Cyclic AMP Inhibits Both Nicotine-induced Actin Disassembly and Catecholamine Secretion from Bovine Adrenal Chromaffin Cells*

(Received for publication, March 25, 1987)

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As part of our studies on the functional role of the cytoskeleton in exocytosis we have reported (Cheek, T. R., and Burgoyne, R. D. (1986) FEBS Lett. 207, 110–114) that a calcium-independent transient disassembly of cortical actin filaments occurs on activation of the chromaffin cell nicotinic receptor but not when the cell is exposed to 55 mM K+. In order to determine whether this actin disassembly is required, in conjunction with a rise in intracellular Ca²⁺, to elicit a maximum secretory response from these cells, we have examined the relationship between actin disassembly, the elevation in intracellular Ca²⁺, and secretion in detail.

The results show that the dose dependence of nicotine-induced secretion and actin disassembly are essentially identical with maximal effects at a dose of nicotine that produced a submaximal rise in intracellular Ca²⁺. Intracellular cAMP, elevated by three independent means, did not inhibit 55 mM K⁺-induced secretion but inhibited nicotine-induced secretion. Forskolin inhibited actin disassembly while not affecting the rise in intracellular Ca²⁺. These results demonstrate that a close inter-relationship exists between the secretory response and actin disassembly and provide further evidence suggesting that actin disassembly could be required in addition to the rise in intracellular Ca²⁺ in order to elicit a maximal secretory response in chromaffin cells. In addition, the results point to a role for cAMP in the regulation of stimulus-induced actin disassembly.

Activating the nicotinic receptor of the bovine adrenal chromaffin cell results in the exocytotic release of catecholamines. It is thought that this process is triggered by the rise in [Ca²⁺]i (1, 2) which occurs following the influx of Ca²⁺ (3) through voltage-dependent Ca²⁺ channels (4).

The observations that nicotinic agonist-induced secretion cannot be elicited in the absence of extracellular Ca²⁺ (5, 6) and that secretion from permeabilized cells can be stimulated by raising [Ca²⁺], in the absence of nicotinic agonists (7) appear to indicate that a rise in [Ca²⁺]i may be the only absolute requirement for catecholamine release. However, there is other evidence which is strongly suggestive that Ca²⁺ may not be the only signal generated by nicotinic agonists in chromaffin cells; high K⁺ (6) and the Ca²⁺ ionophore A23187 (2) raise [Ca²⁺], to a level similar to or higher than that due to nicotinic agonists but result in a secretory response that is both smaller and shorter lived than that elicited by nicotine (2, 6, 8–11, 17, 26). Furthermore, a study using single chromaffin cells that had been microinjected with securinin has shown that the magnitude and duration of the Ca²⁺ rise elicited by high K⁺ is identical to that elicited by nicotine, and yet high K⁺ released only 30% of the catecholamines released by nicotine (10).

In the search for an additional signal we have observed that, independently of extracellular calcium, the dense network of actin filaments at the chromaffin cell periphery (12) becomes transiently disassembled on activation of the nicotinic receptor, but not in response to depolarization by high K⁺ (13). It has been suggested for a number of secretory cell types, including chromaffin cells, that such actin filament reorganization or disassembly may be required in order to allow the secretory granules access to the sites of exocytosis (14–18, 26). In order to determine whether the transient actin disassembly is required in conjunction with a rise in intracellular Ca²⁺ to elicit a full secretory response in chromaffin cells, we have examined the relationships between actin disassembly, the elevation in intracellular Ca²⁺, and secretion in detail.

The results demonstrate that the nicotinic dose dependence of the actin disassembly is very similar to that of catecholamine secretion, inferring a close inter-relationship between the two events, and show that cyclic AMP (cAMP) is able to inhibit both nicotine induced disassembly of actin and secretion while leaving the intracellular Ca²⁺ rise intact. These results support the notion that actin filament disassembly is required in conjunction with a rise in intracellular Ca²⁺ in order to elicit a maximum secretory response in adrenal chromaffin cells.

MATERIALS AND METHODS

Chromaffin cells were isolated from bovine adrenal medullas by enzymatic digestion (9) in Ca²⁺-free Krebs-Ringer buffer consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM Hepes, pH 7.4 (buffer A), washed in buffer A, and preincubated for 1 h in buffer A containing 3 mM CaCl₂ and 0.5% bovine serum albumin.

Catecholamine release was measured as described (19). Briefly, secretion was stimulated by adding aliquots of cells to centifuge tubes containing the appropriate concentration of secretagogue. After incubation of the cells at room temperature for 6 min, the reaction was terminated by the addition of an equal volume of ice-cold buffer A containing 20 mM EGTA. Released catecholamine was determined fluorometrically as described (19). The catecholamine content of the chromaffin cells was in the range of 46–145 nmol/10⁶ cells.

In order to determine the cellular content of F-actin, aliquots of
cells were added to tubes containing buffer or nicotine. Unstimulated and stimulated cells at 0 and 15 s after challenge were diluted into an excess of ice-cold buffer containing 20 mM EGTA and centrifuged in a microcentrifuge (MSE Scientific Instruments, Crawley, U.K.) 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. (20) exactly as modified by Sheterline et al. (21). In each assay the cells were diluted so that the actin content of 1 × 10^6 cells was determined.

In order to examine the effect of elevated cAMP levels on both catecholamine release and cellular F-actin content, cells were incubated with either forskolin, cholera toxin, or 8-bromocyclic AMP for the times indicated.

For the assay of cAMP levels, cells were incubated without or with either cholera toxin or forskolin as shown. After pelleting by centrifugation the cells were lysed by resuspension in 4 mM EDTA, 5 mM Tris-HCl, pH 7.5, boiled, sonicated, and recentrifuged, and the supernatant was taken for assay of cAMP using a cyclic AMP assay kit (Amersham International, Bucks, United Kingdom) as described in the manufacturers' instructions.

For the determination of [Ca^{2+}](i), the quin 2 technique (22) was carried out as described previously (11, 19). In order to determine the effect of forskolin on [Ca^{2+}](i), cells were incubated with various concentrations of the drug as shown.

RESULTS

Fig. 1 shows the nicotine dose dependency of cortical actin disassembly, catecholamine secretion, and the rise in intracellular Ca^{2+}. The figure demonstrates that the nicotine dose dependence of both actin disassembly and catecholamine secretion are remarkably similar, maximal secretion and actin disassembly occurred at about 10 μM nicotine, when the intracellular Ca^{2+} response was still submaximal. Interestingly, at higher doses of nicotine (50 μM) the extent of both catecholamine secretion and actin disassembly was reduced by about 50% while leaving the increase in the intracellular Ca^{2+} rise unimpaired.

Intracellular Ca^{2+} increased further in response to supramaximal doses of nicotine (>100 μM) and appeared to be still increasing, albeit at a reduced rate, in response to 500 μM nicotine, which was the highest dose tested. Earlier studies of the Ca^{2+} response in chromaffin cells have shown a similar phenomenon (2, 23, 26).

We have previously reported that treatment of chromaffin cells with the adenylate cyclase activator forskolin led to an increase in intracellular cAMP and resulted in an inhibition of catecholamine release elicited by nicotine, but not of that elicited by high K^+, and that this inhibition was at a step distal to Ca^{2+} entry (11). It has recently been reported that, in both PC12 cells (24) and rat sympathetic ganglia (25), forskolin can directly inhibit nicotinic acetylcholine receptor-coupled ion channels in a dose-dependent manner unrelated to the activation of adenylate cyclase. In order to confirm that the inhibitory effect of forskolin on nicotine-induced secretion from chromaffin cells was due to elevated cAMP and not nonspecific blockade of receptor channels, chromaffin cells were preincubated with either forskolin, the G-protein (N1) activator cholera toxin or the cAMP analogue 8-bromocAMP prior to stimulation with either nicotine or high K^+.

The results are shown in Fig. 2 and demonstrate that elevation of intracellular cAMP by either cholera toxin or 8-bromocAMP, as well as by forskolin, results in a significant reduction of nicotine but not K^+-induced secretion, thereby suggesting that the inhibitory effect is mediated by a rise in the intracellular level of cAMP. Direct measurement of cellular cAMP levels (Table I) confirmed that forskolin elevated intracellular cAMP to a greater extent than cholera toxin, which is consistent with the reduced inhibitory response seen with cholera toxin. In order to eliminate the possibility that 8-bromocAMP was acting via adenosine receptors, the effect of adenosine on secretion was examined. In contrast to 8-bromocAMP, adenosine enhanced secretion due to both nicotine and high K^+ (Table II).

In order to determine whether or not the inhibitory effect of elevated cAMP on nicotine-induced secretion could be mediated by an inhibition of the actin disassembly, we ex-

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**Fig. 1.** Nicotine dose-response curves for catecholamine release (○), F-actin disassembly (□), and peak increase in the intracellular Ca^{2+}(i). Results are expressed as a percentage of the maximum response after basal. Maximum [Ca^{2+}] was 492 nm, maximum catecholamine released was 13.13% of total cellular catecholamine, and maximum reduction in F-actin content was from 47% of the total cellular actin to 34%. Release data are mean values of triplicate determinations. Actin disassembly error bars indicate the range of two determinations. [Ca^{2+}] values are means of triplicate determinations, except at 10 and 130 μM nicotine, where n = 2.

**Fig. 2.** Effect of forskolin, cholera toxin, and 8-bromocAMP on catecholamine release stimulated by either 10 μM nicotine (○) or 55 mM K^+ (□). Cells were incubated with either 30 μM forskolin at room temperature or preincubated with 500 μg/ml cholera toxin for 3 h at 37 °C or 2.3 mM 8-bromo cAMP (8-Br cAMP) for 4 h at 37 °C prior to stimulation at room temperature. Catecholamine release over a 6-min period was expressed as a percentage of the release elicited by control challenges in the absence of cAMP activators. Control challenge with nicotine released 11.4% (n = 3) of total cellular catecholamine, and control challenge with K^+ released 3.7% (n = 3) of total catecholamine.
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Effect of cholera toxin and forskolin on CAMP levels in bovine chromaffin cells

Aliquots of cells were incubated without or with 30 μM forskolin for 6 min or with 500 μg/ml cholera toxin for 3 h and then pelleted by centrifugation, lysed, and assayed for cAMP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catecholamine release pmol/10^6 cells</th>
<th>Increase above basal</th>
</tr>
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<tbody>
<tr>
<td>Basal (n = 7)</td>
<td>3.1 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Cholera toxin (n = 8)</td>
<td>9.6 ± 1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Forskolin (n = 8)</td>
<td>15.7 ± 0.7</td>
<td>5.1</td>
</tr>
</tbody>
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Effect of adenosine on catecholamine release stimulated by either nicotine or high K+

Freshly isolated chromaffin cells were incubated with 100 μM adenosine for 4 h at 37 °C, washed, and then stimulated with either 10 μM nicotine or 55 mM K+ for 6 min at room temperature. Control release due to nicotine or high K+ is expressed as 100%. Nicotine released 11.03% of the total cellular catecholamine and high K+ released 3.42% of total catecholamine.

Effect of 8-bromo-cAMP on the amount of filamentous actin in chromaffin cells

Cells were challenged for either 0 s (control) or 15 s under the conditions described before lysis and assay of actin content by the DNase I inhibition assay. In the case of 8-bromo-cAMP, cells were pretreated with 2.5 mM drug for 4 h at 37 °C before stimulation at room temperature.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of total actin in filamentous form %</th>
<th>Reduction in F-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine 0 s (n = 3)</td>
<td>42.0 ± 1.2</td>
<td>54</td>
</tr>
<tr>
<td>Nicotine + 8-bromo-cAMP 0 s</td>
<td>39.0 ± 1.2</td>
<td>3</td>
</tr>
<tr>
<td>Nicotine + 8-bromo-cAMP 15 s</td>
<td>38.0 ± 3.5</td>
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DISCUSSION

As a continuation of our studies on the possible involvement of the cytoskeleton in secretion from adrenal chromaffin cells, we present here results consistent with the notion (13) that disassembly of cortical actin filaments may be required in conjunction with a rise in cytosolic Ca²⁺ in order to elicit a maximum secretory response due to nicotinic agonists.

Consistent with the hypothesis that the actin network of the cell periphery acts as a barrier to the secretory granule by preventing fusion when the cell is at rest (17, 18, 26), there is evidence that the actin network becomes transiently disassembled in response to nicotinic agonists (13) and that actin-stabilizing drugs reduce both basal and Ca²⁺-induced secretion from permeabilized chromaffin cells (27). The remarkable similarity between the nicotine dose dependence of secretion and actin disassembly, as shown in Fig. 1, confirms that a close inter-relationship exists between secretion and actin disassembly. It is noteworthy that depolarization with high K⁺, which results in a secretory response of consistently no more than 30% of the catecholamines released by nicotine drugs, does not result in cortical actin disassembly (13). It is

![Fig. 3. Concentration dependence of inhibition of nicotine stimulated release (○), actin disassembly (⊙), and peak intracellular Ca²⁺ rise (△) by forskolin. The concentration of nicotine used in all experiments was 10 μM. Maximum [Ca²⁺]₉ rise was 175 nM, maximum catecholamine released was 6.40% of total cellular catecholamine. The initial and final levels of F-actin were 45% of total cellular actin and 25%, respectively. Error bars indicate S.E. of at least three determinations.](image)
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possible that high K* may result in a Ca2+-dependent inhibition of actin cross-linking proteins (17, 18, 26), thereby reorganizing the actin filaments in a way which would be undetectable with the techniques used here. Alternatively, high K* may only trigger exocytosis of those granules already close to the plasma membrane.

We have previously reported that increased intracellular cAMP, elevated by the adenylate cyclase activator forskolin, resulted in inhibition of both nicotine and carbamylcholine, but not K*+-induced secretion at a step distal to Ca2+ entry (11). Fig. 2 confirms that forskolin is able to inhibit nicotine but not K*+-induced secretion. However, it has recently been shown that forskolin can directly block nicotine-ecytolamine receptor ion channels in PC12 cells (24) and rat sympathetic ganglia (25). That the inhibitory effect of forskolin on secretion from chromaffin cells was mediated by elevated intracellular cAMP was confirmed when similar results were obtained from cells preincubated with either chola toxin, a direct activator of N., or 8-bromo-cAMP, a membrane-permeable analogue of cAMP.

Preincubation of cells with adenosine resulted in a slight enhancement of the secretory response elicited by both nicotine and high K*, thereby demonstrating that 8-bromo-cAMP did not activate the chromaffin cell adenosine receptors. Indeed, the slight enhancement of secretion by adenosine receptor activation is consistent with an action of adenosine on A1 receptors which inhibit cAMP production (28). The inhibitory site of action of cAMP in the secretory process is unknown, but because its target seemed to be those intracellular events associated with secretion elicited by cholinergic agonists, we have investigated the possibility that elevated cAMP may inhibit the nicotine-evoked actin disassembly, thereby reducing the secretory response. Nicotine-induced actin disassembly and secretion are inhibited by similar extents over the same forskolin concentration range, with substantial inhibition being found at concentrations of forskolin that had little effect on intracellular Ca2+. This result is again indicative of a close relationship between secretion and actin disassembly and suggests that the inhibiting effect of cAMP on secretion involves the prevention of actin disassembly.

At higher forskolin concentrations (40–100 μM), the intracellular Ca2+ rise becomes inhibited, half-maximal inhibition occurring at about 50 μM forskolin. It is likely that, at these high doses, the intracellular Ca2+ rise is inhibited as a consequence of a blockade of the nicotine receptor-linked ion channels, as has been described in other cell types (24, 25).

The finding that elevation of cAMP inhibits both agonist-induced secretion and actin disassembly while the Ca2+ rise is still intact may be significant for several reasons. First, it provides more evidence suggesting that actin disassembly may be required along with an increase in cytosolic Ca2+ to bring about a full secretory response in these cells and second, it raises the attractive possibility that a cAMP-dependent inhibitory pathway mediated by a prevention of actin disassembly could function during the normal secretory events in chromaffin cells to terminate the response.

With reference to this latter possibility, several recent reports have shown that the time course for catecholamine release from these cells is very rapid, release being complete within 1 min after stimulation (29, 30) and, consistent with its proposed role in the secretory process, the disassembled subplasmalemmal actin network becomes reassembled also after about 1 min (13). The results presented here show that nicotine-induced secretion is half-maximally inhibited at 10 μM forskolin, a concentration which has been shown to raise intracellular cAMP in chromaffin cells to a level similar to that which occurs in chromaffin cells following nicotinic stimulation (11). However, although the rise in cellular cAMP triggered by nicotinic receptor activation is slower than secretion (11), the question of whether or not it is responsible for the transient nature of both secretion and actin disassembly/ reassembly remains to be tested.

Acknowledgment—We thank B. A. Houghton for typing the manuscript.

REFERENCES