Nicotine-evoked disassembly of cortical actin filaments in adrenal chromaffin cells

Timothy R. Cheek and Robert D. Burgoyne*

MRC Secretory Control Research Group, The Physiological Laboratory, University of Liverpool, PO Box 147, Liverpool L69 3BX, England

Received 28 August 1986

Using rhodamine-phalloidin staining it was found that actin filaments are concentrated in the cortex of resting chromaffin cells. Cortical actin filaments were disassembled 15 s after stimulation by nicotine and had reassembled 30 s later. Actin filament disassembly following nicotinic stimulation was also detected using the DNase I inhibition assay. Disassembly was independent of external calcium, insensitive to trifluoperazine and was not elicited by high K^+, muscarinic agonists or phorbol ester. Disassembly of cortical actin filaments may allow access of secretory granules to exocytotic sites and act in conjunction with a rise in intracellular free calcium to bring about the full secretory response due to nicotinic agonists.

Chromaffin cell *Actin filament* *Secretion* *Cytoskeleton* *Adrenal medulla*

1. INTRODUCTION

Many cell types possess a dense network of actin filaments at the cell periphery [1,2]. The idea that these cortical actin filaments have to be reorganised or disassembled to allow access of secretory granules to exocytotic sites was first proposed for the pancreatic β-cell [3]. In recent years this idea has been reiterated many times for a variety of secretory cell types [4–12] with the suggestion that reorganisation of the cytoskeleton occurs through the action of calcium-dependent actin-regulatory proteins [8–13]. However, no direct evidence has yet been presented for changes in actin-filament assembly or organisation following cell stimulation in support of such a model for the secretory process. Here we demonstrate directly that nicotinic stimulation of adrenal chromaffin cells results in a rapid, transient disassembly of cortical actin filaments. Actin-filament disassembly was independent of external calcium and unaffected by trifluoperazine. Disassembly was not elicited by 55 mM K^+, TPA or a muscarinic agonist. These results suggest that actin filament disassembly following nicotinic stimulation is brought about by the activation of an unidentified second messenger pathway and that actin filament disassembly is required in conjunction with a rise in intracellular free calcium ($[Ca^{2+}]_i$) for a maximal secretory response.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

For rhodamine-phalloidin staining bovine adrenal chromaffin cells were dissociated and maintained in culture on coverslips in multiwell trays for 24 h as described [31]. The cells were washed in Krebs-Ringer buffer containing 3 mM $CaCl_2$ and incubated at 20°C for 45 min prior to stimulation by the addition of nicotine to 50 μM. At the appropriate time the buffer was rapidly removed and cells fixed by addition of 2% formaldehyde in saline and incubated for 1–2 h. The cells were washed in Tris-buffered saline (130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) permeabilised by incubation in Tris-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

buffered saline containing 0.1% Triton X-100, 0.3% BSA, and incubated in 2.5×10^{-8} M rhodamine-phalloidin for 15 min in the dark. After washing, the coverslips were mounted in PBS and examined using a Zeiss Universal microscope with epi-fluorescent illumination using a 63 × oil immersion objective.

In order to determine the cellular content of F-actin freshly isolated chromaffin cells [29] were incubated for 60 min in Krebs-Ringer buffer containing 3 mM $CaCl_2$ pelleted and resuspended in buffer with or without $CaCl_2$ or EGTA, and aliquots added to tubes containing buffer or nicotine. Unstimulated cells and stimulated cells at 5, 15, 30 or 60 s after challenge were diluted into an excess of ice-cold buffer containing 20 mM EGTA and centrifuged in an MSE microcentaur centrifuge for 15 s. The cell pellets were resuspended in lysis buffer and G-actin and total actin assayed by the DNase I inhibition assay of Blikstad et al. [17] exactly as modified by Sheterline et al. [32]. In each assay the cells were diluted so that the actin content of 1×10^5 cells was determined. The results are shown as percentage of total actin that is in a polymerised form.

3. RESULTS AND DISCUSSION

Secretory granules in resting chromaffin cells have been shown to be linked within a cytoskeletal network [14] and bundles of actin filaments underlying the plasma membrane have been observed by electron microscopy [15]. We examined the distribution of filamentous actin, at the light microscope level, in chromaffin cells in culture using the fluorescent probe rhodamine-phalloidin which binds to filamentous but not monomeric actin [16]. In resting cells (fig.1a,b) rhodamine-phalloidin fluorescence was most intense at the cell periphery indicating that the majority of filamentous actin in chromaffin cells is concentrated in the cell cortex. Virtually no binding of rhodamine-phalloidin was detectable in cells stimulated for 15 s with 50 μM nicotine prior to fixation (fig.1c). 45 s after stimulation (fig.1d) rhodamine-phalloidin staining was indistinguishable from that of resting cells. These results indicate that cortical actin filaments in chromaffin cells are rapidly disassembled and reassembled following receptor stimulation.

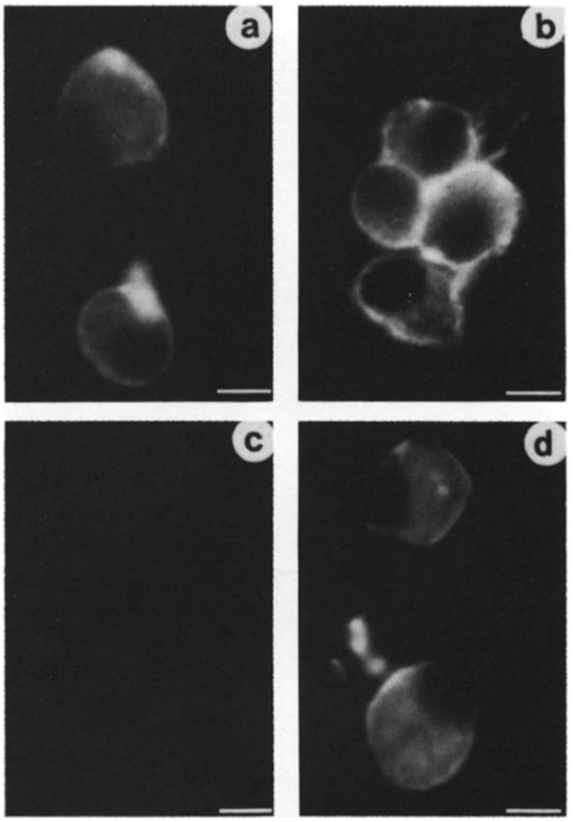

Fig.1. Rhodamine-phalloidin staining of chromaffin cells. An intense ring of fluorescence around the cell periphery is present in resting cells (a,b) and cells fixed 45 s after stimulation with 50 μM nicotine (d). Cells fixed 15 s after stimulation (c) were virtually unstained by rhodamine-phalloidin. Scale bars, 10 μm.

The results from rhodamine-phalloidin staining were confirmed using an independent technique. The relative amounts of filamentous and monomeric actin can be determined in cell homogenates using the DNase I inhibition assay [17]. In the freshly isolated resting cells in fig.2a, 48 ± 2.9% (mean ± SE of 7 determinations) actin was in the filamentous form. Stimulation by 50 μM nicotine resulted in a reduction in the proportion of actin that was polymerised within 5 s (fig.2a). By 60 s after stimulation the level of filamentous actin had returned towards resting levels. In other experiments the proportion of filamentous actin was no different from controls after stimulation for 2 or 10 min. The dose-responses for nicotine-induced disassembly after

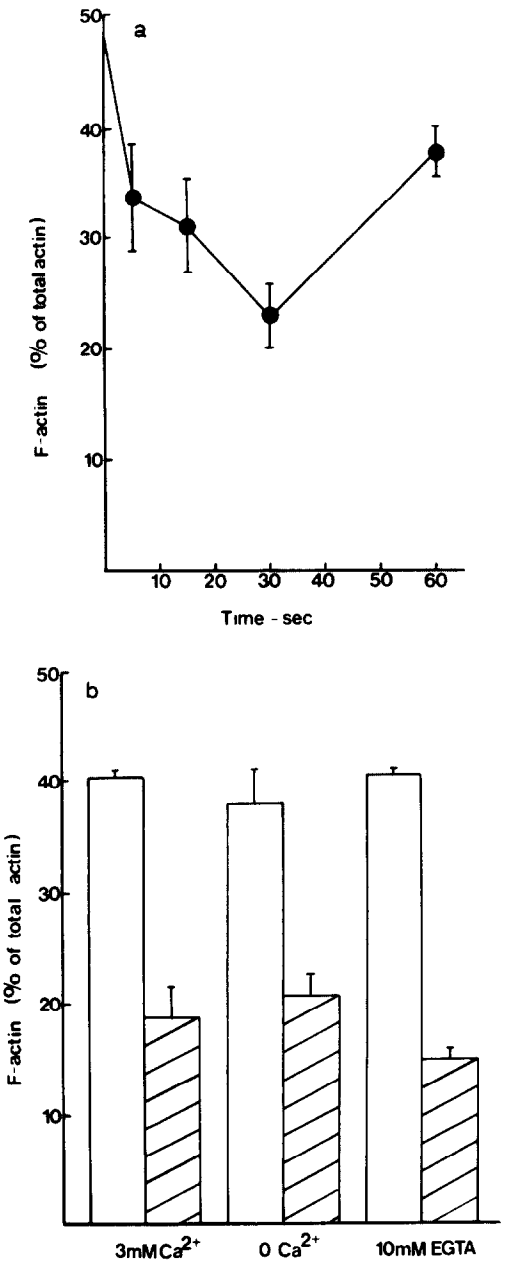

Fig.2. Effect of nicotine on the proportion of F-actin in chromaffin cells using the DNase I inhibition assay. (a) Time course showing cells lysed at various times after stimulation with 50 μM nicotine in the presence of 3 mM $CaCl_2$. (b) Effect of stimulation with nicotine in the presence or absence of external calcium on F-actin content of chromaffin cells. Cells were incubated for 30 s without (□) or with (□) 50 μM nicotine in the medium containing 3 mM $CaCl_2$, no $CaCl_2$ or no $CaCl_2$ plus 10 mM EGTA as indicated.

30 s and nicotine-induced secretion were virtually identical; both effects were half-maximal at 3 μM nicotine (not shown). From the DNase I assay the degree of disassembly of actin following stimulation was not as marked as expected from the rhodamine-phalloidin staining. This difference may be due to differences inherent within the two techniques or to differences between freshly isolated cells and cells grown in culture. The finding that disassembly of filamentous actin reversed within 1 min is consistent with the time course of secretion from chromaffin cells monitored by continuous assay of ATP release [18,19]; these studies showed that secretion was complete within 1 min.

We examined the calcium dependency of actin disassembly by stimulating cells in medium without added calcium with or without 10 mM EGTA. From these experiments (fig.2b) it was clear that nicotine-induced actin disassembly was independent of extracellular calcium. Under these conditions no change in $[Ca^{2+}]_i$ in response to nicotine can be detected using quin2 [20]. The nicotine-induced disassembly of actin was also apparent (table 1) in cells pretreated with 25 μM trifluoperazine at a concentration that abolishes secretion [21–23], and would be expected to inhibit both calmodulin and protein kinase C. Trifluoperazine itself had no effect on the level of polymerisation of actin.

The findings that filamentous actin is concentrated in the cortical region of chromaffin cells and that its stimulus-dependent disassembly is calcium-independent and trifluoperazine-resistant are entirely consistent with an earlier electron microscopical study [23]. In resting cells a 300–400 nm wide region of the cell cortex contained few secretory granules. Stimulation of cells with carbamylcholine resulted in an increase in granules in this region and this increase was more marked in cells where exocytosis was blocked by trifluoperazine. The increase in granules at the cell periphery was also independent of extracellular calcium.

The effects of other agents on the state of assembly of actin was examined. Depolarisation with 55 mM K^+ raises $[Ca^{2+}]_i$ [24] and stimulates a low level of secretion. The phorbol ester TPA which activates protein kinase C potentiates calcium-induced secretion in leaky chromaffin

Table 1

Effect of trifluoperazine (TFP), 55 mM K^+, TPA and methacholine on the amount of filamentous actin in chromaffin cells

Experimental treatment	External Ca^{2+} (mM)	% reduction in filamentous actin
50 μM nicotine	3	51 ± 3 (14)
50 μM nicotine, 25 μM TFP	3	45 ± 2 (4)
50 μM nicotine, 25 μM TFP	0	45 ± 1 (4)
55 mM K^+	3	−5 ± 7 (3)
55 mM K^+	0	3 ± 5 (3)
20 nM TPA	3	4 ± 2 (6)
200 nM TPA	3	4 ± 3 (10)
400 nM TPA	3	3 ± 3 (4)
0.3 mM methacholine	3	−7 ± 7 (4)
10 mM methacholine	3	6 ± 2 (5)

Cells were challenged for 30 s under the conditions described before lysis and assay of actin content by the DNase I inhibition assay. TPA and TFP were used as stock solutions in DMSO and ethanol, respectively. In the case of TFP cells were pretreated for 10 min prior to challenge with nicotine. Results are shown as the percentage reduction in filamentous actin content compared to control resting cells

cells [25]. Activation of muscarinic receptors stimulates polyphosphoinositide breakdown [26,27] and a small rise in $[Ca^{2+}]_i$ [20,28] but does not itself elicit secretion [20]. Treatment with 55 mM K^+, TPA (20–400 nM) or the muscarinic agonist methacholine did not result in actin disassembly (table 1).

The nicotine-induced actin disassembly is not itself sufficient for secretion to occur since secretion in response to nicotinic agonists is blocked by removal of external calcium or treatment with trifluoperazine; under these conditions actin disassembly is still apparent. Furthermore, some secretion can occur without detectable actin disassembly in response to high K^+. We [24] and others [29] have found that high K^+ and calcium ionophore [30] consistently elicits 50% or less of the level of secretion elicited by nicotine [5], under the conditions used here, despite a large elevation in $[Ca^{2+}]_i$ [24]. It is conceivable that actin disassembly is necessary for the full secretory response elicited by cholinergic agonists and is due to the activation of a second messenger pathway additional to the rise in $[Ca^{2+}]_i$. High K^+ may trigger exocytosis of only those secretory granules already close to the plasma membrane [23] or may result in some reorganisation of cortical actin filaments such as through a calcium-dependent inhibition of cross-linking [4,8,11] that would be undetectable with the techniques used here.

The mechanism by which nicotinic stimulation results in cortical actin-filament disassembly is unclear but does not appear to involve a rise in $[Ca^{2+}]_i$, activation of protein kinase C or breakdown of polyphosphoinositides which is known to be mediated by muscarinic [26,27] but not by nicotinic agonists in bovine chromaffin cells. The disassembly of actin is clearly separate from the reorganisation of fodrin [8] in stimulated chromaffin cells which is dependent on external calcium and is much slower than both actin disassembly/reassembly and secretion which have similar time courses.

Disruption of actin filaments by cytochalasin B potentiates secretion from pancreatic B-cells in response to glucose [3] and, of particular relevance for the findings reported here, cytochalasin B markedly reduces the calcium requirement for exocytosis in neutrophils [5]. Therefore, the disassembly of cortical actin may be a widespread mechanism in secretory cells for the enablement or enhancement of calcium-regulated exocytosis.

ACKNOWLEDGEMENTS

This work was supported by a project grant from the MRC. T.R.C. was in receipt of an MRC Research Studentship. We wish to thank Dr P. Sheterline for suggesting the use of rhodamine-phalloidin in these experiments.

REFERENCES

[1] Stossel, T.P. (1982) Philos. Trans. R. Soc. London Ser. B299, 275–289.
[2] Bray, D., Heath, J. and Moss, D. (1986) J. Cell Sci. suppl.4, 71–88.
[3] Orci, L., Gabbay, K.H. and Malaisse, W.J. (1972) Science 175, 1128–1130.
[4] Fowler, V.M. and Pollard, H.B. (1982) Nature 295, 336–339.

[5] Lew, P.D., Monod, A., Waldvogel, F.A., Dewald, B., Bagglioni, M. and Pozzan, T. (1986) J. Cell Biol. 102, 2197–2204.
[6] Friedman, J.E., Lelkes, P.I., Rosenheck, K. and Oplatka, A. (1980) Biochem. Biophys. Res. Commun. 96, 1717–1723.
[7] Friedman, J.E., Lelkes, P.I., Rosenheck, K. and Oplatka, A. (1986) J. Biol. Chem. 261, 5745–5750.
[8] Perrin, D. and Aunis, D. (1985) Nature 315, 589–592.
[9] Langley, O.K., Perrin, D. and Aunis, D. (1986) J. Histochem. Cytochem. 34, 517–525.
[10] Bader, M.F., Trifaro, J.-M., Langley, O.K., Thierse, D. and Aunis, D. (1986) J. Cell Biol. 102, 636–646.
[11] Burgoyne, R.D., Cheek, T.R. and Norman, K.-M. (1986) Nature 319, 68–70.
[12] Drenckhahn, D. and Mannherz, H.G. (1983) Eur. J. Cell Biol. 30, 167–176.
[13] Cheek, T.R., Richards, C.R., Hesketh, J.E. and Burgoyne, R.D. (1986) Biochim. Biophys. Acta 887, 164–172.
[14] Kondo, H., Wolosewick, J.J. and Pappas, G.D. (1982) J. Neurosci. 2, 57–65.
[15] Cooke, P. and Poisner, A.M. (1976) Cytobiologie 13, 442–450.
[16] Wieland, T. (1977) Adv. Enzyme Regul. 15, 285–399.
[17] Blikstad, I., Markey, F., Carlsson, K., Persson, T. and Lindberg, U. (1978) Cell 15, 935–943.
[18] Livett, B.G. and Boksa, P. (1984) Can. J. Physiol. Pharmacol. 62, 467–476.
[19] Rojas, E., Pollard, H.B. and Heldman, E. (1986) FEBS Lett. 185, 323–327.
[20] Cheek, T.R. and Burgoyne, R.D. (1985) Biochim. Biophys. Acta 846, 167–173.
[21] Baker, P.F. and Knight, D.E. (1981) Philos. Trans. R. Soc. London Ser.B 296, 83–103.
[22] Kenigsberg, R.L., Cote, A. and Trifaro, J.M. (1982) Neuroscience 7, 2277–2286.
[23] Burgoyne, R.D., Geisow, M.J. and Barron, J. (1982) Proc. R. Soc. London Ser.B 216, 111–115.
[24] Burgoyne, R.D. and Cheek, T.R. (1985) FEBS Lett. 182, 115–118.
[25] Knight, D.E. and Baker, P.F. (1983) FEBS Lett. 160, 98–100.
[26] Fisher, S.K., Holz, R.W. and Agranoff, B.W. (1981) J. Neurochem. 37, 491–497.
[27] Forsberg, E.J., Rojas, E. and Pollard, H.B. (1986) J. Biol. Chem. 261, 4915–4920.
[28] Kao, L.-S. and Schneider, A.S. (1985) J. Biol. Chem. 260, 2019–2022.
[29] Knight, D.E. and Baker, P.F. (1983) Q. J. Exp. Physiol. 68, 123–143.
[30] Burgoyne, R.D. (1984) Biosci. Rep. 4, 605–611.
[31] Burgoyne, R.D. and Norman, K.M. (1985) Neuroscience 14, 955–962.
[32] Sheterline, P., Rickard, J.E. and Richards, R.C. (1984) Eur. J. Cell Biol. 34, 80–87.