Effects of pertussis toxin on adenylate cyclase responses to prostaglandin E₂ and calcitonin in human breast cancer cells

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(Received 5 June 1984/Accepted 16 August 1984)

Both calcitonin and prostaglandin E₂ (PGE₂) stimulate adenylate cyclase activity in the human breast cancer cell line (T 47D). The maximum cyclic AMP response to calcitonin exceeds that of PGE₂. When maximal concentrations of the two hormones were added simultaneously to the cells, the amount of cyclic AMP generated was less than that seen with calcitonin alone. When cells were treated with the protein toxin of *Bordetella pertussis* (islet-activating protein; IAP) which inactivates the inhibitory regulatory component (N_i) of adenylate cyclase, there was no change in basal or calcitonin-responsive adenylate cyclase in intact cells. However, the PGE₂ response was augmented at all dose levels, and this effect was dependent on the concentration of IAP. Moreover, in cells pretreated with IAP, simultaneous addition of PGE₂ and calcitonin resulted in additivity rather than in inhibition of cyclic AMP production. The additivity of the response to calcitonin and PGE₂ after IAP treatment implies activation of separate pools of adenylate cyclase catalytic subunit by the two hormones. These data are consistent with a model in which calcitonin acts on adenylate cyclase in T 47D cells through stimulatory regulatory components alone, whereas PGE₂ acts on the same cells through both stimulatory and inhibitory components. The N_i input can limit the maximum effect of PGE₂ and is capable of limiting calcitonin effects when the two agonists are used simultaneously.

Control of adenylate cyclase is achieved by transfer of information from receptor-agonist complexes to catalytic subunits of the adenylate cyclase enzyme by means of nucleotide regulatory units (Rodbell, 1980). Stimulation of adenylate cyclase results from activation of a guanine nucleotide-binding regulatory protein (N_s) (Cooper *et al.*, 1979; Northup *et al.*, 1980), which is composed of a 35 kDa + 45 kDa heterodimer (Northup *et al.*, 1983a); the 45 kDa component possesses GTP binding and GTPase activity, and is activated by ADP-ribosylation caused by cholera toxin (Johnson *et al.*, 1978; Northup *et al.*, 1983b; Suidan *et al.*, 1983). Adenylate cyclase inhibition is mediated by an analogous regulatory protein complex (N_i) (Hildebrandt *et al.*, 1982; Jakobs *et al.*, 1983), composed of a 35 kDa + 41 kDa heterodimer (Bokoch *et al.*, 1983). The

41 kDa component of N_i also binds GTP and is associated with GTPase activity (Bokoch *et al.*, 1983). IAP, prepared from *Bordetella pertussis* toxin, causes ADP-ribosylation of N_i (Katada & Ui, 1982), leading to its inactivation, and preventing its inhibitory input to the adenylate cyclase (Bokoch *et al.*, 1983). The concept of dual regulatory control (stimulatory and inhibitory) of adenylate cyclase arose from observations that hormone-induced decreases in cellular cyclic AMP levels in several responsive systems could be ascribed to direct effects on adenylate cyclase (Meintel *et al.*, 1978; Giannattasio *et al.*, 1981).

Hormones have been regarded as stimulating adenylate cyclase via N_s units, e.g. β -adrenoreceptor agonists, or inhibiting via N_i units, e.g. muscarinic, α -adrenoreceptor agonists, and PGE₁ (Nuzeki & Ui, 1981; Katada & Ui, 1981; Garcia Sainz, 1981). In the present paper we report the interaction of two stimulators of adenylate cyclase, calcitonin and PGE₂, in a human breast cancer cell

Abbreviations used: PG, prostaglandin; IAP, islet-activating protein.

line, T 47D (Findlay *et al.*, 1980). In these cells the maximum adenylate cyclase response to calcitonin is substantially greater than that to PGE_2 . The present data, obtained with IAP from pertussis toxin as an inhibitor of N_i activity, support the model of adenylate cyclase activation developed from recent studies of the structure of the enzyme complex (Bokoch *et al.*, 1983). However they introduce the further possibility that a stimulator of adenylate cyclase can produce its net effect by acting through both N_s and N_i subunits, thus adding an important dimension to the hormonal regulation of cellular response via the cyclic AMP pathway.

Materials and methods

The human breast cancer cell line, T 47D, was obtained from the Breast Cancer Task Force, Washington, DC, U.S.A., and used in these experiments between the 85th and 100th culture passages. The T 47D cells were maintained routinely as previously described (Lamp *et al.*, 1981), by growing in RPMI 1640 medium containing 10% (v/v) foetal bovine serum with 10^{-7} M-insulin (Novo; crystalline) and 10^{-7} M-hydrocortisone hemisuccinate. The latter hormones were omitted from cultures at the time of subculture, 2–3 days before experiments.

Intact cell adenylate cyclase responses were assayed by measuring cyclic $[^3\text{H}]\text{AMP}$ production by cells prelabelled with $[^3\text{H}]\text{adenine}$, as described by Rodan *et al.* (1983). In this method, cellular ATP pools were labelled with ^3H by incubation with $[2,8\text{-}^3\text{H}]\text{adenine}$ ($0.5\text{--}2\text{ }\mu\text{Ci/ml}$) for 2 h at 37°C in 12-well culture dishes in RPMI 1640 medium containing 0.1% bovine serum albumin. When the amount of $[^3\text{H}]\text{adenine}$ used in prelabelling cells was varied between 0.2 and $3\text{ }\mu\text{Ci/ml}$, the concentrations of $[^3\text{H}]\text{adenine}$ correlated linearly with hormone-stimulated cyclic $[^3\text{H}]\text{AMP}$ generation. After incubation of cells with $[^3\text{H}]\text{adenine}$ they were washed twice with serum-free medium and incubated for a further 20 min in medium containing 0.1% bovine serum albumin and 1 mM-isobutylmethylxanthine, which completely inhibited degradation of generated cyclic AMP by endogenous phosphodiesterase activity. Hormone treatments were then added (in $100\text{ }\mu\text{l}$ of the latter incubation medium) as indicated in individual experiments and incubations were carried out at 37°C for 10 min unless otherwise indicated. Incubations were terminated by removing medium and adding $100\text{ }\mu\text{l}$ of 20% (w/v) trichloroacetic acid at 4°C , followed by $800\text{ }\mu\text{l}$ of 5 mM solution of ATP, ADP, AMP, cyclic AMP and adenine. Under these conditions, less than 3% of cyclic AMP generated in cells in response to stimulation was released into the

medium. Results obtained when medium was not removed before addition of trichloroacetic acid were virtually identical with those using cells alone. After being left at 4°C for 1 h, generated cyclic $[^3\text{H}]\text{AMP}$ was measured by subjecting the supernatants to sequential chromatography on Dowex and alumina (Salomon *et al.*, 1974). Recovery of cyclic AMP was assessed by addition of cyclic $[^{32}\text{P}]\text{AMP}$ (approx. 4000 c.p.m.). Radioactivity was counted in Beckman Ready-Solv EP scintillation cocktail in a Beckman 2800 scintillation counter. Experiments were carried out to determine whether treatment of T 47D cells with IAP influenced the labelling of cellular ATP pools with ^3H . Cellular ATP specific radioactivity was assayed (Schweinsberg & Loo, 1980) in cells incubated with $[^3\text{H}]\text{adenine}$ for 2 h, stopping the reaction as above, and separating cellular nucleotides by high pressure liquid chromatography, measuring unlabelled ATP by A_{254} against standards, and radioactivity by liquid-scintillation counting.

IAP, purified from *Bordetella pertussis* vaccine, was a gift to Dr. R. G. Larkins from Dr. M. Yajima, Shiga, Japan. Synthetic salmon calcitonin (potency 4700 units/mg) was a gift from the Armour Pharmaceutical Co., Kankakee, IL, U.S.A. Prostaglandins E_1 , E_2 , $\text{F}_{2\alpha}$, thromboxane B_2 and the thromboxane analogue U46619 were gifts from Dr. John E. Pike, Upjohn Co., Kalamazoo, MI, U.S.A. $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, cyclic $[^{32}\text{P}]\text{AMP}$, $[^3\text{H}]\text{adenine}$ and cyclic $[^3\text{H}]\text{AMP}$ were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Culture media and foetal bovine serum were obtained from Flow Laboratories, plastic culture bottles and dishes from Costar, and isobutylmethylxanthine from Sigma Chemical Co. All other chemicals were of reagent grade and from standard suppliers.

Results

Effects of IAP on cyclic AMP formation in T 47D cells in response to salmon calcitonin and PGE_2

In intact cells, salmon calcitonin and PGE_2 treatment led to a dose-dependent increase in cyclic $[^3\text{H}]\text{AMP}$ production by T 47D cells prelabelled for 2 h with $[^3\text{H}]\text{adenine}$ (Fig. 1). The maximum response to salmon calcitonin was a 40-fold stimulation, with a $K_{0.5}$ in several experiments of $10^{-9}\text{--}10^{-10}$ M. PGE_2 caused a 5- to 12-fold stimulation, with $K_{0.5}$ of $10^{-9}\text{--}10^{-8}$ M. We have shown previously that calcitonin stimulated adenylate cyclase in homogenates of T 47D cells to a greater extent than did PGE_2 (Lamp *et al.*, 1981). Fig. 1 also shows that pretreatment of T 47D cells with IAP potentiated the cyclic AMP response of

Fig. 1. Effect of IAP (pertussis toxin) pretreatment on cyclic AMP formation in response to PGE₂ and calcitonin in intact T 47D cells

T 47D cells growing in complete culture medium were treated with or without IAP (20ng/ml) in 12-well dishes for 16h at 37°C before experiments. Cyclic [³H]AMP generated in response to treatments was assayed in cells prelabelled with [³H]-adenine (1.8μCi/ml) as described in the Materials and methods section. Salmon calcitonin with (Δ) and without (▲) IAP pretreatment; PGE₂ with (○) and without (●) IAP pretreatment. Results are means ± s.e.m. of triplicate determinations.

intact cells to PGE₂ at all concentrations tested, while having no effect either on basal cyclic AMP accumulation or on the calcitonin dose-response. Thus the maximum response to PGE₂ increased from 7-fold to 35-fold, but the K_{0.5} for PGE₂ was similar for the two treatments (4 × 10⁻⁸M and 8 × 10⁻⁸M).

Measurement of intracellular specific radioactivity of ³H-labelled ATP pools confirmed that treatment of cells with IAP during the [³H]adenine labelling period did not result in any change in ATP labelling. Incubation of cells with [³H]-adenine (1.8μCi/ml) for 2h yielded ATP specific activities of 54.4 × 10³ d.p.m./nmol of ATP and 58.2 × 10³ d.p.m./nmol of ATP for control and IAP-treated cells respectively.

Time course of IAP effect on PGE₂ response

When T 47D cells were treated with IAP (20ng/ml) for increasing periods up to 24h, it was noted again that treatment resulted in no change in basal cyclic AMP production. Enhancement of response to a maximally effective PGE₂ concentration was apparent at all times examined. The

Table 1. Effect of IAP (pertussis toxin) treatment of T47 D cells on cyclic AMP generation in intact cells in response to calcitonin, PGE₂, and the two treatments together
T47 D cells were preincubated for 16h in complete culture medium alone or in medium containing IAP (20ng/ml). Assays were carried out of the cyclic [³H]AMP generated in response to salmon calcitonin (10⁻⁷M), PGE₂ (10⁻⁶M) and the two agonists together. Cells were prelabelled with [³H]adenine (0.5μCi/ml). Values are means ± s.e.m. of triplicate determinations.

Treatment	Cyclic [³ H]AMP (c.p.m./10 ⁶ cells)	
	Control	IAP-treated
None	254 ± 8	221 ± 12
PGE ₂ (10 ⁻⁶ M)	12754 ± 121	93742 ± 206
Calcitonin (10 ⁻⁷ M)	87924 ± 224	91102 ± 241
PGE ₂ + calcitonin	55171 ± 92	185106 ± 280

maximum effect of IAP treatment in repeated experiments was consistently obtained after 5–6h (results not shown). For the other experiments reported in this paper a standard treatment time of 16h was chosen.

Time course of response to calcitonin and PGE₂ after treatment of cells with IAP

Although a high concentration of the phosphodiesterase inhibitor, isobutylmethylxanthine, was used in these experiments, it was necessary to ensure that the observed effects were not due to changes in cyclic AMP degradation. Results in Fig. 2 confirm that IAP treatment results in enhancement of response to PGE₂, but not to calcitonin. Moreover, frequent sampling up to 20min showed that the enhanced PGE₂ response resulted from an increase in cyclic [³H]AMP formation rate, hence reflecting adenylate cyclase activity.

Effects on cyclic AMP formation of simultaneous treatment with calcitonin and PGE₂

When maximally effective concentrations of calcitonin and PGE₂ were added together to T 47D cells, it was noted consistently that the total amount of cyclic AMP generated was less than that seen with calcitonin alone (Table 1). When the effects of the hormones were determined in T 47D cells pre-treated with IAP, the maximum response to PGE₂ was enhanced, with no significant effect on that to calcitonin (Table 1). Most striking, however, was the fact that under these circumstances the effects of the two agonists together were additive.

Fig. 2. Time course of cyclic AMP generation in T 47D cells

T 47D cells growing in complete culture medium in 12-well dishes were treated for 16 h with IAP (closed symbols) or medium alone (open symbols). After incubation for 2 h with [³H]adenine (2 μCi/ml), cells were treated with 10⁻⁶ M-PGE₂, (○, ●) or 10⁻⁷ M-salmon calcitonin (△, ▲), and cellular cyclic [³H]AMP was measured at the indicated time points. Results are means ± S.E.M. of triplicate determinations.

Interactions of other prostanoids

Some other prostanoids were investigated to determine the specificity of the PGE₂ response. In the experiment illustrated in Table 2, PGE₁ was found to produce an effect identical to that of PGE₂, in that its cyclic AMP response was enhanced by IAP treatment, it attenuated the calcitonin response to the same extent as did PGE₂, and after IAP treatment, PGE₁ and calcitonin effects were additive. PGF_{2α}, on the other hand, had only very slight stimulatory effects, which were not enhanced by IAP treatment of cells, nor did PGF_{2α} modify the calcitonin response. In separate experiments (results not shown), thromboxane B₂ and the stable thromboxane analogue, U46619, also failed to produce any significant stimulatory or inhibitory effects on cyclic AMP production.

Concentration-dependence of IAP effects on PGE₂ response

The enhancement of T 47D cell cyclic AMP response to PGE₂ was dependent on the concentration of IAP used to treat the cells (Fig. 3), with half-maximal effectiveness at about 200 pg/ml. Furthermore, in the same experiment, and over the same IAP concentration range, the inhibitory effect of PGE₂ on the calcitonin response was lost, and additivity of the two treatments restored.

Table 2. Effects of various prostanoids and calcitonin on cyclic AMP response in T47 D cells, with and without treatment with IAP (pertussis toxin)

T47 D cells growing in complete culture medium were preincubated for 16 h with or without IAP (20 ng/ml), were labelled with [³H]adenine (1.5 μCi/ml), and the generated cyclic [³H]AMP was determined, as described in the Materials and methods section, in response to various prostanoids and salmon calcitonin. Values are means ± S.E.M. of triplicate determinations.

Treatment	Cyclic [³ H]AMP (c.p.m./10 ⁶ cells)	
	Control	IAP-pretreated
Basal	1260 ± 175	1563 ± 19
PGE ₂ (10 ⁻⁶ M)	11 510 ± 339	44 693 ± 732
PGE ₁ (10 ⁻⁶ M)	11 234 ± 222	44 586 ± 821
PGF _{2α} (10 ⁻⁶ M)	1889 ± 166	1553 ± 361
Calcitonin (10 ⁻⁷ M)	31 631 ± 672	38 946 ± 1520
Calcitonin + PGE ₂	24 069 ± 1747	98 827 ± 2788
Calcitonin + PGE ₁	23 046 ± 385	78 322 ± 5522
Calcitonin + PGF _{2α}	30 676 ± 784	39 382 ± 1307

Concentration-dependence of PGE₂ effects on calcitonin response

The effect of increasing concentrations of PGE₂ on cyclic AMP generation was determined in

Fig. 3. Effect of increasing concentrations of IAP (pertussis toxin) on cyclic AMP generated in response to calcitonin and PGE₂ in intact T 47D cells

T 47D cells growing in complete culture medium were incubated with increasing concentrations of IAP in 12-well dishes for 16h before measurement of cyclic [³H]AMP production in response to control solution (■), salmon calcitonin (3×10^{-9} M; ○), PGE₂ (3×10^{-8} M; □), and both calcitonin and PGE₂ (●). Cells were prelabelled with [³H]adenine (1 μ Ci/ml). Results are means $\pm$ S.E.M. of triplicate determinations.

T 47D cells stimulated half-maximally by calcitonin (Fig. 4). In cells which had not been treated with IAP, PGE₂ inhibited the calcitonin response in a dose-dependent manner. On the other hand, when calcitonin and PGE₂ were added simultaneously to cells pre-treated with IAP, additive effects were observed, which also increased with the PGE₂ concentration.

Discussion

The active component of pertussis toxin, IAP, has provided a useful means of investigating molecular mechanisms of adenylate cyclase action. It has been shown clearly to inactivate the N_i input to adenylate cyclase (Bokoch *et al.*, 1983), thus removing an inhibitory influence. The data reported here show in an intact cell system that N_i inhibition has resulted in enhancement of cyclic AMP response to one agonist, (PGE₂) but not to another (calcitonin) in the same cells. Thus the effect of IAP on the PGE₂ response in T 47D cells resembles that reported for the IAP effect on the lipolytic response to β -adrenergic stimulation in rat

Fig. 4. Effect of PGE₂ on cyclic AMP formation in intact T 47D cells in response to calcitonin, with and without IAP treatment

T 47D cells preincubated for 16h with (open symbols) or without (closed symbols) IAP (20 ng/ml) were labelled with [³H]adenine (1.2 μ Ci/ml) for 2h and the generated cyclic [³H]AMP was measured, as described in the Materials and methods section. Upper panel: all incubations contained 10⁻⁹ M salmon calcitonin, with increasing amounts of PGE₂. Lower panel: incubations contained medium alone, with increasing amounts of PGE₂. Results are means $\pm$ S.E.M. of triplicate determinations.

adipocytes (Moreno *et al.*, 1983). In considering possible explanations for this observation, the model we have tested is one in which the two adenylate cyclase stimulators, calcitonin and PGE₂, have different inputs to the adenylate cyclase catalytic subunit via the nucleotide regulator subunit transducers, N_s and N_i. The augmentation of the PGE₂ response was consistent with the view that PGE₂ had a significant N_i input to its effects on adenylate cyclase catalytic activity, in addition to its N_s input. Calcitonin, on the other hand, with its response unaffected by IAP treatment, would be considered to act through N_s alone.

The results obtained upon using the two agonists simultaneously were of particular interest. When PGE₂ was included together with calcitonin at maximally effective concentrations, the total amount of cyclic AMP generated was less than that seen with calcitonin alone. This observation in

intact T 47D cells is relevant to those made some years ago in rat epididymal fat pad and rat adipocytes (Butcher & Baird, 1968), in which PGE_1 inhibited cyclic AMP production by adrenaline, and in human fat cells (Burns *et al.*, 1972), in which α -adrenergic blockade enhanced the cyclic AMP elevating effect of adrenaline. Our interpretation is that removal of N_i action by IAP treatment negates the ability of PGE_2 to inhibit the calcitonin response when the two agonists are used together. It implies that N_i units invoked in the action of PGE_2 on T 47D cells are available to attenuate the action of the alternative agonist, calcitonin, in addition to attenuating its own action.

No evidence was obtained in these experiments for a prostanoid acting solely via N_i units in these cells. PGE_1 , the only other prostanoid causing significant increases in cyclic AMP production, also had a dual effect on N_s and N_i , as deduced from experiments with IAP.

The differences between N_s and N_i appear to reside in their 45 kDa and 41 kDa components respectively. Their characterization of the adenylate cyclase complex had led Northup *et al.* (1983b) to suggest, on the basis of equilibrium criteria, that N_s stimulation of adenylate cyclase occurs by dissociation of the N_s doublet and interaction of the 45 kDa component with the cyclase catalytic subunit. Inhibition by N_i , however, appears rather to take place via the 35 kDa subunit, released from N_i and interacting with and preventing the 45 kDa subunit of N_s from stimulating the cyclase. On the other hand the observation that N_i inhibition occurs in the absence of N_s (in S49 cyc⁻ cells) is not consistent with the latter proposal (Johnson *et al.*, 1978; Bokoch *et al.*, 1983), but rather favours the possibility that the 41 kDa component of N_i might inhibit directly. Based on the model of nucleotide regulatory subunit involvement in adenylate cyclase activation, the calcitonin- PGE_2 interaction is interpreted as follows.

Two hormones regarded as stimulators of adenylate cyclase in the breast cancer cell line, T 47D, appear to produce their net effects by different mechanisms. Rather than adhering to classical concepts of hormones acting by discrete stimulatory or inhibitory pathways, we have produced evidence that PGE_2 can involve simultaneously both N_s and N_i units in achieving its net effects on cyclic AMP production. Calcitonin, on the other hand, acts through N_s alone. Furthermore, the N_i units invoked in PGE_2 action are capable of attenuating not only the PGE_2 response, but also that to calcitonin. When the inhibitory component of the PGE_2 response is blocked, allowing expression only of stimulatory pathways, additivity of the cyclic AMP response to the two effectors becomes

evident. The molecular mechanisms by which PGE_2 can utilize both N_s and N_i units in these cells remain to be determined. The prostaglandin might achieve this through a single receptor, but, despite the apparent specificity of the response to PGE_2 and PGE_1 , we cannot exclude the possibility that there may be separate prostaglandin receptors mediating the N_s and N_i effects.

The mechanisms proposed draw upon several recent studies of isolated components of the adenylate cyclase system. Our data are consistent with the model we propose, and provide support for its concepts in a living cell system. The further implication of these data and this model is that if a target cell adenylate cyclase responds to more than one hormone, the net effect should not be assumed to be additive, but will depend on the interaction of stimulatory and inhibitory inputs to the adenylate cyclase complex. If such a model is applicable to other hormones, tissues and cell types, it may be relevant to the combined effects of circulating (e.g. peptide hormones, catecholamines) and of local (e.g. prostaglandin) stimulators of adenylate cyclase in individual tissues.

This work was supported by a program grant from the National Health and Medical Research Council. The expert technical assistance of Mrs. S. Omond is gratefully acknowledged.

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