NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

Phrenic Nerve Hemidiaphragm Assay

In vitro (ex *vivo*) assessment of botulinum neurotoxicity

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Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. There are three separate functional regions of the toxin responsible for specific binding, translocation and enzymatic activities respectively. Loss of any one of these activities will result in an inactive toxin molecule, as assessed by a fully functional model such as the mouse LD_{50} test. Following the three R's principle, alternative fully functional assays are required. Use of the ex-*vivo* phrenic nerve hemidiaphragm assay therefore, full-fills this criteria.

Electrical stimulation of the phrenic nerve causes twitch responses in the focally innervated mouse diaphragm muscle. The diaphragm muscle consists of a thin sheet which remains viable for hours in an organ bath, allowing the inhibitory effects of botulinum neurotoxin to be directly measured. Toxin potency is directly measured by the degree of muscular paralysis induced by the toxin acting on the pre-synaptic nerve terminal. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity.

Materials / Methods

Equipment

Computer: Dell Optiplex with 1.25GB RAM

PowerLab/4SP 4 channel recorder (ADInstruments, UK)

Bridge Amp ML110 (ADInstruments, UK)

4 Channel Dual Impedence Stimulators (ADI, Digitimer, UK)

Isometric force transducers, GM 2 type (FMI, Germany)

Hemidiaphragm Equipment / tissue baths / holders (FMI, Germany)

Thermocirculator (Harvard Apparatus, UK)

95% Oxygen/5% Carbon dioxide Gas cylinder (BOC, UK)

Gas Regulator (BOC, UK)

Extractor Fan / filter Kit 2000 with hood and lamp (Nederman/ VWR International)

Low energy light bulb 20w (equivalent to 100w) fitted to lamp (Various)

Small tissue clips / green braided polyester suture 2 metric 3-0 UPS (FMI, Germany)

Calibration weight 5g (Ohaus).

Black thread (Various)

Krebs Gelatine Ringer solution

Gelatin / NaCl / KCl / KH_2PO_4 stock (4X store sterile at 4-8°C, warm to RT before use)
27.6g/L or 55.2g/2L NaCl
1.44g/L or 2.88g/2L KCl
0.64g/L or 1.28g/2L KH_2PO_4
8g/L or 16g/2L gelatin
Heat until all the gelatin is completely dissolved, adjust volume with ion-exchanged, distilled water to exactly 1 or 2L, mix, aliquot 4X 500ml and autoclave.

Sodium hydrogen carbonate stock / $NaHCO_3$ (833mM) – 21g per 300ml

Magnesium sulphate heptahydrate stock / $MgSO_4.7H_2O$ (120mM) – 2.96g per 100ml

Calcium chloride 2-hydrate stock / $CaCl_2$ $.2H_2O$ (250mM) – 3.68g per 100ml

D-glucose / $C_6H_{12}O_6$ (2.22M) – 39.6g per 100ml

Store all stock solutions at 4-8°C.

Make up 1L of fresh Krebs solution daily from the stock solutions:

To exactly 250ml of the gelatin stock add approximately 600ml distilled water whilst mixing on a magnetic stirrer, then add 30ml of the Sodium hydrogen carbonate stock solution and 10ml of each of the Magnesium sulphate heptahydrate stock and the Calcium chloride 2-hydrate stock solutions and 5ml of the D-glucose stock (2.22 M) or 1.98g and adjust the pH to 7.5 if required and finally the volume to exactly 1L.

Krebs with the final composition should be produced:

Gelatine (0.2%), NaCl (118mM), KCl (4.83mM), KH_2PO_4 (1.19mM), $NaHCO_3$ (25mM), $MgSO_4.7H_2O$ (1.2mM), D-glucose / $C_6H_{12}O_6$ (11.1 mM), $CaCl_2$ $.2H_2O$ (2.54mM).

This solution is continuously oxygenated with carbogen (95% Oxygen/5% Carbon dioxide) gas.

Method

(i) Turn on PowerLab - The left most indicator on the front panel, the Power indicator, should glow green. The second indicator from the left of the front panel, the Status indicator, should flash yellow 4 times (indicating a SCSI ID number of 4). The status indicator will then turn off (If not consult Powerlab manual). Turn on computer and monitor.

(ii) Access chart recorder

Double click the mouse on; 4 channels icon, Chart v5 should open. Settings saved should be: 400 samples/sec. High pass = DC, Low pass = 200Hz, Range = lmV. Digital filter = Low pass / 40Hz Cut off frequency.

Data pad settings: Time, Comment text, Ch1 freq, Ch2 freq, Ch1 Avg, Ch2 Avg, Ch3 freq, Ch4 freq, Ch3 Avg, Ch4 Avg, Comment time.

(iii) Calibration

Click mouse on (set up) and go to (zero all inputs). Start chart. Using the 5g Ohaus calibration weight, attach for a short time to each transducer. Stop the trace. Select the area of trace for channel 1 to include the 5g weight area. Click on (channel 1) and go to (units conversion). Select area of the base line and click the boxed arrow pointing to zero units. Select area representing the 5g displacement and click on the boxed arrow pointing to 5 units. Click on (apply), then (O.K.)

Repeat the procedure for all remaining channels.

(iv) Dissection / set up.

Wrap an ice block with blue roll. Place some Krebs (gassed with 95% O_2 / 5% CO_2) on a separate ice block. Obtain 20-30g (male) MF1 mice from the B.S.S. (order at ~ 20-25g). Transfer the mice in a spare cage to the dissection area. Kill the mice by cervical dislocation (A schedule 1 method). Avoid over stretching the mice as this may damage the diaphragms neuromuscular junction / nerve or severe the phrenic nerves. Place the mice under the lamp/extractor hood on the ice block.

Lay the animal on its back and remove the fur and skin covering the upper part of the abdomen and thorax. Remove the muscle layers covering the chest and expose the rib

cage. Lift the rib cage along the midline with a pair of forceps and make an incision half way between the xiphisternum and the neck. Examine inside the thoracic cavity to ensure that the phrenic nerves are not adhering to the chest wall as this occasionally happens. If this is the case, gently dislodge the nerves. Make lateral cuts on either side of the midline incision, parallel to the ribs, and fill the cavity with cold gassed Krebs solution. Lift the caudal edge of the incised rib cage and remove all the ribs except the one attached to the diaphragm on the animals left side. Carefully free the left phrenic nerve of connective tissue / fat and tie a short length of dark coloured thread around the uppermost part of the nerve and cut the nerve above the attached thread. Cut the wall of the abdomen to free the rib cage. The diaphragm and phrenic nerve should be irrigated regularly with cold gassed Krebs. Holding the xiphisternum with forceps cut straight down through the diaphragm to the spine, taking care not to cut or stretch the phrenic nerve. Cut the diaphragm laterally from its attachment to the body wall so freeing the preparation from the animal. Place the nerve and muscle in a petri dish containing gassed Krebs and trim the ribs / diaphragm to a suitable size to fit in the tissue bath. Attach a small tissue clip with an attached loop of thread to the tip of the tendonous tissue at the apex of the diaphragm preparation for later attachment to the transducer.

Quickly but gently locate the centre of the rib on the platinum spike electrode of the tissue holder and impale to secure the preparation. Gently pull the thread attached to the nerve through the ring electrode. Hook the loop from the tissue clip / diaphragm onto the force transducer and lower the mounted preparation into the organ bath containing gassed warmed Krebs. Adjust the tension to give a baseline tension of 1 - 1.5g (15mN). Gently pull the thread attached to the nerve until the tip of nerve is pulled through the ring electrodes. The nerve may be held in place by either suspending a small weight or attaching the thread with Blue tack. Start recording and stimulation of the nerve (~3V, 1Hz, 0.2ms). Regularly adjust the baseline tension to ~ 1.5g (15mN) as required. Ensure that the preparation is adequately aerated, but over aeration will produce excessive frothing. Nerve stimulation should be maintained throughout the experiment, except during washing or direct stimulation. Tissue preparations are stimulated indirectly (via the nerve) using a supramaximal voltage

(~3V). A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) may also help the tissue to stabilise.

Wash the preparation with ~6ml fresh Krebs/gelatine at least twice. Adjust the tension as required until the baseline tension is consistent and no longer needs adjustment. Once a consistent twitch and baseline tension are produced without change over a period of greater than 30min the tissue is ready for toxin addition. No more adjustments should be made to the baseline tension for the rest of the experiment. If the twitch response is decreasing after this time or the preparation is producing responses of less than 0.5 gram tension then the preparation should either be given longer to stabilise or be discarded. The twitch response should be regular and consistent, if not the tissue should again either be given longer to stabilise or be discarded. No visible change to the baseline tension or twitch size should be found 30min prior to adding toxin.

Tissue baths are maintained at 37°C and contain 6ml of Krebs / gelatine oxygenated with 95% O_2 + 5% CO_2. A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) is applied shortly (5-10min) before toxin addition and at the end of the experiment as an added control.

(v) Experimental protocol

Reconstitute toxin vials (therapeutic or reference preparations) typically with 1ml of gassed Krebs / gelatine. Dilute to the desired concentration (typically between 1 – 20 LD_{50} U/ml) to give a total volume of 6ml Krebs / gelatine in a labelled bijou, mix gently and incubate at 37°C for 30 min. Stop the chart and drain the bathing Krebs solution, quickly but carefully add the toxin solution (pouring the toxin solution down the side of the tissue bath and not directly onto the tissue or thread connected with the force transducer) and immediately start the chart (Stimulation is automatically stopped when controlled by the PowerLab if the chart is not recording). Appropriately label the toxin addition on the chart. Click on the *, located at the bottom left of the chart, if a single channel is to be labelled then type the channel number and click add. If all channels are to be labelled then enter * and label the chart appropriately and click on add.

Typically after up to three and a half hours after adding the toxin, directly (muscle) stimulate the tissue and stop the chart. Click the mouse onto the (save) icon and appropriately label the file and save.

Discard or remove tissue and store for further studies. Discard remaining Krebs. Lower tissue holders back into the tissue baths and wash out thoroughly with tap water followed by distilled water. Once every month or as necessary clean the system with dilute (10%) sodium hypochlorite solution. Ensure all hypochlorite is thoroughly washed out of the system with several washes of distilled water and spills have been thoroughly washed off (as it will kill the tissue and is highly corrosive). The gas should not be turned off during cleaning or washing to prevent the back flow of liquid into the gas pipe. Tissue baths and tissue holders should be bathed and washed with fresh Krebs/gelatine thoroughly before further use.

Antibody neutralisation of toxin is assessed by premixing a fixed toxin concentration with the antitoxin and incubating for 30 minutes at 37°C before addition to the tissue at T0. An identical dose cycle is used to that shown above.

(vi) Data analysis:

Measure the average twitch height (10-20 twitches) before toxin addition and at 10 min intervals following toxin addition. Express as a percentage of twitch height before toxin addition. Plot the percentage twitch height against time and calculate the time to 50% inhibition.

Following toxin addition the twitch size should vary by less than 10% over the first 30 min. Any data falling outside these limits should be either excluded or if the decrease occurs in the first 10 min due to tissue movement but then remains stable, the data may be recalculated with 100% contraction set at 30min post toxin addition. (It is, however, preferable to have 5% or less deviation if possible.).

Using a reference toxin preparation with a known potency perform a dose response curve using at least 4-6 replicates per dose. Unknown toxin concentrations can then be calculated by repeatedly testing (at least 4 times) at a suitable dilution which can be

read off the linear part of the dose response curve. Other suitable methods of calculating the relative potency such as parallel line analysis may also be considered.