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PERTUSSIS TOXIN INHIBITS STIMULATED MOTILITY INDEPENDENTLY OF THE ADENYLATE CYCLASE PATHWAY IN HUMAN MELANOMA CELLS

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Summary: The human melanoma cell line, A2058, has previously been shown to respond to an autocrine motility factor (AMF). We have studied biochemical pathways that may be involved in the generation of such a motile reponse. Pertussis toxin (PT) caused a profound, rapid decrease in stimulated motility that was both dose and time-dependent. Preincubation of cells for 2 hr with as little as lng/ml of PT significantly inhibited motility. A concentration of PT (0.5 μg/ml) that completely eliminated migration after a 30 min. preincubation had a markedly reduced effect when added 1 hr after the start of the assay. In contrast, agents which selectively modulate or have a role in the adenylate cyclase pathway, e.g., cholera toxin, forskolin, the cAMP analogue 8-bromoadenosine 3':5'-cyclic monophosphate and the cyclase inhibitor 2',5'-dideoxyadenosine, all had negligible effect upon motility. These data are consistent with the presence of a receptor coupled to a PT sensitive G protein initiating motility independently of the adenylate cyclase system.

In addition to uncontrolled replication, malignant cells rapidly invade surrounding tissue and metastasize by entering into and exiting conduits such as the vascular and lymphatic systems. This metastatic dissemination requires active locomotion of the tumor cells (1,2). However, the biochemical signals that initiate and regulate tumor cell motility are among the least understood aspects of invasion. We have recently described the isolation and partial characterization of an autocrine motility factor (AMF) from a human melanoma cell line, A2058(3). This protein induces random and directed cellular motility in these highly metastatic melanoma cells. In the present report, we have used AMF as a model system to study the second messenger pathways

Abbreviations: A2058, a human melanoma cell line; AMF, the autocrine motility factor from A2058 cells; 8-Br cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; CT, cholera toxin; 2',5'-DDA, 2',5'-dideoxyadenocine; DMEM, Dulbecco's Modified Eagle Medium with 4.5 mg/ml glucose; PT, Pertussis toxin.

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involved in tumor cell locomotion. We have used pharmacologic agents that affect G protein transduction as well as the cAMP metabolic pathway. While pertussin toxin had a profoundly inhibitory effect on stimulated motility, the results essentially rule out adenylate cyclase as part of the biochemical cascade that initiates locomotion in A2058 tumor cells.

Methods

Materials: Pertussis toxin and cholera toxin were from List Biological Laboratories, Inc. (Campbell, CA). Forskolin and 8-bromoadenosine 3':5'-monophosphate (8-Br cAMP) were purchased from Sigma Chemical Company (St. Louis, MO). The Nuclepore membranes (polyvinylpyrrolidone-free) as well as the 48-well chemotaxis chamber were purchased from Neuro Probe, Inc. (Cabin John, MD). The 2',5'-dideoxyadenosine (2',5'-DDA) was from Pharmacia P-L Biochemicals (Piscataway, NJ).

Cell culture: The human melanoma cell line A2058 was maintained as previously described (4).

Production of autocrine motility factor: This was accomplished essentially as previously described (3) with the following modifications. A2058 cells were incubated for 48 hr in Dulbecco's Modified Eagle Medium (DMEM) without any protein supplement. The conditioned medium was concentrated using a Centricon ultrafiltration assembly, molecular weight cut off 10,000 daltons.

Cell motility assay: The assay used to determine cell motility was a modification of a previously described technique (3,5). In brief, A2058 melanoma cells that were approximately 75-90% confluent were harvested with trypsin-EDTA and allowed to recover at room temperature in DMEM supplemented with 10% heat-inactivated fetal calf serum for a least 1 hr. The cells were then resuspended at 2 x 10^6/ml in DMEM with 1mg/ml bovine serum albumin. All assays except the time course for pertussis toxin were performed in triplicate using 48-well micro-chemotaxis chambers (5) with 8 μm Nuclepore membranes coated with Type IV collagen (6). The pertussis toxin time course was performed in duplicate using larger single blind well chambers with identically treated Nuclepore membranes. The chambers were incubated at 37°C for 4-5 hr, stained and placed onto glass slides with the original cell side up so that the cell pellet could be wiped from the surface. Cells that had migrated through the pores were trapped between glass and membrane and could be easily counted by light microscopy under high power field (500x).

Chemical agents were preincubated with the cells for various periods of time, added at the start of the assay, or added later. Cells from every treatment group were tested for their motility response to the A2058 conditioned medium vs. DMEM alone. For the control cells, unstimulated random migration was <30% of stimulated migration. Results are expressed as percent of control motility with random migration subtracted for each treatment group.

Results

Effect of dose and incubation time of pertussis toxin upon cell motility: Guanine nucleotide-binding proteins (G proteins) were first described as regulatory components of adenylate cyclase (reviewed in 7). Different G proteins may either stimulate or inhibit enzymes responsible for formation of

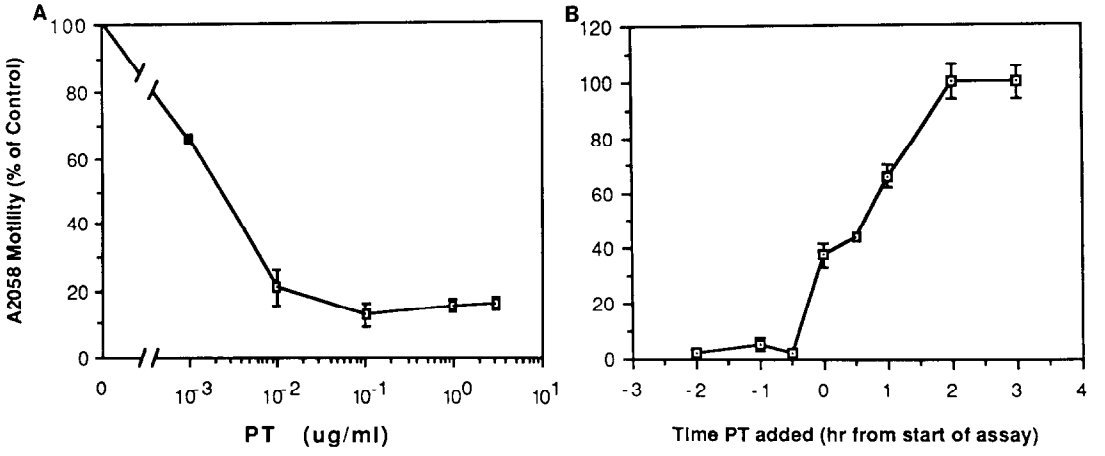

Fig 1. A. Effect of different doses of petussis toxin on A2058 motility. Pertussis toxin was added to the cells at the indicated concentrations for 2 hr prior to and during the assay. B. Time course of the pertussis toxin effect on A2058 motility. Pertussis toxin (0.5 μg/ml) was added to the cells for various periods of preincubation or after the start of the assay. All data is expressed as mean +/- S.E.M.

second messengers. The islet activating protein, pertussis toxin (PT), ADP-ribosylates and thereby blocks activity of the adenylate cyclase inhibitory G protein (G_i) (8) as well as several related G proteins that act on other second messengers.

In order to determine whether such a G protein plays a role in stimulated motility in a human cancer cell line, we added pertussis toxin to A2058 cells at various doses (Fig 1A) or for various periods of time (Fig 1B). Cell viability was unimpaired at any dose of PT used. Two hr preincubation of the cells with any of the tested PT doses (1ng/ml-3μg/ml) resulted in significant reduction in cell motility. Brief preincubation (30 min) at 500ng/ml virtually abolished locomotion and addition of the same dose at the start of the assay resulted in only partial response (40% of control motility). When pertussis toxin was added to the cell compartment at increasing time periods after the start of the assay, there was a successive recovery to full cellular response.

Effect of dose and incubation time of cholera toxin and other agents of cAMP system upon cell motility: Because pertussis toxin, known to inhibit G_i, had such a profound effect in our system, we sought to determine whether acti-

Table 1. Effect of Agents of the cAMP Pathway upon A2058 Stimulated Mobility[a]

Agent	Dose [b]	A2058 Motility (% of Control)	SEM
Cholera Toxin	100μg/ml	64.9	9.3
	30	85.4	7.9
	10	86.9	7.4
	1	102.6	8.5
Forskolin	100μM	95.2	7.4
	30	93.4	9.1
	10	110	9.8
	1	110	10.8
8 Br-cAMP	3μM	87.8	6.1
	1	86.8	6.4
	0.1	92.9	7.6
2',5'-DDA	100μM	105	9.2
	10	96.1	8.0
	1	100	8.1

[a] Unstimulated motility is subtracted from all values. It did not exceed 30% of stimulated motility in untreated cells. None of these agents produced a significant change in the unstimulated motility of the cells.

[b] All agents were preincubated with the cells for 2 hr prior to the start of the assay.

vation of adenylate cyclase would have a similar effect. Cholera toxin is thought to act by ADP-ribosylation of the stimulatory G protein (G_s) in an active configuration (8). Forskolin stimulates adenylate cyclase directly without acting through an intermediary G protein (9). The cAMP analogue, 8-Br cAMP, is able to enter intact cells and act as a cAMP agonist (10).

These three agents were preincubated with the cells for 2 hr prior to and during the AMF stimulated motility assay. The dose response of cholera toxin (Table I) indicates that even 100 μg/ml resulted in only a 35% reduction in motility while doses from 10ng/ml to 30 μg/ml had little effect (>85% of control motility). Similarly, tested doses of forskolin (0.01-100 μM) and 8Br-cAMP (1 μM-3mM) caused at the most only a slight reduction in the response (>85% of control motility).

Overnight treatment of cells with any of the 3 agents (cholera toxin at 10 μg/ml, forskolin at 10 μM, or 8Br-cAMP at 1mM) resulted in a diminished motility response to the AMF activity of A2058 serum free conditioned medium, although the effect was never complete (45-70% of control stimulation).

In contrast, cholera toxin (1-100 μg/ml added at the start of the assay had minimal effect (>90% of control motility).

The adenosine analogue, 2',5'-dideoxyadenosine (2',5'-DDA), directly inhibits adenylate cyclase (11). Preincubation of this agent with the cells for 2 hr at doses from 0.01-100 μM had no significant effect on the motility response to AMF from A2058 serum-free conditioned medium (Table I).

Discussion

The A2058 cell line is a highly metastatic melanoma line that has been shown to have a chemokinetic and chemotactic response to a protein factor (AMF) in its own conditioned medium. These cells do not respond to such extensively characterized chemotactic factors as fMet-Leu-Phe, complement-derived factor C5a, or platelet derived growth factor. The biochemical events that accompany the A2058 response are largely unknown although previous work has indicated that phospholipid methylation may play a role (3). In the present study, this model system has been employed to study the role of G proteins and cyclic nuleotides in the stimulated motility of a human cancer cell line.

Pertussis toxin inhibited stimulated motility in a dose and time-dependent manner (Fig 1A and 1B). The dose response of pertussis toxin was consistent with previously described inhibitory doses for G proteins. The time course showed a much diminished effect when PT was added at a time presumably too late to affect the initiation of motility since the toxin requires approximately 1 hr to enter the cell membrane. These data are consistent with a receptor-mediated system of stimulated cell motility which requires a G protein to initiate the locomotive response.

Since pertussis toxin is known to act by inhibition of the G_i regulatory unit of adenylate cyclase, other agents that act through the cAMP pathway were also studied. Cholera toxin stimulates adenylate cyclase through the G_s protein, forskolin stimulates the enzyme directly, and 8-Br cAMP acts as a cAMP analogue. Although prolonged preincubation or high doses of cholera toxin resulted in variable reduction in the motility response, a 2 hr pre-

incubation with a wide range of doses of either forskolin or 8-Br-cAMP had virtually no effect (Table I). These data suggest that stimulation of cAMP production is not on the direct biochemical pathway of the motility response in A2058 cells. The adenosine analogue 2',5'-DDA, which blocks adenylate cyclase independently of G_i, had a negligible effect. Therefore, the profound inhibitory effect of pertussis toxin does not appear to result from altered basal inhibition of adenylate cyclase (12, 13).

G proteins have been demonstrated to act through a number of second messenger pathways including phospholipase A_2 (14, 15), phospholipase C (16, 17), cGMP phosphodiesterase (8), potassium channels (18, 19), and calcium channels (20). Pertussis toxin is inhibitory in several of these systems. In particular, neutrophil response to the chemoattractant fMet-Leu-Phe includes activation of phospholipase A_2 (15) and phospholipase C (16), both of which are sensitive to pertussis toxin. Finally, pertussis toxin has been proposed to act in the γ-aminobutyric acid system by decreasing receptor affinity for its ligand (23).

In summary, stimulated motility in the human melanoma cell line A2058 has been shown to be inhibited by the islet activating protein, pertussis toxin. This effect appears to be independent of adenylate cyclase. These data are consistent with a receptor-mediated motile response which requires direct participation of a G protein. This process in tumor cells may be analogous to that already defined in the neutrophil (16).

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