Botulinum toxin types A, B and D inhibit catecholamine secretion from bovine adrenal medullary cells

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Evoked catecholamine secretion from cultured bovine adrenal medullary cells is inhibited by commercially available botulinum toxins – types A, B and D (10^4 10^6 MLD/ml of culture medium). Basal secretion is also inhibited. The catecholamine content of such toxin-treated cells is larger than that of control cells and may in part be a result of the inhibition of basal release. The onset of action of botulinum toxin types A and D can be neutralised by their respective antisera. Concentrations of botulinum toxins A, B or D that inhibit secretion leave unaffected the $^{45}Ca^{2+}$ influxes normally associated with secretion. These data provide further evidence to support the idea [(1985) Nature 317, 719–721] that botulinum toxins block secretion by acting downstream of the Ca^{2+} transient at or near the site of exocytosis.

(Adrenal medulla) *Botulinum toxin* *Exocytosis* *Ca^{2+}* *Catecholamine*

1. INTRODUCTION

Transmitter release from peripheral cholinergic nerve endings is blocked by botulinum toxins, leading to muscular weakness and paralysis [2–4]. Little is known though of the mechanism by which these toxins act and this is perhaps in part due to the lack of a suitable in vitro cholinergic preparation. One type of botulinum toxin however (type D) has also been reported to block catecholamine secretion from cultured bovine adrenal medullary cells [1]. The value of this adrenergic preparation to elucidate the mechanism of cholinergic block is not certain though because inhibition of catecholamine secretion was reported to occur only with type D toxin, whereas botulinum toxin types A and B are, in the main, equally effective at blocking in vivo cholinergic synapses [2,3]. This difference could arise because the chromaffin cell has receptors sensitive only to toxin type D, or that only type D is effective intracellularly, or simply that the concentrations of the other toxins used were not high enough. There was also the remote possibility that the inhibitory effect seen with the sample of D toxin was not in fact due to botulinum toxin at all but rather to some contaminant. This paper describes a series of experiments that set out to reexamine the effects of a range of commercially available botulinum toxins on both basal and evoked catecholamine secretion. The experiments show that types A, B and D are equally effective in blocking catecholamine secretion, and in a manner that is entirely consistent with the earlier report that these toxins may inhibit secretion by acting downstream of the Ca^{2+} transient and at or near the site of exocytosis.

2. MATERIALS AND METHODS

Cells were isolated, cultured, incubated with toxins and challenged with carbamylcholine as described before [1,5,6], the only difference being that the cells were cultured in Dulbecco's modified Eagle's Medium containing 10% fetal calf serum, 100 $U \cdot ml^{-1}$ penicillin G and 5 μM cytosine arabinoside. Botulinum toxin types A, B, C and D were obtained from Wako Pure Chemical Industries Ltd, Japan. Toxins were diluted for use in-

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to glycerol phosphate buffer, pH 6.0, and 50 μl added to each 1 ml culture well. The potencies of the toxins were calibrated in terms of mouse lethal doses (MLD), Wistar mice (average weight 23 g) being used. Type A, B, C, D and E botulinum antitoxin sera were used in aliquots of 50 μl (each containing 5 international units of antitoxin). The antitoxins were mixed with 50 μl toxin for 3 min before the mixtures were added to 1 ml culture medium. ^{45}Ca influx experiments were performed as described elsewhere [1]. The effect of toxin on basal release was determined by first incubating over 3 days the cultured cells with botulinum toxin together with 100 μM ascorbate and 1.4 μCi of [^{3}H]noradrenaline per ml of culture medium (equivalent to 3×10^{-8} M noradrenaline). Additional [^{3}H]noradrenaline and ascorbate (3×10^{-8} M and 100 μM, respectively) were added after 24 and 48 h. Cells were then washed 7 times in culture medium at 37°C over a 5 min period and incubated with 0.5 ml culture medium per culture well over a 5 h period at 37°C. The 0.5 ml of culture medium was changed every 30 min, and the amount of label released into these 0.5 ml samples measured. At the end of the 5 h period the cultured cells were challenged at 37°C with culture medium containing carbamylcholine, and the amounts of ^{3}H label and cold catecholamine released over a 30 min period measured. Toxin-treated cultured cells were also incubated for a short 20 min period with [^{3}H]noradrenaline and ascorbate before being rapidly washed and challenged with carbamylcholine as before.

3. RESULTS

The measured potencies of Wako toxin types A, B and D agreed well with those reported by the company, i.e. 2×10^7 MLD per mg of type A or type B proteins, and 2×10^8 MLD per mg of type D protein. Type C toxin however was found to be over 2 orders of magnitude less potent than that measured by the company 1 month earlier. It is possible therefore that the C toxin decomposed during this one month period. Some aliquots of B toxin also appeared unstable and lost their potency, but this was not so marked as with the C toxin. The highest concentrations of toxins used in the experiments with adrenal cells were 10^6, 10^6 and 10^7 MLD, respectively, for type A, B and D toxins, and 5×10^2 MLD for type C toxin.

Botulinum toxin types A, B and D inhibited evoked catecholamine secretion in a dose-dependent manner (figs 1 and 2). Fig.1 shows that incubation with 1–5 μg/ml of the toxins, corresponding to 2×10^4–2×10^5 MLD, for 6 days, inhibited evoked secretion by 50%. The potency is time dependent as in the same experiment the amounts of toxin needed to give half-maximal inhibition after only 3 days of incubation were approx. 5 times the amounts needed for the 6 day incubation. Botulinum toxin type C, when used at its highest concentration for 6 days, also had a small inhibitory effect on evoked secretion (fig.1).

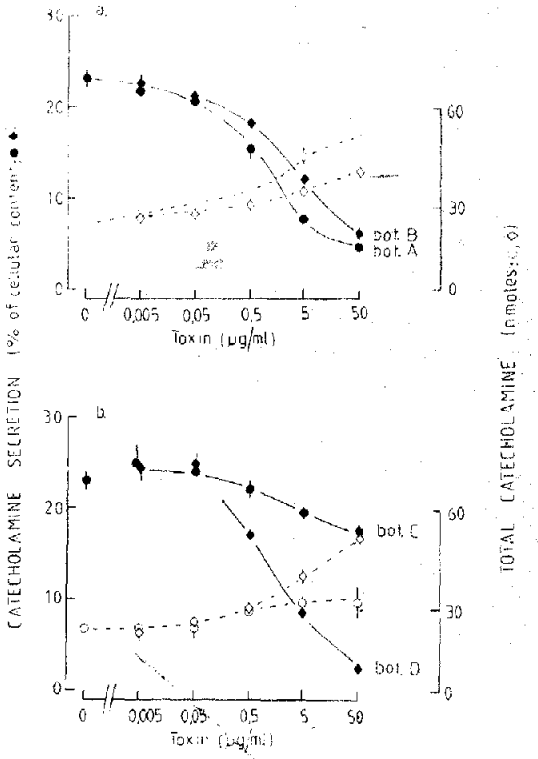

Fig.1. Inhibition of evoked catecholamine secretion by botulinum toxins. Cells were incubated with the various quantities of toxin shown for 6 days before being washed with physiological saline containing 3 mM Ca^{2+}, and challenged with 5×10^{-4} M carbamylcholine for 15 min at 20°C. Secretion, closed symbols; total catecholamine in the culture wells, open symbols. Data points are means of 3 determinations (error bars = SE). (a) Botulinum toxin types A (●,○) and B (◆,◇). (b) Botulinum toxin types C (●,○), and D (◆, ◇).

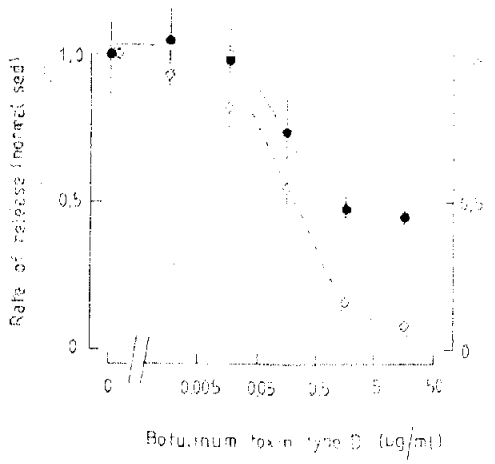

Fig.2. Inhibition of basal and evoked release by botulinum toxin type D. Release is shown normalised to that occurring in the absence of the toxin, the data points being the means of 4 determinations (error bars = SE). (●) Basal release of ^3H measured over 5 h from unstimulated cells that had been preincubated with toxins and [^3H]noradrenaline for 3 days. Release in the absence of the toxin was 1.5% of the cellular content per hour; (○) evoked release of ^3H from the same cells over 30 min at 37°C by 5×10^{-4} M carbamylcholine, the evoked release in the absence of toxin was 6%; (◇) evoked release as above but from cells preincubated with [^3H]noradrenaline for only 20 min prior to being challenged to secrete, the amount released in the absence of the toxin was 11%.

The botulinum toxins that are effective at blocking secretion also increase the total catecholamine content of the cultured cells, the increase occurring over the same toxin concentration range and time course as inhibition of secretion. Fig.1b shows for example that after 6 days of incubation with type D toxin the evoked secretion is inhibited by over 90% and the catecholamine content of the cells is double that of control cells. Whilst a component of this increase might be attributed to an increase in catecholamine synthesis, another explanation could be that the basal rate of secretion is also inhibited by botulinum toxins. Measurement of basal catecholamine release over a period of hours was unsuccessful using the standard fluorimetric trihydroxyindole assay [5,7] because of the small amounts of catecholamine secreted and the instability (oxidation) of the amine at pH 7.4 and 37°C. Measurements of basal release from cells preloaded with [^3H]noradrenaline proved possible however. Fig.2 shows the basal release of ^3H over a 5 h period. The data clearly show that cells incubated with botulinum toxin type D release less label than do control cells, the dose dependence of the toxin being very similar to that for inhibition of evoked release of ^3H label or of endogenous catecholamine. The extent of the inhibition however seems to be somewhat different, evoked release being inhibited by over 90% whereas basal release from the same set of cells is only inhibited a little over 50%. The rate of basal release decreased from 1.5%/h (SE 0.25%/h, 4 determinations) for control cells, to 0.69%/h (SE 0.05%/h, 4 determinations) for toxin-treated cells.

The inhibition of evoked and basal secretion could be interpreted not only as a reduction in the extent of exocytosis but also, during the days of toxin treatment, as a slow redistribution of catecholamine within the cell into compartments that are not immediately associated with the secretory process. The data of fig.2 however show that the toxin dose-dependence for inhibition of evoked release is very similar for cells exposed to [^3H]noradrenaline either for 3 days or for only 20 min before being challenged with a secretagogue. Such data showing that the potency of the toxin is independent of the time that the secretory product is in the cell give no support to the idea of a slow redistribution of secretory product away from granules undergoing exocytosis.

Even though very high concentrations of the toxins are necessary to block catecholamine secretion, the cause of the block is almost certainly by action of the botulinum toxins, rather than by some foreign contaminant, as the potency of the toxins is lost when preincubated with botulinum toxin antisera prepared independently. Fig.3 shows that botulinum toxin type A potency is removed by preincubating the toxin with anti-A toxin antiserum, and that of type D toxin by preincubating with either types C or D antisera. These latter results are consistent with reported C and D type toxin-antitoxin crossreactivity [8].

So far, no conditions have been found to reverse the botulinum induced inhibitory effect. In one experiment for example, secretion was inhibited by 89% after 4 days incubation with 10^6 MLD/ml of botulinum toxin type D. Repeated washing and in-

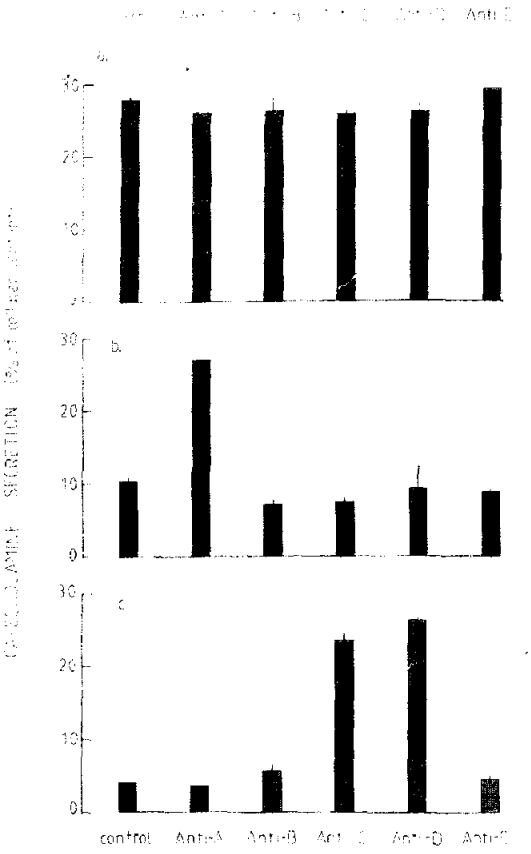

Fig.3. Toxin-antitoxin neutralisation. 15 μg of type A and D toxins were incubated with 5 units of antitoxin for 3 min before being added to 1 ml cell cultures. After 3 days the cells were washed and challenged with 5×10^{-4} M carbamylcholine for 15 min at 20°C. Data are means of 3 determinations (error bars = SE). (a) No toxin used; (b) botulinum toxin type A; (c) botulinum toxin type D.

cubation with an excess amount (5 units) of anti-D-toxin for a further 14 days was not sufficient for the cells to recover, the secretory response still being depressed by 72% compared with control cells of the same age not initially treated with toxin. There is strong evidence to suggest that a rise in intracellular Ca^{2+} triggers catecholamine secretion, and that such a rise is brought about by a Ca^{2+} influx into the cells in response to depolarising agonists [9–11]. Fig.4 shows that this Ca^{2+} influx normally associated with secretion remains unaltered in cells treated with botulinum toxins A, B and D, whereas secretion from the same cells is inhibited.

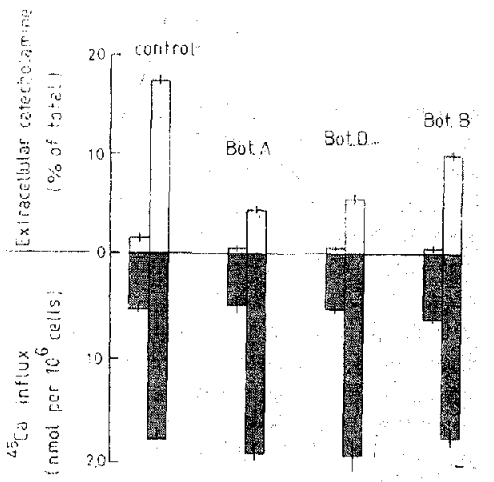

Fig.4. The effect of botulinum toxins on ^{45}Ca influx. Cells were cultured at 1.5×10^6 cells per ml for 3 days with 15 μg per ml of botulinum toxin types A, B and D, before being challenged with 5×10^4 M carbamylcholine for 12 min. The extracellular fluid was removed, the plates washed 5 times over 3 min with ice-cold physiological saline containing 0.5% bovine serum albumin and the ^{45}Ca associated with the cells determined. The second bar of each pair corresponds to measurements made on stimulated cells. The data are means of 3 determinations (error bars = SE). (Open bars) Catecholamine in the extracellular fluid; (closed bars) ^{45}Ca associated with the same cells.

4. DISCUSSION

In an earlier paper [1] it was reported that of the toxins tested only botulinum toxin type D inhibited evoked catecholamine secretion from bovine adrenal medullary cells. The results presented here not only support this earlier finding that botulinum toxin type D inhibits catecholamine secretion from bovine chromaffin cells, but in addition show that type A and B botulinum toxins, when used in the same concentration range as type D toxin, also inhibit evoked release. Botulinum toxin also inhibits basal release from chromaffin cells and this can account for the rise in cellular catecholamine that seems to be associated with toxin-treated cells. The data here are evidence that the botulinum toxins that inhibit acetylcholine release at the mouse neuromuscular junction also inhibit catecholamine secretion from bovine

chromaffin cells in vitro. Furthermore the data are entirely consistent with the earlier proposal [1] for the mechanism of action of botulinum toxin on adrenal cells, i.e. by the toxin acting downstream of the Ca^{2+} transient possibly at or near the site of exocytosis. The amount of toxin necessary to inhibit catecholamine secretion from this in vitro preparation however is 4–5 orders of magnitude greater than that needed to block acetylcholine release in vivo. If botulinum toxins act from within the cell [2–4], then it is unclear, at the moment, whether this difference in potency is because of a difference in the penetration into the cytosol of the two preparations, or whether it is because of a difference in the potency of the toxins once inside. If it is simply the case of a difference in penetration then it may be possible to increase the sensitivity of the chromaffin cell to the toxins by altering the surface properties of the cell [12], or to bypass the plasma membrane altogether by introducing small amounts of toxin directly into the cytosol by either microinjection, endocytosis, or by making use of the 'leaky' cell technique [13,14].

In summary, it looks very likely that the botulinum toxins inhibit secretion by acting at or near the site of exocytosis.

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