusters of all amplitudes is dramatically reduced and only clusters from receptors with zero or one α-Btx-sensitive subunit were present (Fig. 6). For receptors with one α-Btx-sensitive subunit, the extent of reduction is ~85%, which approaches the 95% reduction expected if a single occupancy prevented channel opening and occupancy by 50 nM α-Btx was 95%. Thus for the 1:4 transfection ratio in the presence of α-Btx, receptors with more than one α-Btx-sensitive, low conductance subunit are completely blocked and receptors with one α-Btx-sensitive subunit open very rarely.

For the 1:3 ratio, in the absence of α-Btx, the majority of channel openings originated from receptors with either zero or one α-Btx-sensitive, low conductance subunit and the two amplitude classes occurred with a similar frequency (Fig. 6). After incubation with α-Btx, however, virtually all channel openings originated from receptors with five α-Btx-resistant, normal conductance subunits (Fig. 6). Furthermore, the frequency of these openings was essentially the same as that in the absence of α-Btx, which would be expected if the number of α-Btx-resistant receptors was equivalent under the two conditions, and the number of patches was sufficient to minimize patch-to-patch variability in the number of α-Btx-resistant receptors. Furthermore, by achieving a population enriched in...
receptors with zero or one wild-type, low conductance subunit, we find that α-Btx blocks the vast majority of receptors with a single α-Btx-sensitive site, despite the presence of four sites that remain accessible to the agonist.

Discussion

We employed an electrical fingerprinting approach to determine the stoichiometry for α-Btx block of α7-AChR channel opening. The results reveal that α-Btx completely blocks receptors with more than one α-Btx-sensitive subunit, and greatly suppresses receptors with just one α-Btx-sensitive subunit. These observations are striking because with one or two α-Btx molecules bound, multiple sites remain unoccupied and available to the agonist, yet in the absence of α-Btx, agonist occupancy of a single site elicits robust channel openings with normal durations. The collective structural and single channel biophysical studies suggest that α-Btx immobilizes the site to which it is bound, which in turn, either alters the interaction between agonist and the remaining sites, interferes with coupling of agonist binding to channel opening, or induces a non-native conformation refractory to channel opening. Thus, although snake α-neurotoxins compete against agonist binding, their dominant mechanism of antagonism is non-competitive, originating from conformational arrest of the binding sites.

Previous studies showed that α-Btx blocked spontaneous channel opening by both the muscle AChR and an α7 receptor with mutations at the 9′ position of the second transmembrane domain. In common with a conformational arrest mechanism, α-Btx block of spontaneous channel opening likely resulted from immobilization of the receptor in the closed channel conformation. However, a distinguishing insight from this work is that conformational arrest by α-Btx impairs the ability of the remaining unoccupied sites to either bind agonist or couple binding to channel opening. Analogously, a conformational arrest mechanism may explain competitive antagonism by methyllycaconitine (MLA). Following MLA antagonism of α7 and washout of the drug, recovery of agonist-induced macroscopic currents showed a sigmoid time course best explained by requiring MLA dissociation from all five sites before agonist-induced activation could occur. Thus although MLA and α-Btx interact very differently at the α7-binding site, binding of a single MLA molecule appeared enough to inhibit α7.

While our results show that a single bound α-Btx molecule greatly suppresses channel opening, it is important to consider the extent of suppression. For the concentration of α-Btx used in the present experiments (50 nM, ~20 times the Kᵦ), at equilibrium, occupancy by α-Btx is expected to be 95%. Thus, if a single α-Btx was enough to fully block channel opening, for receptors with a single α-Btx-sensitive subunit, the maximum suppression of channel opening is expected to be ~95%. For the 1:4 transfection ratio, the frequency of openings from receptors with a single α-Btx-sensitive subunit is reduced by more than 85% and for the 1:3 transfection ratio, the reduction approaches 100%. These values bracket the expected suppression of 95% and may reflect limitations of sampling. Thus, we favour the interpretation that occupancy by a single α-Btx is enough to fully block channel opening.

Given this interpretation, the ability of a single α-Btx molecule to block agonist-induced channel opening distinguishes independent from interdependent conformational changes of the α7 subunits (Fig. 7). In the independent mechanism, an individual subunit can change conformation regardless of the conformations of the other subunits and agonist occupancy of a single binding site opens the channel regardless of the conformations of the remaining subunits. However, given that a single α-Btx molecule blocks agonist-induced channel opening, we can discount the independent mechanism, as it incorrectly predicts that binding of five α-Btx molecules would be required.

By contrast, our findings are compatible with an interdependent mechanism, where a single agonist occupancy opens the α7 channel, but only when all five subunits can adopt an active conformation (Fig. 7). In the interdependent mechanism, occupancy by a single α-Btx molecule arrests all five subunits in the inactive conformation, and the energy gain from binding multiple agonist molecules is not enough to overcome conformational arrest by α-Btx. Yet, in the absence of α-Btx, occupancy by a single agonist is enough to produce the activated conformation of all five α7 subunits. Clearly the energy landscape of α7 is exquisitely poised to take advantage of modest changes in energy from occupancy by a single agonist. At the same time, α-Btx exploits the fact that the energy gained from agonist binding is modest compared with conformational arrest imposed by a single bound toxin.

In early models of protein allostery, a key postulate was that on activation, individual subunits in an oligomer maintain conformational symmetry. Maintenance of symmetry predicts that arresting a single site in an inactive conformation will thwart activation of the entire oligomer, which is consistent with a single α-Btx-sensitive subunit imparting toxin-sensitivity to α7. Although we cannot distinguish whether subunit conformational changes occur in a sequential or concerted fashion, our results suggest that in the open channel state, interdependent conformational changes maintain symmetry of the five α7 subunits, which allows a single bound α-Btx to block channel opening.

Methods

Materials. [125I]-labelled α-Btx was from Perkin Elmer, while unlabelled α-Btx and 1-(5-Chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea, known as PNU-120596, were from Tocris Biosciences. All other chemicals, including acetylcholine chloride (ACh) and nicotine (Nic), were from Sigma-Aldrich.

Site-directed mutagenesis. Mutant α7 cDNAs were constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and then confirmed by sequencing the entire coding region. On the basis of previous findings with a chimeric α7(5-HT3A receptor23, the α-Btx-resistant mutant contains two substitutions within the α7 ligand-binding domain (F187K and E189N; Fig. 1). The low conductance form of α7 (WT8) results from substitution of three arginines...
Expression of human 5-HT3. BOSC 23 cells, modified HEK 293 cells, were transfected by calcium phosphate precipitation. Expression of human 5-HT3a receptor26. In the absence of calcium at 100 nM ACh and 1 μM PNU, ACh and PNU were added directly to pipette solutions from 1000 x concentrated stock solutions of ACh in pipette solutions, and PNU in dimethylsulphoxide. Pipette solutions were stored in aliquots at -80 °C until immediately before each experiment. Patch pipettes were pulled from glass capillary tubes (No. 7052, Precision Glass) and coated with Sylgard (Dow Corning). Single-channel currents were recorded and low pass filtered at 10 kHz using an Axopatch 200 B patch-clamp amplifier (InstruTECH ITC-16 interface (Heka Elektronik). For experiments in which nicotine was added to the bath solution (Fig. 2), cells were baked in 0.7 ml of bath solution, and a G2E seal was formed in the cell-attached patch configuration. The patch pipette contained 10 μM PNU in pipette solutions. After 5 min of recording, 0.3 ml of 333 mM nicotine in bath solution was added to the external solution surrounding the patch. For all 5-HT3 experiments, cells in 35-mm culture dishes were aspirated of their surrounding media and gently washed with 1.0 ml of bath solutions, and PNU in dimethylsulphoxide. Pipette solutions were added drop wise to the external solution surrounding the patch after 5 min of recording, 0.3 ml of 333 mM nicotine in bath solution was added to the external solution surrounding the patch. The patch clamp electronics were tuned so that these artifacts were both minimized and recognizable as brief (1 ms) negative baseline deflections, which allowed them to be identified and subsequently removed for presentation purposes. The data were analysed before removal of these artifacts.

Single-channel analysis. Single-channel analysis was performed using the program TAC 4.2.0 (Bruxton), which digitally filters the data (Gaussian response), interpolates the digitized points using a cubic spline function and detects events (Figs 5, 6). Where ’baseline’ is the combined number of all clusters neighbouring baseline, for each condition; ’total’ is the combined time, in minutes, for all recordings for a given condition; ’G2E’ is the combined number of observed clusters, including those superimposed on one another, for each condition; and ’G2Ebaseline’ is the combined number of clusters neighbouring baseline, for each condition. The resulting amplitude histogram gives a measure of the frequency of events with specified amplitudes, and allows for comparisons of cluster frequency across conditions (that is, with and without 5-HT3a subunit). A tacit assumption is that channels in the patch open independently of one another and therefore the frequency of superimposed openings classifies the amplitude distribution if it were possible to accurately measure the amplitudes of all openings, including those that are superimposed.
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Kinetics of [125I]-a-Btx dissociation. To measure the time course of [125I]-a-Btx dissociation (Fig. 3), a cell suspension, prepared as described above, was mixed with 50 nM [125I]-a-Btx and incubated for 1 h at 21°C. The suspension was then centrifuged at 1000g for 1 min, the supernatant was removed and the cells were resuspended in 20 ml of potassium Ringer’s solution. Aliquots of the cell suspension were then rapidly filtered through type A/E glass fibre filters (Gelman Sciences) at specified times after resuspension. Unbound toxin was removed by successive washes with 140 mM NaCl in 10 mM HEPES, pH 7.4. To determine non-specific binding, the same procedure was applied to cells transfected with the human muscle β1 subunit. Specific binding is the difference between total and non-specific binding.

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Author contributions
C.J.B.d. and S.M.S. designed the research; C.J.B.d., C.R.F. and S.M.S. performed the research; C.J.B.d. Analysed the data; S.M.S. supervised the research; and C.J.B.d. and S.M.S. wrote the paper.

Additional information
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