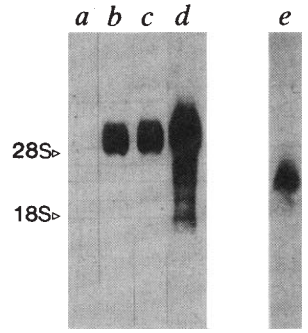

Fig. 4 Analysis of BSF-2 mRNA. Poly(A)$^+$ RNA was prepared from various kinds of cells. RNA (10 μg) was denatured and subjected to blotting analysis[26]. *a*, Poly(A)$^+$ RNA from non-stimulated tonsillar mononuclear cells; *b*, poly(A)$^+$ RNA from tonsillar mononuclear cells stimulated with phytohaemagglutinin (0.1%) and tetradecanoyl phorbol 13-acetate (5 ng ml^{-1}) for 40 h; *c*, poly(A)$^+$ RNA from TCL-Na1 cells; *d*, poly(A)$^+$ RNA from T 24 bladder cell carcinoma; *e*, total RNA from cardiac myxoma cells[10].

BSF-1 for similarities. Only human G-CSF[14] showed significant similarity to BSF-2. Amino acids 28–91 of BSF-2 and amino acids 20–85 of G-CSF match at 17 residues (25.7% of all residues) (Fig. 3*c*). Note that cysteine residues at positions 44, 50, 73 and 83 of BSF-2 match those at positions 39, 45, 67 and 77 of G-CSF exactly. This suggests that BSF-2 is distantly related to G-CSF. If so, intramolecular disulphide bonds would be important in the structure of these proteins.

In Northern blot analysis (Fig. 4) pBSF2.38 DNA hybridized with a single species of messenger RNA ~1,300 nucleotides long which was extracted from activated lymphocytes and TCL-Na1 cells but not from unstimulated control lymphocytes, showing that, like other T-cell-derived lymphokines, BSF-2 mRNA is induced upon mitogenic stimulation.

We have previously reported[10] that cardiac myxoma cells produce high levels of a molecule with BSF-2 activity which was specifically absorbed by the anti-peptide antibody. It has also been shown that bladder cell carcinoma, T24 cells produce a molecule with BSF-2 activity[15]. To show that these cells produce BSF-2 itself, we performed Northern blot analysis. As shown in Fig. 4 (*d*, *e*), BSF-2 mRNA was transcribed in T24 cells and myxoma cells at a much higher level than activated lymphocytes, although the mRNA in myxoma cells was larger than that of lymphocytes. These data indicate that under certain conditions, the BSF-2 gene can be expressed in non-lymphoid tissues. Because patients with cardiac myxoma frequently show several kinds of autoimmune disease-like symptoms[16], it was suggested[10] that deregulated production of BSF-2 might be involved in autoimmune pathogenesis. The fact that BSF-2 gene expression in myxoma cells is high further strengthens this argument.

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Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells

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The clostridial neurotoxins tetanus and botulinum toxin type A are known to block transmitter release from nerve terminals[1–3], probably by interfering with some essential process controlling exocytosis[3,4] after the entry of Ca^{2+} ions. Although exocytosis occurs in many secretory cells, these toxins show a high specificity for neurones and the secretory response of cultured bovine adrenal medullary cells is not inhibited by exposure to medium containing tetanus or botulinum toxin type A (although it is by botulinum toxin type D)[4]. Here we report that when tetanus toxin and botulinum neurotoxin type A are injected intracellularly into chromaffin cells they strongly inhibit secretion, as revealed by the measurement of cell capacitance[5]. These results indicate that these toxins are normally ineffective in chromaffin cells because they are not bound and internalized, so do not reach their site of action. Furthermore, we have localized the secretion-blocking effects of the toxin to a fragment comprising the light chain covalently linked to part of the heavy chain, suggesting that this part of the molecule contains the active site.

We examined cultured bovine adrenal chromaffin cells, isolated as described[6], using the whole-cell configuration of the patch-clamp technique[7]. This allows one to monitor exocytosis using membrane capacitance as a measure of the cell surface area. Capacitance and surface area change considerably as chromaffin granules fuse with the membrane or endocytotic retrieval of the membrane occurs[5]. Secretion was stimulated by dialysing the cells through a patch-pipette with a solution buffered to a free Ca^{2+} concentration of 1 μM. The total membrane capacitance of control cells (typically 4–8 pF) started to increase immediately upon establishment of the whole-cell configuration, eventually leading to a 2–3-fold capacitance increase within 15–25 min (Fig. 1*a*).

When cells were preloaded with either tetanus toxin or botulinum neurotoxin type A the secretory response to the same challenge (dialysis of cells with Ca^{2+}-containing solution) was effectively removed. In fact, cells pretreated with tetanus toxin consistently showed a slight decrease in total membrane capacitance (Fig. 1*c*), suggesting that in these cells there was more endocytotic than exocytotic activity. This 'reversal effect' was observed only once in cells pretreated with botulinum neurotoxin type A ($n = 10$) which may reflect genuine differences in the ability of the two toxins to inhibit secretion or, as has been suggested[8,9], different sites of action. The typical response to increased intracellular Ca^{2+} levels in cells preloaded with botulinum neurotoxin type A was a slight initial increase in cell

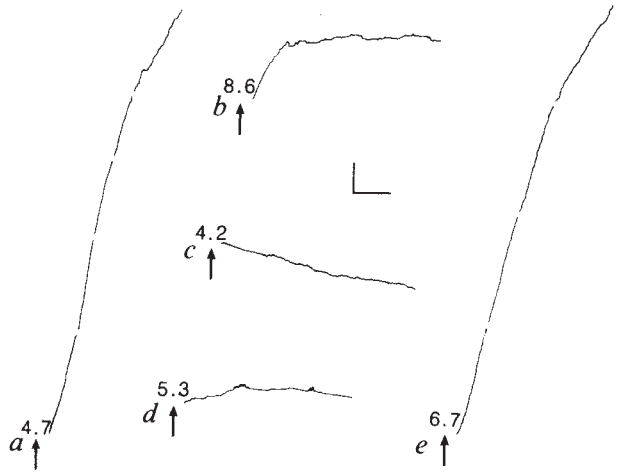

Fig. 1 Effects of clostridial toxins on the secretory responses (measured as capacitance changes) of bovine chromaffin cells stimulated by elevation of the intracellular Ca^{2+} concentration. *a*, Typical time course of capacitance changes recorded from a stimulated control cell. *b*, Typical inhibitory effect of botulinum A toxin on the response of a stimulated cell. *c*, Inhibition of exocytosis by tetanus toxin. *d*, Inhibition of exocytosis in cells preloaded with fragment B at 25 $\mu g\ ml^{-1}$ in the pipette. *e*, Capacitance increase recorded from a cell preloaded with fragment C at 25 $\mu g\ ml^{-1}$ in the pipette. Capacitance measurements were performed according to the method described by Neher and Marty[5]. Briefly, adrenal medullary chromaffin cells isolated as described[6] and kept in short-term culture (1–5 days) were voltage clamped at −70 mV and subjected to a sinusoidal voltage command of 18 mV r.m.s. at 795 Hz. The resulting current was fed into a lock-in amplifier which delivered the capacitive component of the signal when tuned to the desired phase angle with respect to the reference signal. For the large capacitance changes of the control and fragment C traces, these had to be reconstructed from several segments (interruptions) since the lock-in measurement requires recompensation after large deflections[5]. In all traces establishment of the whole-cell recording configuration is indicated by arrows. Numbers given at the start of each trace correspond to initial cell capacitance values (in pF) as determined by the dial setting of the EPC-7 amplifier (List Electronic, Darmstadt). Average capacitance values were 5.9 ± 0.3 pF (mean ± s.e.m., $n = 25$), series resistance values, as read from the EPC-7 settings, were 3.9 ± 0.5 MΩ. *a*, To stimulate secretion a pipette filling solution of the following composition was used (mM): K glutamate 135, NaCl 20, $MgCl_2$ 4, ATP 0.5, EGTA 10, $CaCl_2$ 9 (free Ca^{2+} 1 μM), HEPES 10 (*p*H 7.2). The bath solution contained (mM): NaCl 140, KCl 2.8, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10 (*p*H 7.2). The cell underwent the pretreatments described in *b* except that toxin was omitted from the pipette when 'loading' the cell. *b*, The cell was loaded with the toxin in the whole-cell configuration for 3 min and incubated for 1 h at 37 °C. Botulinum toxin ($LD_{50} = 8\ ng\ kg^{-1}$, mouse s.c.) was used at a concentration of 50 $\mu g\ ml^{-1}$ in a pipette filling solution containing (mM): K glutamate 135, NaCl 20, $MgCl_2$ 4, EGTA 1, HEPES 10 (*p*H 7.2). Both the procedure of loading and the actual test for the secretory response were carried out at 30 °C. *c*, Cells were pretreated, incubated and challenged as in *b* except when loading the cells 50 $\mu g\ ml^{-1}$ tetanus toxin ($LD_{50} = 3\ ng\ kg^{-1}$, mouse s.c.) was present in the electrode filling solution. Vertical scale bar, 0.2 pF; horizontal scale bar, 1 min.

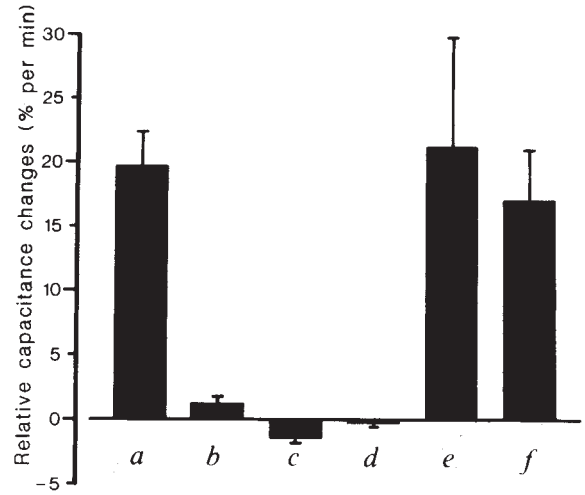

Fig. 2 Effects of clostridial toxins on secretion from bovine adrenal medullary cells. *a*, Control (mean ± s.e.m. of 14 cells); *b*, botulinum toxin A (10 cells); *c*, tetanus toxin (5 cells); *d*, fragment B (7 cells); *e*, fragment C (4 cells); *f*, denatured tetanus toxin (3 cells). The measure of release chosen was the relative capacitance increase (% of the initial value) per unit time (min). Care was taken to assure relatively uniform cell sizes so that differences in initial capacitance values for each set of experiments were small. To determine the release rates capacitance values were measured 2–4 min after transition from cell-attached to whole-cell configuration which usually corresponded to the time in which the capacitance increase was linear (see Fig. 1*a* and *e*).

capacitance, followed by a phase in which no major alteration was seen (Fig. 1*b*). Cells preloaded using pipettes containing no toxin or denatured (boiled) toxins showed a normal secretory response. The rates of release resulting from an elevation of the intracellular Ca^{2+} of control and toxin-treated cells are shown in Fig. 2.

Both secretion and tetanus toxin effects are temperature dependent. Lowering the temperature slows, and can even prevent, the inhibitory effects of tetanus toxin at nerve terminals[10]. We therefore carried out experiments at different temperatures. Whereas secretion at 30 °C proceeded at an average rate of 19.7% per min (see Fig. 2), at 20 °C it was reduced to 7.5 ± 1.4% per min (mean ± s.e.m., $n = 6$). At 20 °C the release rate of cells pretreated with tetanus toxin (5.7 ± 1.5% per min, mean ± s.e.m., $n = 11$) was not significantly different from that of control cells. This finding clearly indicates that the temperature-dependent step in the action of tetanus toxin is subsequent to binding and internalization. Combined with the data obtained using boiled toxins, these results strongly support the conclusion that the inhibitory actions of tetanus and botulinum A toxin on the release mechanism are highly specific and not simply due to the intrusion of a protein of high relative molecular mass (M_r) into the cell.

We used the same system to test for intracellular effects of fragments B and C of tetanus toxin. Fragment C is a part of the toxin's heavy chain of M_r 50,000 which is thought to mediate binding of the toxin to cell membranes[11,12]. Fragment B consists of the light chain covalently linked to the remaining part of the heavy chain (M_r 100,000). These fragments are obtained by enzymatic treatment with papain, and are non-toxic at doses of 10 $mg\ kg^{-1}$ when injected into mice (the LD_{50} for tetanus toxin is 3 $ng\ kg^{-1}$, mouse subcutaneous (s.c.)[13]. Fragment C did not inhibit the responses of chromaffin cells to increased intracellular Ca^{2+} levels (Figs 1*e* and 2), but fragment B effectively suppressed the secretory response (see Figs 1*d* and 2), suggesting that this fragment bears the active site for blocking exocytosis. We have thus shown that a fragment of tetanus toxin that is otherwise non-toxic is able to block secretion.

It is not known whether fragmentation of the toxin into B and C occurs fortuitiously or whether it is integral to the inhibitory actions of tetanus toxin. It appears that some fragmentation occurs *in vivo*, but most of the toxin extracted from the spinal cord (the destination of toxin after uptake into motor nerve terminals and retrograde transport) is indistinguishable from native toxin[14]. This suggests that the proposed uptake mechanism of clostridial neurotoxins[2] (drawn by analogy with diptheria toxin and Semliki Forest virus) by receptor-mediated endocytosis followed by expulsion of the toxin's light chain through a 'channel' formed by the toxin's heavy chain into the cytoplasm may not apply. From our method of toxin administration it is clear that, even if this described uptake mechanism is used, any specific structural changes occurring during uptake are not absolutely required for the actions of the toxins. If changes that are required for activity occur, our results suggest that they are more likely to happen within the cytoplasm.

These results support the hypothesis that tetanus and botulinum A toxin act beyond the rise of intracellular Ca^{2+}

concentration, interfering with an as yet unknown step late in the chain of events leading to exocytosis. Our data show that this crucial step is not restricted to the release process from nerve terminals, but may be quite generally associated with vesicular release. Clostridial neurotoxins may serve as valuable tools in studying late steps in exocytosis. The whole-cell recording technique combined with capacitance measurement is useful because it allows the toxins to reach their site of action directly in cells which lack the prerequisite membrane binding and internalization features and allows tests of fragments that are not normally internalized. Experiments using smaller fragments may allow the characterization of a 'minimal toxin' small enough to be introduced into high-voltage[15] or drug-permeabilized[16,17] cells.

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The essential light chains constitute part of the active site of smooth muscle myosin

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Myosin, a major contractile protein, characteristically possesses a long coiled-coil α-helical tail and two heads. Each head contains both an actin binding site and an ATPase site and is formed from the NH_2-terminal half of one of the two heavy chains (relative molecular mass, M_r, 200,000) and a pair of light chains; the so-called regulatory and essential light chains of approximately M_r 20,000 each. Recently we have identified[1] Trp 130 of the myosin heavy chain from rabbit skeletal muscle as an active-site amino-acid residue after labelling with a new photoaffinity analogue of ADP, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate (NANDP)[2]. Nonspecific labelling was eliminated by first trapping NANDP at the active site with thiol crosslinking agents[3]. Exclusive labelling of the heavy chains with no labelling of the light chains agreed with previous findings[4,5] that the heavy chains alone contain the actin-activated Mg-ATPase activity of rabbit skeletal myosin. Here we report similar photolabelling experiments with smooth muscle myosin (chicken gizzard) in which ^{3}H-NANDP is trapped at the active site with vanadate[6] and which show that both the heavy chains and the essential light chains are labelled. The results indicate that both chains contribute to the ATP binding site and represent the first direct evidence for participation of the essential light chains in the active site of any type of myosin.

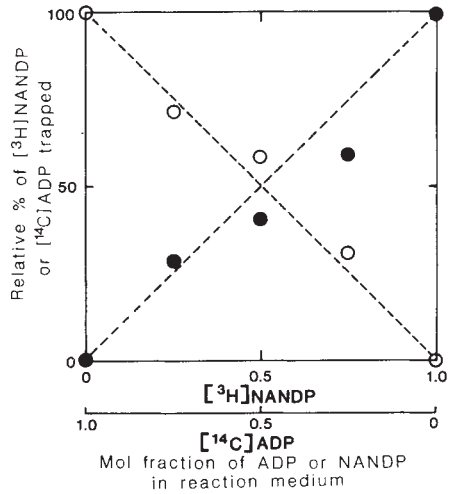

Fig. 1 The relative loss of Ca^{2+} and K^+-EDTA ATPase activities of gizzard myosin as a function of vanadate-trapped [^{3}H]NANDP. Gizzard myosin (4 mg ml^{-1}) in 0.5 M KCl, 2.0 mM $MgCl_2$, 20 mM Tris-Cl, *p*H 8.0, was incubated with 0.7 mM vanadate and varying concentrations of [^{3}H]NANDP[1,2] for 2 h at 0 °C. Aliquots (0.9 ml) were passed over Sephadex G-25 (PD-10) columns to remove vanadate and untrapped [^{3}H]NANDP. The peak containing myosin-[^{3}H]NANDP-vanadate was assayed for protein (biuret method) and for Ca^{2+} (○) and K^+-EDTA (●) ATPase activities[18]. The [^{3}H]NANDP content was determined by scintillation counting. Actin-free gizzard myosin was prepared from fresh chicken gizzards essentially by the procedure of Ebashi[30] and was in the dephosphorylated form. In addition, all experiments were performed at high ionic strength where all the myosin molecules are in the 6S unfolded state[16]. Vanadate solutions were prepared and analysed according to Goodno[31].

It is of primary importance in photoaffinity labelling studies to eliminate nonspecific labelling. The discoveries that nucleotides and nucleotide analogues could be trapped in the active site of myosin either by crosslinking two reactive thiols, SH_1 and SH_2 (refs 3, 7) or by forming a stable nucleotide complex with vanadate[6] have given highly effective ways to achieve specific photolabelling. Both approaches lead to an $\sim 10^4$ decrease in the off-rate of bound nucleotide and give complexes with lifetimes of several days at 0 °C. It is likely that both types of trapping yield quite similar active-site structures since we have found that binding of NANDP can be stabilized at the active site of cardiac myosin by either method (Y.O. and R.G.Y., manuscript in preparation). Photolysis in each case resulted in 50–60% covalent photolabelling of a heavy-chain peptide which was almost identical in sequence to that obtained from rabbit skeletal myosin[1]. Similar studies with other ATP photoaffinity analogues all indicate that heavy chains of skeletal myosin and not light chains are modified[7,8]. Earlier studies by Szilagyi *et al.*[9] with an analogue containing a nitroarylazide group on the ribose ring of ATP also photolabelled predominantly the heavy chains although a small amount of labelling on the light chains could not be ruled out. These studies did not, however, utilize either of the two trapping methods mentioned above.

It was of interest to extend our studies to smooth muscle myosin because these molecules, unlike myosins from skeletal and cardiac muscles, exhibit myosin-linked regulation of contraction[10,11]. In addition, gizzard myosin is known to undergo