Pertussis toxin alters the growth characteristics of Swiss 3T3 cells

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Pertussis toxin (islet-activating protein) treatment of intact Swiss 3T3 cells causes a time- and dose-dependent loss of availability of a 40 kDa membrane protein for toxin-catalyzed ADP-ribosylation in subsequent incubations with [^{32}P]NAD. In parallel, [^{3}H]thymidine uptake by quiescent cells stimulated with fresh serum, cell doubling time and cell saturation density are all decreased 30–50%. These results cannot be accounted for by the potential effect of the toxin on cell cAMP levels. They suggest that a pertussis toxin substrate, probably G_i, modulates some component of growth regulation in Swiss 3T3 cells.

G-protein *Cell growth* *Pertussis toxin* *Cell culture* *Growth inhibition* *cyclic AMP*

1. INTRODUCTION

A family of membrane-bound GTP-binding proteins mediates the coupling of many hormone and neurotransmitter receptors to their respective second messenger enzymes [1]. One of these proteins, G_i, is a trimer with a 40 kDa α-subunit which is a substrate for ADP-ribosylation by the toxin produced by *Bordetella pertussis* called islet-activating protein or IAP [2–7]. G_i mediates inhibition of adenylyl cyclase [2–9], but it may regulate other second messenger systems as well [10–14]. Recently, Murayama and Ui [15] described the presence of this protein in the contact inhibited Swiss 3T3 cell line and the fact that IAP, which uncouples G_i from receptor activation [2,3,9], blocks the effects of many hormones which activate arachidonic acid release and calcium uptake in these cells. Here it is shown that IAP, apparently through the ADP-ribosylation of G_i, alters the growth characteristics of these cells.

2. MATERIALS AND METHODS

IAP and cholera toxin were obtained from List Biologicals. Swiss 3T3 cells were obtained from the American Type Culture Collection (ATCC) and were grown as described by Rozengurt et al. [16]. [^{3}H]Thymidine (20 Ci/mmol) and [α-^{32}P]ATP (2000 Ci/mmol) were obtained from New England Nuclear. [^{32}P]NAD was synthesized as described by Cassel and Pfeuffer [17].

Crude membranes were prepared from cells grown to confluence in T150 flasks (Corning). Cells were washed three times with PBS, scraped from the flask with a rubber policeman into 10 ml PBS and centrifuged at 1000 × *g* for 5 min. The pellet was resuspended in 1 ml homogenizing buffer (20 mM Na-Hepes, pH 8.0, 1 mM EDTA, 150 mM NaCl) and disrupted with a Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernate was centrifuged at 100000 × *g* for 30 min and the pellet resuspended in homogenizing buffer and stored at −80°C until use. IAP-catalyzed ADP-ribosylation of membrane proteins was carried out as described [18]. Densitometry of autoradiograms of [^{32}P]ADP-ribosylated samples separated by SDS-polyacrylamide gel electrophoresis was performed using a Joyce Loebel Magiscan II (Nikon).

Cell growth rates were determined by plating 10^4 cells in 35 mm dishes, or 3×10^4 cells in 60 mm

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dishes, culturing them for various times and then counting the cells with a haemocytometer after trypsinization into 0.5 ml of 0.1% trypsin in calcium and magnesium-free PBS. [^{3}H]Thymidine uptake experiments used cells plated in 24-well plates that had been grown to confluence and then maintained for 1 week without a change of medium. The procedure described by Rozengurt et al. [16] was used to determine trichloroacetic acid-precipitable radioactivity, except that Dulbecco's MEM was used instead of a mixture of Dulbecco's MEM and Weymouth's medium.

3. RESULTS AND DISCUSSION

Crude membranes prepared from Swiss 3T3 cells and subsequently incubated with [^{32}P]NAD contained a single 40 kDa protein specifically labelled in the presence of IAP (not shown). The presence of this protein in these cells and its ability to mediate inhibition of adenylyl cyclase have been described by Murayama and Ui [15]. This protein has all the properties associated with G_i [2–7]. If intact cells were cultured in the presence of IAP for 24 h prior to preparing membranes, subsequent incubations with [^{32}P]NAD and IAP failed to label the 40 kDa protein. This suggests that Swiss 3T3 cells contain a receptor for IAP that allows the toxin to enter the cells and endogenously ADP-ribosylate the G_i prior to preparing membranes. Fig.1 shows the dose-response relationship for the loss of IAP-mediated labelling of the 40 kDa protein in membranes as a function of the amount of IAP used for treating the intact cells. These results are entirely compatible with those reported by Murayama and Ui [15].

Fig.1 also shows the effects of 24 h exposure of the cells to different IAP concentrations on cell growth as measured by both [^{3}H]thymidine uptake by confluent, serum-starved cultures and increase in cell number during 3 days of growth following IAP treatment. Both parameters are decreased by a maximum of 30–50% with a dose-response relationship similar to that for loss of availability of G_i for ADP-ribosylation by IAP in membranes. In long-term experiments (fig.2), where increase in cell number was followed until the cells reached confluence and stopped growing, it was apparent that IAP decreased both the growth rate of the cells and their confluent density.

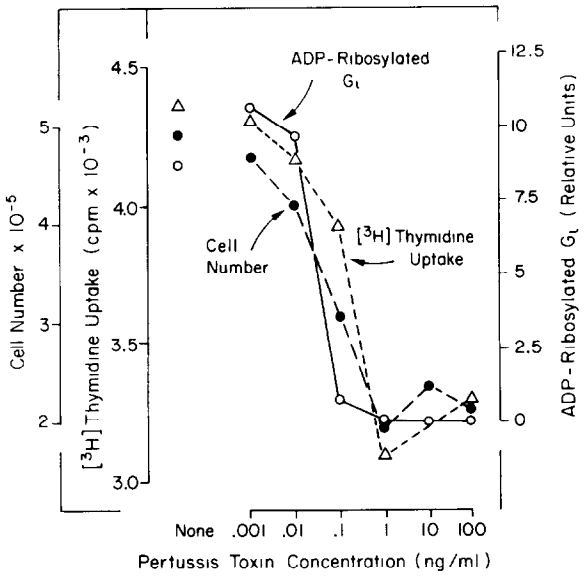

Fig.1. Dose-response relationships for the effects of pertussis toxin (IAP) on Swiss 3T3 cells. In all cases pertussis toxin treatments were for 24 h. Procedures were as described in section 2 except that increase in cell number was determined by plating 3×10^4 cells in duplicate 60 mm dishes. The cells were allowed to attach for 3 h, then IAP was added at the indicated concentrations. The medium was changed 24 h later, and cell number determined 2 days thereafter. [^{3}H]Thymidine uptake was determined in triplicate wells of 24-well plates, and G_i available for ADP-ribosylation by IAP in the presence of [^{32}P]NAD determined in duplicate. In all cases standard errors are less than 10% of the means; in most cases less than 5%.

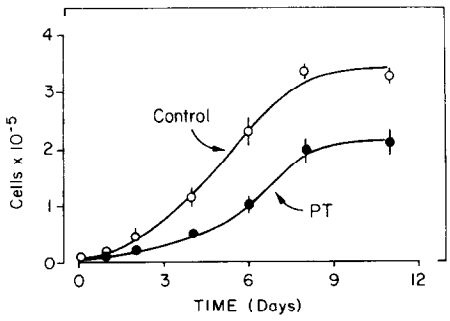

Fig.2. Effect of pertussis toxin on growth rate and saturation density of Swiss 3T3 cells. Cells were grown in 35 mm dishes, initially 10^4 cells, in the absence (control) or presence (PT) of 10 ng/ml IAP. Duplicate dishes were counted on the days indicated. The medium was changed every 2 days. Error bars indicate SE.

Although the mechanism of IAP inhibition of growth in these cells probably involves its inactivation of G_i, the mechanisms by which G_i might affect growth are less clear. Since G_i in permeabilized Swiss 3T3 cells mediates inhibition of adenylyl cyclase [15], one might predict that IAP treatment could elevate cAMP levels. Increased intracellular cAMP does not appear to explain our results, however, since IAP and cholera toxin, which also would raise cellular cAMP levels, have different effects on [^{3}H]thymidine uptake in these cells (fig.3). Cholera toxin treatment does not affect [^{3}H]thymidine uptake under optimal conditions (10% serum), where IAP has its most prominent effect; but does increase uptake under limiting conditions (1% serum), where IAP has little effect. In addition, cAMP has been previously shown to stimulate mitogenic responses of Swiss 3T3 cells [19], in agreement with the cholera toxin effects demonstrated in fig.3. Several other second messenger systems have been implicated as sites of regulation by G_i [10–15], but it is unclear which, if any, of these are affected by G_i in these cells. However, this is the first demonstration that G_i mediates the effects of factors which influence cell growth. By identifying hormones whose growth-promoting effects in Swiss 3T3 cells are blocked by IAP, protocols can be devised for isolating cellular mutants with altered expression of G_i. Such an approach may be of fundamental importance for determining which second messenger systems are regulated by G_i.

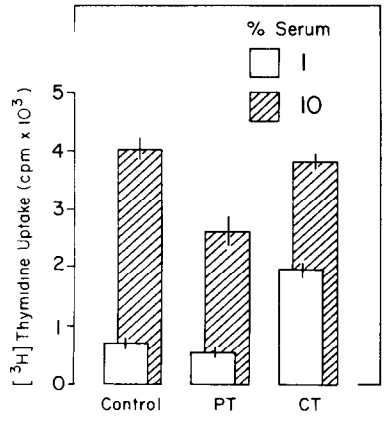

Fig.3. Effect of 100 ng/ml cholera toxin (CT) or 10 ng/ml pertussis toxin (PT) on [^{3}H]thymidine uptake of quiescent Swiss 3T3 cells in the presence of low (1%) or high (10%) serum. Error bars represent SE of triplicate determinations. Pertussis toxin or cholera toxin were added to the depleted medium 24 h before changing to medium with 1 or 10% fresh serum containing 0.1 μCi [^{3}H]thymidine. The plates were processed 48 h later.

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