Abstract. Insertion of a crude preparation of cyclic AMP (cAMP)-dependent protein kinase inhibitor (PKI) into a cloned mouse anterior pituitary cell line (AtT-20/D16-16) blocked cAMP-mediated hormone release. This was accomplished by developing a technique to incorporate PKI into multicellular cultures. The technique involved the encapsulation of the PKI into liposomes coupled to Protein A (a bacterial protein that binds to the Fc portion of antibodies). Application of such liposomes to AtT-20 cells targeted by pre-treatment with an antiserum against neural cell adhesion molecule (a cell surface glycoprotein expressed by these cells) resulted in the attachment of the liposomes onto the cell surface followed by the delivery of the liposome content into the cells. The AtT-20 cells respond to cAMP-promoting agents such as forskolin by secreting the hormone adrenocorticotropin (ACTH). Liposomes containing PKI and coupled to protein A specifically blocked cAMP-mediated ACTH release from cells treated with anti-N-CAM antibodies. In contrast, the ACTH release response to K+ or phorbol esters does not appear to involve cAMP and was not reduced by such manipulations. The specificity of PKI to block hormone release initiated by one but not by other secretagogues directly links cAMP-dependent protein kinase with the ACTH release process but suggests that there are other mechanisms also involved in stimulus-secretion coupling in corticotrophs.

SECOND messengers are believed to mediate the physiologic effects of hormones and neurotransmitters. One such second messenger is cyclic AMP (cAMP).1 This cyclic nucleotide is found in most mammalian cells and increases in its intracellular levels accompany activation of cell surface hormone receptors (22, 32). cAMP stimulates protein kinases that in turn catalyze the phosphorylation of different cytoplasmic and membrane-bound proteins (22). These phosphoproteins are considered to be crucial in mediating the physiologic actions of cAMP.

Demonstration of the biologic role of these phosphorylation events has proven difficult to accomplish since simultaneous measurement of cAMP-dependent protein kinase activity or cAMP-mediated protein phosphorylation and the biologic response ascribed to cAMP is not easy to perform. One approach has used pressure injection of the catalytic subunit of cAMP-dependent protein kinase into individual Aplysia neurons was shown to both facilitate the phosphorylation of several distinct proteins and alter K+ conductance (6, 10, 12, 22, 38). In contrast, injection of the PKI protein into these Aplysia neurons reduced the ability of cAMP and agonists that stimulate adenylate cyclase activity to affect the electric activity of these cells (1).

This experimental approach is limited to studies on single cells. Physiologic responses such as hormone secretion can not be easily examined from single cells, thus effectively excluding similar determination of the intracellular mechanisms involved in stimulus-secretion coupling. It would therefore be advantageous to develop techniques to incorporate into multicellular systems substances normally impermeable to cell membranes such as PKI to bypass the use of injecting electrodes. This would aid in the determination of the relative importance of various second messengers in the physiology of the cell.

Liposomes have been used to target pharmacologic agents such as methotrexate (MTX) or folic acid to cells in vitro (13, 22). For example, injection of the catalytic subunit of cAMP-dependent protein kinase into individual Aplysia neurons was shown to both facilitate the phosphorylation of several distinct proteins and alter K+ conductance (6, 10, 12, 22, 38). In contrast, injection of the PKI protein into these Aplysia neurons reduced the ability of cAMP and agonists that stimulate adenylate cyclase activity to affect the electric activity of these cells (1).

1. Abbreviations used in this paper: cAMP, cyclic AMP; CF, carboxyfluorescein; DME, Dulbecco's modified Eagle's medium; MTX, methotrexate; N-CAM, neural cell adhesion molecule; PKI, protein kinase inhibitor.

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The procedure involves the encapsulation of the active molecule into phospholipid vesicles which are specifically directed to target cells via monoclonal antibodies covalently coupled to their outer membrane. Alternatively, the liposomes can be coupled to *Staphylococcus aureus* protein A which will confer upon the liposomes the ability to bind to cells pre-treated with antibodies with specificity for a particular antigen expressed at the cell surface. In the present study, we report the adaptation of this method to study the intracellular processes of stimulus–secretion coupling. In particular, it is shown that a crude preparation of PKI can be entrapped into liposomes and targeted to a tumor cell line (AT-20/D16-16) derived from the mouse anterior pituitary using anti-neutral cell adhesion molecule (N-CAM) antibodies. These cells secrete adrenocorticotropic hormone (ACTH) and both cAMP and cAMP-dependent protein kinase have been implicated in the receptor-mediated release of this hormone (3, 8, 11, 16, 21, 42). Once inside the AT-20 cells, the PKI blocks the ACTH release response to cAMP and cAMP-promoting agents but not to secretagogues that do not activate cAMP-dependent protein kinase activity.

**Materials and Methods**

Forskolin was purchased from Calbiochem-Behring Corp. (Los Angeles, CA). 8-Bromo-cAMP, isobutylmethylxanthine, KCl, human serum albumin, and PKI (crude, from rabbit muscle) were from Sigma Chemical Co. (St. Louis, MO). Phorbol 12,13-dibutyrate was a gift of Dr. S. Jaken, National Cancer Institute, Bethesda, MD. Dulbecco's modified Eagle's medium (DME) (4,500 mg/liter of glucose) was obtained from GIBCO (Grand Island, NY). Fetal calf serum was from North American Biologicals (Miami, FL) and human ACTH (synthetic) and anti-serum were gifts from the National Pituitary Agency (Baltimore, MD). 125I-labeled human ACTH was from Immuno Nuclear Corp (Stillwater, MN) and goat anti-rabbit immunoglobulin was from Cappel Laboratories (Coopersville, PA). The cAMP immunoassay kits were from Boonton-Dickinson and Co. (Rutherford, NJ).

**Cell Culture Technique**

Mouse AT-20/D16-16 tumor cells (originally subcloned by S. Sabol, National Institutes of Health) were grown and subcultured in DME with 10% fetal calf serum as previously described (9). Cells were plated in 96-well flat-bottom tissue culture clusters at an initial density of 5 x 10^4 cells per well. The cells were grown for 48–96 h to near confluency in 100 μl of culture medium.

**Anti-N-CAM Antibodies**

Polyclonal anti-N-CAM antibodies were obtained from a rabbit immunized with an antigen preparation purified as previously described on a monoclonal H28 antibody column; Pharmacia Fine Chemicals). The labeled fractions (1 ml, 100 μCi) were pooled and stored at 4°C until used.

Since molar concentrations of PKI as large as those used for CF and MTX cannot be practically attained, liposomes with larger internal volumes and entrainment efficiencies were prepared for the PKI studies. Thus, reverse-phase evaporation vesicles were made according to Sroka and Papahadjopoulos (40). Dimyristoyl phosphatidyl choline (21.6 μmol, Avanti Polar Lipids, Inc.), dipalmitoyl phosphatidyl serine (4 μmol, Avanti Polar Lipids, Inc.), cholesterol (14 μmol), and N-[3-(2-pyridyl)dithiopropionyl] dipalmitoyl phosphatidyl ethanolamine (0.4 μmol) were dissolved in 2 ml diethyl ether. An emulsion between the lipid ethereal solution and an aqueous phase (1 ml) containing PKI (6.5 mg, 2 x 10^9 cpm) was emulsified by vortexing for 10 min. The emulsion was then extruded under reduced pressure (0.5–0.8 atm) at room temperature in a rotary evaporator. The liposome suspension was extruded sequentially through 0.4- and 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA) as described (24). PKI and CF not entrapped were removed by gel filtration on a Sepharose 4B column (1.6 x 30 cm). The liposome peak contained 6% of the total inhibitor (9 x 10^9 cpm) and 6.8% of the total CF.

Protein A was coupled to PKI-containing liposomes as described above; N-succinimidyl 3-(2-pyridyl)dithiopropionate-modified Protein A, activated by reduction with dithiothreitol (465 μg, 1.5 x 10^9 cpm, in 1 ml phosphate-buffered saline) was reacted with 3 ml of the liposome preparation (18 μmol lipids, 180 μg PKI, 5 x 10^9 cpm) for 24 h at room temperature. Liposomes were then separated from uncoupled Protein A by gel filtration on Sepharose 4B (1.6 x 30 cm). The percentage of total coupled Protein A was 5%. In experiments with no entrapped PKI (control liposomes with no PKI but coupled to Protein A), this percentage was more precisely measured to be ~10%. The final preparation contained 35 μg/ml PKI and 0.23 mM CF and ~9 μg/ml Protein A with ~3 mM total lipids.

**MTX-containing Liposomes**

To test the endocytic potential of the N-CAM antigen at the surface of AT-20 cells, we prepared small unilamellar vesicles containing CF (Eastman Kodak Co., Rochester NY; purified by crystallization and filtration over Sephadex LH20 according to Ralston et al. [27]) and MTX (Sigma Chemical Co.). Briefly, an organic solution containing 17 μmol dipalmitoyl phosphatidyl choline (Avanti Polar Lipids, Inc., Birmingham, AL), 10 μmol cholesterol (Sigma Chemical Co.; recrystallized in ethanol), and 1 μmol of N-[3-(2-pyridyl)dithiopropionyl] dipalmitoyl phosphatidyl ethanolamine (synthesized as described by Barbet et al. [5]) was evaporated to dryness under argon, then 3 ml of an aqueous solution of CF (50 mM) and MTX (25 mM) was added, heated to 50°C and vortexed for ~5 min. The resulting turbid suspension of multilamellar liposomes was then sonicated for 30 min at 50°C using a W-375 probe sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The clear liposome preparation was centrifuged (2,000 x g, 10 min) to remove titanium particles, and chromatographed on Sephadex G50 (0.9 x 30 ml) in phosphate buffer to separate liposomes from solutes not entrapped.

Small unilamellar vesicles containing CF and MTX were then coupled to *S. aureus* Protein A (Pharma Fine Chemicals) as described by Leserman et al. (14). Protein A (1 mg; 8.7 x 10^9 cpm of 125I-labeled Protein A in 300 μl of phosphate buffer) was reacted with a 1:10 fold molar excess of N-succinimidyl 3-(2-pyridyl)dithiopropionate (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h at room temperature. Then, dithiothreitol (Sigma Chemical Co.) was added to the reaction mixture at a 50 mM final concentration. After a 20-min incubation at room temperature, the mixture was chromatographed on a PD10 column in phosphate-buffered saline (pH 8.0). The thiol-derivatized Protein A (600 μg in 0.5 ml) was added to the liposome preparation (9 μmol total lipids in 1.5 ml) and incubated for 24 h at room temperature. Liposomes were separated from uncoupled Protein A by chromatography on Sepharose 4B (Pharma Fine Chemicals; column, 1.6 x 30 cm). Liposomes eluted in the void volume (as demonstrated by the presence of encapsulated CF) together with 21% of the total Protein A (evaluated from recovered 125I).

**PKI-containing Liposomes**

The PKI was dissolved in distilled water to a final concentration of 10 μg/ml and used without further purification. Radioiodination was performed according to Salincki et al. (37). To a small plastic vial containing 9 μg iodogen were added 20 μl of 0.05 M phosphate buffer (pH 7.0), 200 μg PKI diluted in 150 μl phosphate buffer, and 1 ml 125I-sodium iodide. After 10 min of incubation at room temperature, the reaction mixture was diluted with 400 μl of 0.05 M phosphate buffer and applied onto a Sephadex G-25 column (PD10 column; Pharmacia Fine Chemicals). The labeled fractions (1 ml, 100 μCi) were pooled and stored at 4°C until used.

Since molar concentrations of PKI as large as those used for CF and MTX cannot be practically attained, liposomes with larger internal volumes and entrainment efficiencies were prepared for the PKI studies. Thus, reverse-phase evaporation vesicles were made according to Sroka and Papahadjopoulos (40). Dimyristoyl phosphatidyl choline (21.6 μmol, Avanti Polar Lipids, Inc.), dipalmitoyl phosphatidyl serine (4 μmol, Avanti Polar Lipids, Inc.), cholesterol (14 μmol), and N-[3-(2-pyridyl)dithiopropionyl] dipalmitoyl phosphatidyl ethanolamine (0.4 μmol) were dissolved in 2 ml diethyl ether. An emulsion between the lipid ethereal solution and an aqueous phase (1 ml) containing PKI (6.5 mg, 2 x 10^9 cpm transferred into phosphate-buffered saline by PD10 filtration) and CF (40 μm) was made without sonication using two glass syringes connected by a three-way stopcock (18). The emulsion was then evaporated under reduced pressure (0.5–0.8 atm) at room temperature in a rotary evaporator. The liposome suspension was extruded sequentially through 0.4- and 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA) as described (24). PKI and CF not entrapped were removed by gel filtration on a Sepharose 4B column (1.6 x 30 cm). The liposome peak contained 6% of the total inhibitor (9 x 10^9 cpm) and 6.8% of the total CF.
Methotrexate Studies

Before the experiments, cells were washed with DME and incubated for 1 h at 25°C with anti-N-CAM antibodies (1:300 dilution). The cells were then incubated with 50 µl of DME and the liposomes or the free MTX in 100 µl of medium for 3 h at 37°C. After the incubation, 50 µl of DME containing [3H]-deoxyuridine (0.5 µCi; New England Nuclear, Boston, MA, 18 Ci/mmol) were added for 10 h at 37°C. Then the cells were washed and harvested on fiber glass filters and tritium was measured by liquid scintillation spectroscopy.

Procedures for ACTH Release

Before the experiments, the AtT-20 cells were washed twice with 100 µl of DME containing 25 mM Hepes and 2% fetal calf serum. The cells were then incubated with 50 µl of medium with or without the anti-N-CAM antibody (final dilution of 1:300) for 1 h at 25°C. The cells were then washed twice and incubated for 3 h (unless otherwise indicated) at 37°C with 100 µl of medium containing either PKI, liposomes (with or without PKI) either lacking or coupled to Protein A. At the end of this time, the medium was removed and fresh DME containing forskolin, K+ or phorbol 12,13-dibutyrate was applied to the cells and incubated for 30 min at 37°C. An aliquot of the medium was removed and frozen at -20°C for later analysis of ACTH immunoreactivity. Experiments involving 8-bromo-cAMP were for 60 min in order to observe significant stimulation of ACTH release. Forskolin and phorbol 12,13 dibutyrate were dissolved in ethanol and stored as 10 mM stock solutions. The final concentration of ethanol (0.5% in the forskolin experiments) did not affect ACTH release by itself. Experiments were done in triplicate wells and in most cases repeated 3-4 times on different subcultures. The cell density did not vary appreciably between wells on the same plate. For this reason most experiments were performed on a single 96-well plate at a time and ACTH release values were not corrected for differences in cell number. 

ACTH Immunoreactivity

ACTH immunoreactivity was measured as previously described (9). The antibody used was specific for the 14-24 segment of ACTH.

cAMP Determination

Similar procedures as described for ACTH release were used. However, following the liposome pre-treatment, 100 µl of DME (25 mM Hepes) containing 0.5 mM isobutyl methylxanthine with or without forskolin was applied for 30 min. The medium was then removed, 100 µl of 0.5 N HCl was applied, and the tissue sonicated in the well. The samples were then analyzed for cAMP using the Becton-Dickinson radioimmunoassay kit as previously described (28). Values are expressed as pmol/well and are the means ± SEMs of three determinations.

Results

Targeting of Protein A-bearing Liposomes to AtT-20 Cells Using Anti-N-CAM Antibodies

The expression of the neural cell adhesion molecule (N-CAM) at the cell surface of AtT-20 cells was demonstrated by indirect immunofluorescence (Fig. 1, A and B) and immunoprecipitation (not shown). When live AtT-20 cells were labeled with anti-N-CAM antibodies and fluoresceinated goat anti-rabbit antiserum, all the cells in the culture fluoresced brightly. Binding of the protein A-bearing liposomes to AtT-20 cells pre-treated with anti-N-CAM antibodies was easily visualized by fluorescence microscopy since the liposomes contained CF (Fig. 1, C and D). The fluorescent liposomes appeared as small bright grains at the surface of the cells (Fig. 1, C2 and D2). Although the incubations were done at room temperature, perinuclear fluorescence in some cells indicated the endocytosis of some vesicles (Fig. 1, D1). By contrast, cells treated with anti-N-CAM antibodies and then reacted with liposomes not coupled to Protein A did not show cell-associated fluorescence (Fig. 1, E and F) indicating that Protein A mediates the liposome attachment to the antibody. The same absence of binding of the liposome-protein A conjugates was also observed when the cells were preincubated with a preimmune antibody (Fig. 1, G and H), indicating that Protein A-coupled liposomes by themselves cannot bind to the cells and resulting in the necessity of the specific interaction of N-CAM molecules with their antibodies before liposome attachment.

Delivery of the Content of Liposomes Targeted to AtT-20 Cells with Anti-N-CAM Antibodies

To test the capacity of the target N-CAM molecule to mediate internalization and delivery of the liposome content into AtT-20 cells, we prepared liposomes containing MTX coupled to Protein A. MTX has the ability to inhibit the intra-cyttoplasmic enzyme, dihydrofolate reductase, and this effect is readily monitored by measuring the inhibition of [3H]deoxyuridine incorporation into DNA of replicating cells. AtT-20 cells treated with anti-N-CAM antibody and exposed (for 3 h) to MTX-containing liposomes coupled to Protein A showed reduced [3H]deoxyuridine incorporation (Fig. 2). The inhibition of [3H]deoxyuridine incorporation was dependent on the concentration of MTX-containing liposomes. A similar inhibition curve was observed with free MTX (Fig. 2). However, such liposomes containing MTX applied to cells not pre-treated with anti-N-CAM antibodies or pre-treated with an irrelevant antibody did not affect [3H]deoxyuridine incorporation (Fig. 2). These results further confirm the specificity of the technique and demonstrate the endocytic potential of N-CAM as a target antigen.

Incorporation of cAMP-dependent PKI into AtT-20 Cells

Experiments were conducted to determine whether free PKI could be incorporated into AtT-20 cells and block cAMP-mediated ACTH release. When applied to AtT-20 cells, the PKI did not block the ACTH release response (Table I) to forskolin, an agent that activates adenylate cyclase and cAMP.

Figure 1. Immunofluorescent detection of N-CAM antigen expression and liposome binding. Cells were grown on polylysine coverslips. The live cells were washed and reacted with polyclonal anti-N-CAM antibodies at 1:1,000 dilution or preimmune serum at 1:300 dilution in culture medium for 30 min at room temperature. The coverslips were then washed in DME and reacted either with a goat anti-rabbit fluorescein-linked antibody at 1:100 dilution for 30 min (A and B), then washed again and fixed in acid/alcohol for 10 min at 10°C before being mounted in PBS and glycerol, or with different preparations of liposomes at 24 µM CF culture medium (C-H) for 3 h at room temperature. A, C, E, and G are phase-contrast images whereas B, D, F, and H are fluorescent photographs of the same fields. For liposomes, the fixation step was omitted and the cells were immediately examined under the microscope. For C and D, the cells were reacted with anti-N-CAM antibodies and liposomes linked with Protein A and containing CF and PKI. Two different experiments are shown with focus either on the cell surface (C2 and D2) or intracellularly (C1 and D1). The liposomes bound to the surface of the cell appeared like small individual fluorescent dots. For E and F the cells were reacted with the same preparation of liposomes lacking Protein A; for G and H the anti-N-CAM antibody was replaced by preimmune serum. Bars, 10 µm.
dependent protein kinase in homogenates of these tumor cells (8, 16, 21). Pre-treatment of the AtT-20 cells with anti-N-CAM antibodies was not sufficient to allow the PKI to block stimulated ACTH secretion nor was co-treatment of the cells with free PKI and liposomes coupled to Protein A but not containing PKI (Table I). These results indicate that free PKI cannot gain access to AtT-20 cells.

When PKI was encapsulated into the liposomes coupled to Protein A and applied to AtT-20 cells pre-treated with anti-N-CAM antibodies, forskolin-stimulated ACTH release was abolished (Table I). The blockade of forskolin-induced ACTH secretion required both pre-treatment of the AtT-20 cells with anti-N-CAM antibodies and liposomes coupled to Protein A (Fig. 3). Neither the anti-N-CAM antibodies nor the liposomes coupled to protein A but not containing PKI reduced forskolin's effect on ACTH release (Fig. 3). Application of protein A–bearing liposomes containing PKI to AtT-20 cells pre-treated with anti-N-CAM antibodies also diminished 8-bromo-cAMP (0.1 mM)-stimulated ACTH release (control, basal = 0.12 ± 0.01; 8-bromo-cAMP = 0.44 ± 0.05; significantly different from basal, \( P < 0.05 \) using a Student's \( t \) test; treated, basal = 0.14 ± 0.03; 8-bromo-cAMP = 0.21 ± 0.04, values in ng ACTH/well are the means ± SEMs of three experiments done in triplicate wells).

The incorporation of the PKI into AtT-20 cells appeared to specifically block cAMP-mediated ACTH release. Thus, neither K+ nor phorbol esters stimulate ACTH secretion by activating cAMP-dependent protein kinase and treatment of anti-N-CAM antibody labeled AtT-20 cells with Protein A–bearing liposomes containing PKI did not affect the ACTH release response to either of these secretagogues (Fig. 4). Furthermore, the inhibition of forskolin-stimulated ACTH by the liposome–PKI treatment was not due to a blockade of adenylate cyclase activity since forskolin-stimulated cAMP

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**Table 1. Effect of PKI on Forskolin-stimulated ACTH Release**

| Condition | ACTH release (ng/well) | AB (LIPO+PKI) | PKI | AB+ PKI | AB+ (LIPO–PKI) |
|-----------|------------------------|---------------|-----|---------|----------------|
| Basal     | (12) 0.27 ± 0.03*      | (12) 0.30 ± 0.03* | (12) 0.25 ± 0.02 | (3) 0.27 ± 0.03 | (3) 0.23 ± 0.03 |
| Forskolin | (12) 0.60 ± 0.06*      | (12) 0.61 ± 0.06* | (12) 0.30 ± 0.02 | (3) 0.55 ± 0.04* | (3) 0.57 ± 0.04* |

AtT-20 cells were treated for 1 h with DME with or without anti-N-CAM antibodies (AB) at a 1:300 dilution. The cells were then washed and treated with either PKI (0.5 μg/100 μl) liposomes containing PKI (0.18 μg/100 μl) coupled to Protein A (LIPO+PKI), or liposomes coupled to Protein A but not containing PKI (LIPO–PKI). At the end of this time the cells were washed and either basal- or forskolin- (50 μM) stimulated ACTH release was measured. Results are the mean ± SEM of (N) experiments done in triplicate wells.

* \( P < 0.05 \) (using a Student's \( t \) test) different from basal ACTH release from the same treatment.
Figure 4. Liposomes containing PKI specifically inhibit the ACTH release response to forskolin. Cells were exposed for 1 h at RT to DME with 2% fetal calf serum with or without anti-N-CAM antibodies (AB). The cells were then washed and treated for 3 h at 37°C with medium either with or without liposomes containing PKI (0.18 μg PKI) coupled to Protein A. The cells were washed and stimulated for 30 min with either forskolin (50 μM), phorbol 12,13-dibutyrate (1 μM), or K+ (50 mM). The results are the means ± SEMs of four experiments done in triplicate wells. The ACTH release in response to forskolin is significantly different (P < 0.05 using a Student's t test) from basal ACTH release in the control and AB treatments. The ACTH release in response to K+ and phorbol 12,13-dibutyrate is significantly different (P < 0.05, using a Student's t test) from basal ACTH release in the control and AB + LIPO treatments.

Table II. Effect of Protein A-bearing Liposomes Containing PKI on Forskolin-stimulated cAMP Formation

| Condition          | cAMP (pmol/well) |
|--------------------|------------------|
| Control AB         | Basal            |
|                    | 2.2 ± 0.5        |
|                    | 2.6 ± 0.4        |
| AB + LIPO          | 1.2 ± 0.3        |
| Forskolin          | 108.0 ± 15.1*    |
|                    | 77.0 ± 2.8*      |
|                    | 83.0 ± 3.5*      |

Reisine et al. Insertion of PKI Blocks ACTH Release

Discussion

The results of this study further support the role of cAMP in the release of ACTH. Previously, it was shown that cAMP-dependent protein kinase was present in corticotrophs and could be activated by the hormones corticotropin-releasing factor and catecholamines as well as by the direct activator of adenylate cyclase, forskolin (3, 16, 21). However, the link between the activation of cAMP-dependent protein kinase and the ACTH secretory event had not been established. The ability of cAMP-dependent protein kinase to catalyze the phosphorylation of exogenously applied histone proteins and endogenous substrates is blocked by the PKI (21). Encapsulating PKI into liposomes coupled to protein A and applying these liposomes to AtT-20 cells pre-treated with anti-N-CAM antibodies solved the problem of the inaccessibility of PKI to intact cells. The ability of this treatment to prevent forskolin and 8-bromo-cAMP from releasing ACTH indicates that the activation of cAMP-dependent protein kinase is required for stimulus-secretion coupling evoked by forskolin in these tumor cells. Demonstration of the precise manner by which cAMP is involved in ACTH release will have to await the identification and functional characterization of the endogenous substrates for cAMP-dependent protein kinase in corticotrophs.

The inability of PKI to affect the hormone release response to the membrane depolarizing agent K+ or the protein kinase C activator, phorbol ester, indicates that the intracellular...
events initiated by these secretagogues to cause ACTH secretion are distinct from those associated with cAMP. Previously, K+ was shown not to increase cAMP accumulation (28) or cAMP-dependent protein kinase activity (16) in AtT-20 cells. Its mode of stimulating ACTH release may involve changes in calcium influx or mobilization as proposed by Richardson (29) and inferred from electrophysiological data of Adler et al. (2) and Surprenant (39).

Phorbol ester is believed to activate protein kinase C (23), an enzyme present in AtT-20 cells (25). The inability of PKI to inhibit phorbol ester-stimulated ACTH release suggests a distinction between the mechanism by which cAMP-dependent protein kinase and protein kinase C evoke ACTH secretion. Elucidation of the phosphorylation events initiated by the two kinases should aid in further determining the precise manner by which these two enzymes regulate hormone secretion. The ability to introduce substances into endocrine cells that can specifically mimic or antagonize the secretion of hormone induced by cAMP, K+, or phorbol ester should aid in elucidating the means by which the individual second messengers modify hormone secretion. This, in turn, should help to determine the role of these intracellular pathways in transducing the physiological stimuli that regulate secretion.

The procedures used in the liposome technique did not produce artifactual effects on ACTH release from the AtT-20 cells. The anti–N-CAM antibodies did not reduce stimulated hormone secretion and liposomes lacking PKI did not alter ACTH release from control or anti–N-CAM antibody–treated cells. Furthermore, in order for liposomes to attach to and deliver their contents into AtT-20 cells, it was necessary that they be coupled to protein A and that the AtT-20 cells be pre-treated with anti–N-CAM antibodies. This conclusion was initially formulated from the results of the studies with CF and MTX (see also references 5, 13, 15, and 36).

Between 1 and 2 h of liposome treatment was required to incorporate enough PKI into the AtT-20 cells to completely abolish forskolin-stimulated ACTH release. This time course is similar to that observed for the delivery of MTX or CF encapsulated in liposomes targeted to other cell types with specific cell surface antibodies (41). The time lag may be related to the process of endocytosis or to the transport of PKI molecules to the cellular compartments containing cAMP-dependent protein kinase.

Once incorporated into AtT-20 cells, the PKI blocked forskolin evoked ACTH release for several hours. Similar results were obtained in R15 cells of the Aplysia in which PKI was pressure injected into these neurons and the ability of cAMP to increase K+ conductance measured (1, 22). This suggests that PKI is relatively stable in the cell and not rapidly inactivated or excluded.

The delivery of encapsulated materials to targeted cells seems to be best achieved using small or intermediate-sized liposomes (25–200 nm) (17). The formation of such liposomes involves the exposure of the solutes to either sonication, detergents, or organic solvent. The mechanisms by which entrapped solutes gain access to the cell cytoplasm are not well understood. Receptor-mediated endocytosis and subsequent transfer of the liposomes to acidic intracellular vesicles which eventually fuse with lysosomes have been proposed for the targeted delivery of encapsulated MTX or CF (19). This would imply further exposure of the solutes to potentially denaturing environment as well as hydrolytic enzymes. The liposome technique might therefore not be adaptable to all molecules.

With the development of antibodies against unique cell surface antigens, it should be possible using the liposome technique to direct molecules, capable of specifically affecting a variety of physiologic responses, to subgroups of heterogeneous cell populations. Despite the above-mentioned limitations, such a procedure should be applicable to a wide range of neurobiological and endocrine systems in which it is desirable to specifically modify particular intracellular events coupled to physiologic responses only detectable in intact multicellular populations.

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