Volume 298, number 2,3, 118–122 FEBS 10720 February 1992
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The effect of botulinum toxin type D on the triggered and constitutive exocytosis/endocytosis cycles in cultures of bovine adrenal medullary cells

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Received 20 December 1991

The extracellular fluid phase marker, horseradish peroxidase, enters chromaffin cells when triggered to secrete catecholamine. This triggered uptake, like secretion, is abolished in cells pre-incubated with botulinum toxin. Endocytosis of horseradish peroxidase into unstimulated cells is unaffected by botulinum toxin but is inhibited when the temperature is reduced. Once internalised by the unstimulated cells, horseradish peroxidase is released back into the extracellular fluid, the rate of release being temperature sensitive but unaffected by carbamylcholine or botulinum toxin. These results suggest that triggered exocytosis is a necessary event to precede triggered endocytosis, and that botulinum toxin may affect only the triggered exocytosis/endocytosis cycle and not the constitutive cycle.

Endocytosis; Vesicular, Botulinum toxin; Exocytosis; Chromaffin cell; Fluid phase uptake

1. INTRODUCTION

Most cells continuously undergo cycles of exocytosis and endocytosis, and in some cases the turnover of extracellular fluid is quite large. For example, using horseradish peroxidase (HRP) as a fluid phase marker, it has been estimated that about 25% of the cell volume of fibroblasts is taken up over a period of 3–6 h [1–5] and that this fluid phase uptake is balanced by exocytosis, with 20–50% of internalised tracers released back into the extracellular fluid within 3–10 min [3,6,7].

This activity, termed constitutive exocytosis/endocytosis, is distinct from regulated exocytosis/endocytosis which occurs in cells containing specialised secretory vesicles. These cells can release the contents of their vesicles by exocytosis in response to external stimuli. In chromaffin cells, for example, up to 30% of the cellular catecholamine can be released in a short time in response to cholinergic stimulation. The same stimulus triggers not only exocytosis but also endocytosis.

Recent studies, directed towards dissecting the intracellular factors controlling these two events, used horseradish peroxidase (HRP) as a marker of fluid phase uptake, as it allowed the rapid kinetics of endocytosis to be studied [8]. HRP, however, detects all types of endocytosis [9], and so it was possible that cholinergic activation of the cell triggered endocytosis which was independent of triggered exocytosis. The discovery that botulinum toxin type D blocks exocytosis by acting downstream of the calcium transient, at or near the site of exocytosis in chromaffin cells [10], provides a tool to investigate this further.

This paper therefore describes experiments that address two questions: (i) Does botulinum toxin inhibit triggered endocytosis of HRP as well as triggered exocytosis in chromaffin cells? and (ii) what is the effect of the toxin on the basal uptake of HRP into, and subsequent release from, unstimulated cells?

The results show that botulinum toxin blocks triggered endocytosis as well as exocytosis, and are consistent with the idea that triggered exocytosis is a necessary event to precede endocytosis. The data strongly suggest that botulinum toxin does not block untriggered, constitutive fluid phase exocytosis and endocytosis.

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2. MATERIALS AND METHODS

2.1. *Solutions and chemicals*

Physiological saline contained 150 mM NaCl,5 mM KCl, 10 mM glucose, 2.5 mM $CaCl_2$, 1.8 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 10 mM HEPES, pH 7.3.

In solutions containing zero calcium, Ca was replaced by Mg and 5 mM EGTA was added.

All chemicals, except for botulinum toxin, were obtained from Sigma.

Purified botulinum toxin type D was obtained from Wako Co., Japan. In some experiments unpurified D toxin (a gift from Dr E. Schantz, Wisconsin, USA) containing an ADP-ribosyltransferase component was used [11]. The results described in this paper are from experiments using purified toxin. Similar results were obtained using the unpurified form.

2.2. *Preparation of cells and experimental procedures*

Cells were isolated and cultured in multiwell culture plates for 3–5 days at a density of 10^6 cells/well as described previously [12] and in

 Published by Elsevier Science Publishers B.V.

the presence of various concentrations of botulinum toxin [13]. A chromaffin cell suspension, carefully and freshly prepared from medullary tissue, is relatively pure, with non-chromaffin cells occupying only about 20% of the population [14]. Such purity is achieved by using only 'white' medullary tissue free from the obvious darker cortical tissue, and the use of Percoll gradients to further purify the cells, together with the use of cytosine arabinoside to prevent proliferation of contaminating cells in culture, e.g. fibroblasts, ensure that our cell preparation is essentially chromaffin cells [12].

Catecholamine secretion, and horseradish peroxidase (HRP) uptake and release were followed as described previously [12]. Basal secretion of HRP was followed after pre-loading the cells by a 30 min incubation at 23°C with HRP (1 mg/ml) and subsequent removal of extracellular label with three washes in physiological saline.

3. RESULTS

3.1. *Catecholamine release and horseradish peroxidase uptake*

Fig. 1a and b show that carbamylcholine triggers both catacholamine secretion from, and HRP uptake into, cultured chromaffin cells, with the onset of HRP uptake slightly lagging behind the faster onset of catecholamine release. Unstimulated cells also release a small amount of catecholamine, i.e. less than 4% of the cellular content per h. The same sample of unstimulated cells also take up a small amount of extracellular horseradish peroxidase, but here the ratio of basal uptake to basal catecholamine release is markedly greater than the ratio of evoked HRP uptake to evoked catecholamine release. The time-course of basal HRP uptake and basal catecholamine release were similar, both being linear over 20 min.

Fig. 2 shows that the amount of HRP that entered both stimulated and unstimulated cells was linearly dependent on the extracellular concentration of HRP during uptake, indicating that HRP enters the cell predominantly by fluid phase uptake.

Carbamylcholine-evoked catecholamine release and, consequently, HRP uptake were dependent on extracellular calcium. Basal HRP uptake was less so, however. The carbamylcholine-evoked responses were reduced by over 95% when the extracellular calcium was decreased from 3 mM to close to 1 nM (5mM EGTA and

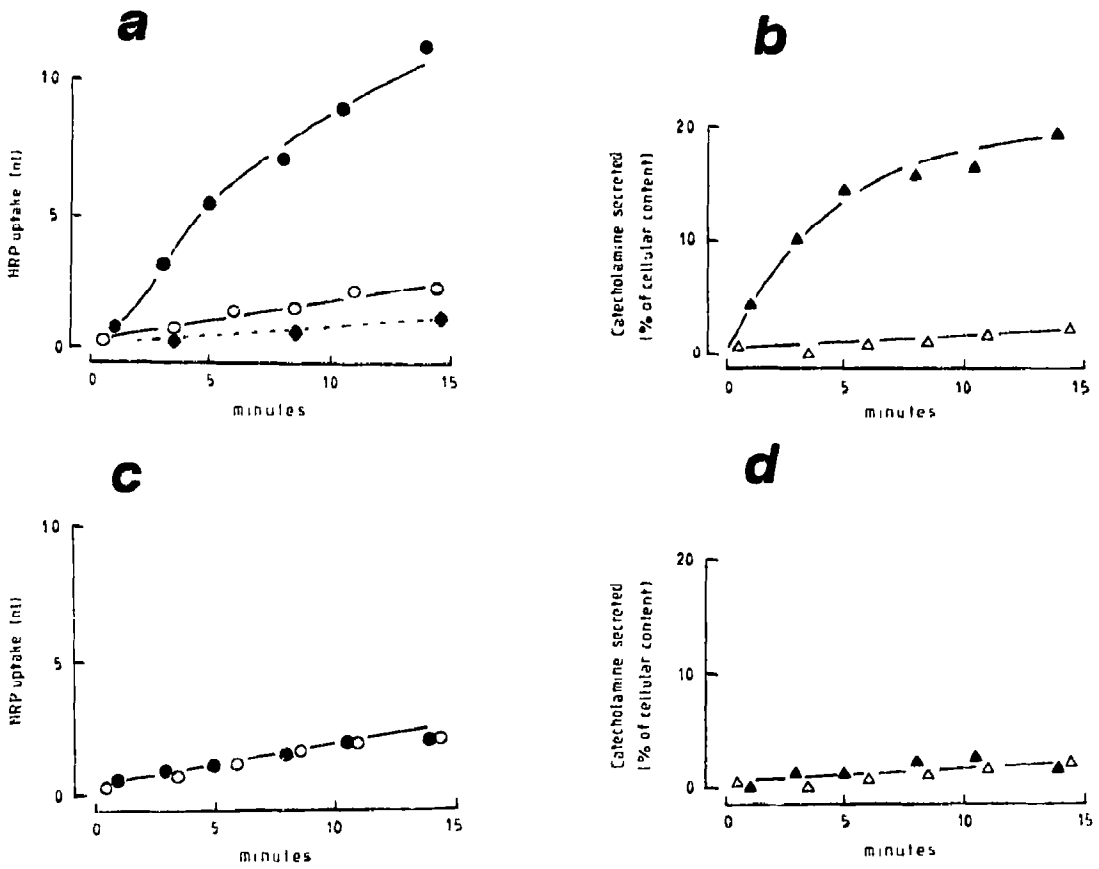


Fig. 1. Botulinum toxin blocks triggered HRP uptake and catecholamine secretion form chromaffin cells. Chromaffin cells were cultured for 5 days alone (a, b) or in the presence (c, d) of 50 μg/ml of botulinum toxin type D. They were then incubated in HRP (1 mg/ml) and challenged with 100 μM carbamylcholine at 23°C. Cells challenged with carbamylcholine (●,▲). Unchallenged cells (○,△). At the various times shown the amounts of HRP taken up into cells (a,c) and the amounts of catacholamine secreted from these cells (b,d) were measured. Some cells (a), not challenged with carbamylcholine, were incubated with HRP at 0°C and the uptake measured (◆). The catecholamine secreted is expressed as a percentage of the total cellular content, and the amount of HRP taken up is expressed as a volume of extracellular fluid (1 ml of fluid corresponding to 1 mg of HRP).

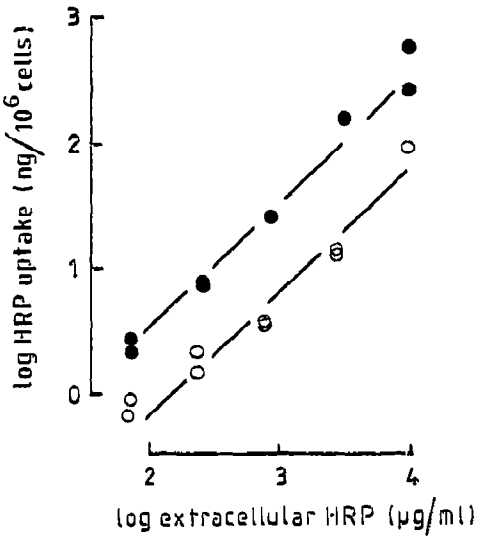


Fig. 2. The HRP taken up into stimulated and unstimulated cells represents fluid phase uptake. The amount of HRP taken up at 23°C over 10 min into unstimulated cells (○) or into cells stimulated with 100 μM carbamylcholine (●), is plotted against the concentration of HRP present in the extracellular fluid during the 10 min incubation.

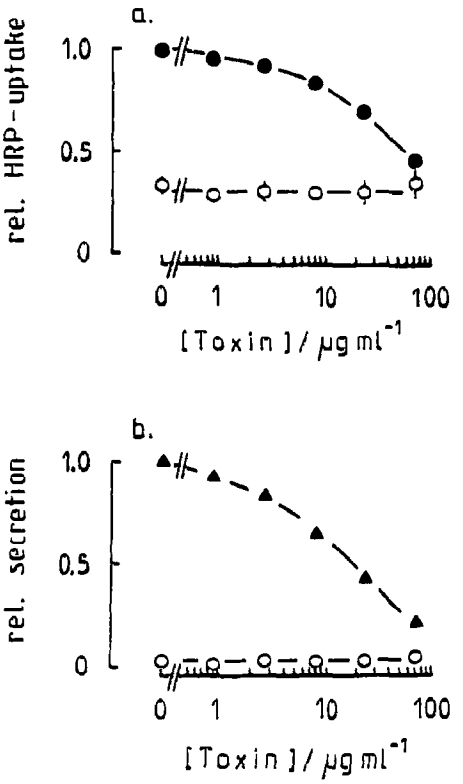


Fig. 3. The sensitivity to botulinum toxin type D of HRP uptake and catecholamine secretion. Cells, pre-incubated for 3 days in various concentrations of toxin, were exposed for 20 min to solutions containing 1 mg/ml of HRP together with (●,▲) or without (○) 100 μM carbamylcholine. Uptake of HRP (a) and secretion of catecholamine (b) are shown. The data are the means (S.E.M.) of 4 determinations and are expressed relative to the levels of HRP uptake and catecholamine secreted in the absence of botulinum toxin. Temperature 23°C.

no added calcium), but the basal uptake of HRP was changed by less than 10% over the same decrease in extracellular calcium.

3.2. *The effect of botulinum toxin on triggered and non-triggered endocytosis*

Fig. 1c and d show that incubation of cells for 5 days with botulinum toxin type D (50 μg/ml) completely abolished the stimulation-dependent components of both catecholamine secretion and fluid phase uptake. The uptake into toxin-treated, stimulated cells was not significantly different ($P > 0.05$) from the basal uptake into toxin-treated or untreated cells (Table 1).

The effect of varying the concentration of toxin on catecholamine secretion and HRP uptake is shown in Fig. 3. Increasing concentrations of toxin reduced the stimulation-dependent components of both catecholamine secretion and HRP uptake. The dose–response curves were very similar. Basal uptake of HRP was not altered by any concentration of toxin used.

Similar results to those shown in Fig. 1 and 3 were obtained with unpurified D toxin, which also contained the ADP-ribosyltransferase component. Again, toxin treatment reduced HRP uptake into stimulated cells to the same level as that seen in unstimulated cells.

We have previously shown that HRP taken up by triggered endocytosis rapidly enters an intracellular pool that can be released back into the extracellular fluid by triggered exocytosis. HRP taken up into unstimulated cells also enters an intracellular pool that is also releasable, but here the exocytosis appears to be constitutive [12]. Fig. 4 is consistent with this and shows that as much as 20% of the endocytosed HRP is released back into the extracellular fluid over a 15 min period, and although temperature-sensitive, the secretory response is unaffected by levels of carbamylcholine that trigger catecholamine secretion. The figure also shows that this component of HRP secretion is unaffected by levels of botulinum toxin that strongly inhibit triggered catecholamine secretion.

Table 1

Rate of fluid phase uptake (measured by uptake of HRP) into bovine chromaffin cells

	Carbamylcholine-stimulated cells	Unstimulated cells	
	+ toxin	+ toxin	− toxin
nl/min uptake (S.D., n=6)	0.109 (0.014)	0.124 (0.014)	0.149 (0.017)

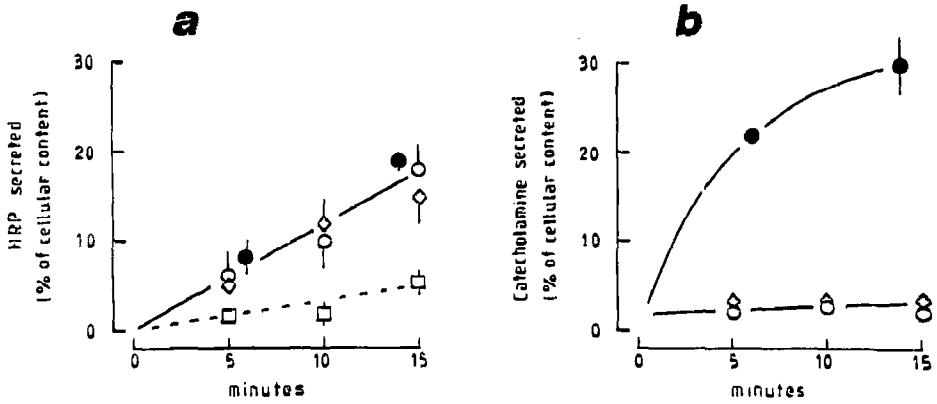


Fig. 4. Release of HRP taken up into unstimulated cells. Unstimulated cells, pre-incubated with or without botulinum toxin, as described in the legend to Fig. 1, were loaded with HRP by a 30 min incubation in 1 mg/ml of the enzyme. Some cells were then challenged with 100 μM carbamycholine, and the evoked and basal release of (a) HRP and (b) catecholamine was followed over 15 min at 23°C. Cells stimulated with carbamylcholine (●); unstimulated cells (○); HRP release from unstimulated cells held at 0°C over the 15 min period (□); unstimulated, toxin-treated cells (◇). The amounts of HRP and catecholamine secreted are expressed as persentages of the total cellular content. Data are means (S.E.M.) of 4 determinations.

4. DISCUSSION

Horseradish peroxidase is a very water-soluble protein with a molecular weight of 40 kDa. Proteins of this size are unlikely to cross biological membranes easily unless they are unfolded and translocated by special transporters. Thus HRP has been used extensively to monitor fluid phase endocytosis [9]. An earlier study provided evidence that chromaffin cells possessed a stimulation-independent constitutive pathway of endocytosis as well as a pathway associated with triggered exocytosis, and that furthermore the continuous removal of membrane area from the cell surface by constitutive endocytosis was balanced by constitutive exocytosis. Although there was evidence that, as far as the triggered exocytosis/endocytosis secretory cycle was concerned, the nature of the endocytosis vesicle was partly made up of secretory vesicle membrane [12], there remained the possibility that some of the retrieved membrane was derived from sources other than secretory vesicle membrane. Thus a scheme could be envisaged whereby endocytosis occurred independently of the exocytotic event, but that was nevertheless triggered by intracellular conditions that also favoured exocytosis.

The observation here that rapid, triggered HRP uptake is botulinum toxin sensitive gives no support to this idea. Botulinum toxin acts downstream of the Ca^{2+} transient, probably at or near the site of exocytosis [10], and the finding that it also abolishes the stimulation-dependent increase of fluid phase uptake is entirely consistent with a model in which triggered exocytosis is a necessary event for triggered endocytosis to occur. This finding therefore lends no support to the idea that in chromaffin cells endocytosis of membrane other than secretory vesicle membrane is triggered by an intracellular signal generated after receptor activation. In particular it is evident that the calcium signal, still present in botulinum toxin-treated cells, is not sufficient to trigger endocytosis. Although increasing concentrations of botulinum toxin reduced triggered HRP uptake to the level found in unstimulated cells, the uptake into these unstimulated cells is unaffected by the toxin. Botulinum toxin may therefore distinguish between constitutive endocytosis and endocytosis associated with triggered exocytosis. The export of the fluid phase marker taken up by constitutive endocytosis is neither inhibited by the toxin nor stimulated by carbamylcholine. A likely explanation to the data therefore is that botulinum toxin does not block the constitutive exocytosis/endocytosis cycle, and that the pools of vesicles undergoing triggered secretory cycles are distinct from the vesicles undergoing constitutive secretory cycles. Basal release of catecholamine is very low, and so inhibition by botulinum toxin is not easy to detect using fluorimetric techniques. In studies using cells labelled with [^{3}H]noradrenaline, levels of toxin that fully inhibited triggered noradrenaline release inhibited about half the basal release [13]. One explanation for this is that about half the basal release arises from catecholamine in a botulinum-insensitive, constitutive pool of vesicles and the other half from a triggered pool of vesicles [15].

The possible coexistence of two distinct secretory systems with differential sensitivity to botulinum toxin within the same cell implies that botulinum toxin may not block all forms of exocytosis. If there are multiple mechanisms for membrane fusion, no statements can be made for the site of action of this toxin exept that it may affect only one type of membrane fusion. If on the other hand there is a common mechanism for membrane fusion then our results suggest that botulinum toxin does not block this membrane fusion step itself but interferes with the triggering mechanism for fusion.

Acknowledgements: We are grateful to Mr. John Gibbs for excellent technical assistance, and to the Medical Research Council (UK) and Wellcome Trust for financial support.

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