Characteristics of selective activation of cyclic AMP-dependent protein kinase isoenzymes by calcitonin and prostaglandin E₂ in human breast cancer cells

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The characteristics of the cyclic AMP-dependent protein kinase isoenzyme response to calcitonin stimulation have been studied in two human breast cancer cell lines, T 47D and MCF 7. Both cell lines possess calcitonin receptors, a calcitonin-responsive adenylate cyclase and the two isoenzymes of the cyclic AMP-dependent protein kinase, types I and II. The adenylate cyclase also responds to prostaglandin E₂. Acute activation of the cyclic AMP-dependent protein kinase isoenzymes was determined by using a modification of a multiple small anion exchange column method [Livesey, Kemp, Re, Partridge & Martin (1982) *J. Biol. Chem.* **257**, 14983–14987]. Control experiments showed that post-extraction activation did not influence the data. Calcitonin caused a rapid, selective activation of isoenzyme II in the T 47D cells with half-maximal response at 10^{-10} M, and persisting for at least 24 h. In MCF 7 cells calcitonin also caused a highly selective activation of isoenzyme II with half-maximal response at 5×10^{-11} M, but the response was transient with a return to basal isoenzyme activity by 4–6 h. At this time further addition of calcitonin did not restimulate the cyclic AMP-dependent kinase activity. In neither cell line did calcitonin treatment result in activation of isoenzyme I. Prostaglandin E₂, on the other hand, the only significant alternative agonist of adenylate cyclase in T 47D cells, activated isoenzymes I and II to an equal extent in these cells, illustrating that two hormones activating adenylate cyclase in the one cell type might exert different effects by their selective actions upon protein kinase isoenzymes.

Hormones which act via cyclic AMP modulate a diverse range of cellular responses (Nimmo & Cohen, 1977). Furthermore, different hormones acting via cyclic AMP in the same cell type control different response patterns (Bruxton & Brunton, 1983). The major, and probably the only, mechanism of cyclic AMP action in mammalian systems appears to involve stimulation of cyclic AMP-dependent protein kinase isoenzymes (Glass & Krebs, 1980).

Most mammalian cells contain two cyclic AMP-dependent protein kinase isoenzymes, referred to as types I and II (Corbin *et al.*, 1975). Studies of the relative levels of these isoenzymes in different cellular states, hypertrophy (Byus *et al.*, 1976), differentiation (Lee *et al.*, 1977), tumour regression (Cho Chung, 1980) and cell cycle (Costa *et al.*, 1978), have suggested different functions of the

two isoenzymes. We have previously reported a new method of assessing the acute hormonal activation of each isoenzyme in the intact cell and have shown that selective isoenzyme activation can occur (Livesey *et al.*, 1982).

The two human breast cancer cell lines, T 47D and MCF 7, possess calcitonin receptors and a calcitonin-responsive adenylate cyclase (Findlay *et al.*, 1980). In the T 47D cells calcitonin has been shown to activate exclusively isoenzyme II of cyclic AMP-dependent protein kinase (Ng *et al.*, 1983). In the present report it is shown that exclusive activation of isoenzyme II is associated with calcitonin treatment of MCF 7 cells, but that the duration of response differs in the two cell lines. Furthermore, it is also shown that two hormones (calcitonin and prostaglandin E₂) elevating cyclic AMP in the one cell type can pro-

duce different patterns of activation of isoenzymes of cyclic AMP-dependent protein kinase.

Materials and methods

Hormones and chemicals

Synthetic salmon calcitonin was a gift from Armour Pharmaceutical Co., Kankakee, IL, U.S.A. Bovine serum albumin was obtained from Armour Pharmaceutical Co.; [γ - 32 P]ATP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Foetal calf serum and culture media were obtained from Grand Island Biological Co. (Australia) and cyclic AMP from Boehringer Mannheim. Synthetic peptide substrate corresponding to part of the phosphorylation site sequence in porcine hepatic pyruvate kinase (Hjelmqvist *et al.*, 1974) was synthesized as previously described (Kemp, 1980). Phosphocellulose paper (P81) and microgranular DE52 ion exchange resin were products of Whatman, Maidstone, Kent, U.K. and CM-Sephadex was obtained from Pharmacia. All other chemicals were reagent grade and from standard suppliers.

Cell culture

Human breast cancer cells (T 47D and MCF 7) were obtained from the Breast Cancer Task Force, Washington DC, U.S.A. Breast cancer cells were grown in 75 cm² plastic culture flasks (Lux Scientific) in RPMI 1640 medium supplemented with 5% (v/v) foetal calf serum, gentamycin (80 mg/l) and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (20 mM). Cells were incubated at 37°C and equilibrated in CO₂/air (1:19). Subculture was performed weekly with 0.0125% trypsin and 0.5 mM-disodium EDTA in phosphate-buffered saline to harvest cells. For protein kinase assays harvested cells were plated in 25 cm² plastic culture flasks at least 48 h prior to assay, each flask representing a single hormone dose or time point. Cells were used when they had reached confluence.

Separation of cyclic AMP-dependent protein kinase isoenzymes after hormone stimulation

The method is a modification of that described previously (Livesey *et al.*, 1982). Some details of the modified procedure have been published previously (Ng *et al.*, 1983). Hormones in RPMI 1640/2% (v/v) foetal calf serum were added to confluent cells in 25 cm² tissue culture flasks. At the end of incubations, cells were washed and scraped from vessel surfaces in an excess of column starting buffer as previously described (Ng *et al.*, 1983), with the aim of minimizing the possibility of post-cell-rupture activation or reassociation of the isoenzymes (Palmer *et al.*, 1980). Dilute cell suspensions were immediately sonicated and

applied to 2 ml DE52 columns, and washed with a minimum of 10 ml of the column starting buffer (Ng *et al.*, 1983). Cell processing time for each column was no greater than 3 min.

The columns, each representing a single hormone dose or time point, were simultaneously eluted with linear gradients of NaCl. Optimal separation of isoenzymes in both cell lines was achieved with a 2 ml DE52 column pre-equilibrated in column starting buffer and elution with a linear gradient (0–0.6 M) of NaCl, total volume 40 ml for each column.

Simultaneous elution was achieved by using a gravity-fed multioutlet gradient mixer and a manifold constructed to carry the columns on the crossbar of an LKB fraction collector. Drop rate was determined by a dummy column set at one-tenth the drop number per test column fraction required; 20-drop (approx. 1 ml) fractions were collected. Variability in fraction size for a single column was less than 2% and between columns less than 10%. All gradients were linear and reproducible.

The free catalytic subunit generated during isoenzyme activation was eluted in the wash fractions of the DE 52 columns. This activity was concentrated and partially purified by CM-Sephadex chromatography and assay as previously described (Ng *et al.*, 1983).

Measurement of cyclic AMP-dependent protein kinase activity

Cyclic AMP-dependent protein kinase activity was measured as described previously (Livesey *et al.*, 1982). Reaction mixtures contained 20 mM-Mes (4-morpholine-ethanesulphonic acid)/NaOH buffer (pH 6.8), 10 mM-magnesium acetate, 1 mM-disodium EDTA, 15 μ M-bovine serum albumin, 0.125 mM- $[\gamma$ - 32 P]ATP (400–500 c.p.m./pmol) and 156 μ M synthetic peptide substrate, total volume 80 μ l. Reactions were commenced by addition of 30 μ l aliquots of column fractions or enzyme samples, incubation time was 30 min at 37°C, and reactions were terminated by spotting 25 μ l of reaction mixture onto Whatman P81 paper discs held in a perspex manifold. Isoenzyme activity was measured in the presence of 6.25 μ M-cyclic AMP. Discs were then washed in four changes of 5 mM-phosphoric acid for 10 min each and were rinsed in ethanol before counting with a toluene scintillant in a Packard automatic scintillation counter.

Results

As was found to be the case with T 47D cells (Ng *et al.*, 1983), the MCF 7 human breast cancer cell line possesses the two isoenzymes of cyclic AMP-dependent protein kinase found in most mam-

malian tissues (results not shown). The only difference in chromatographic profiles between unstimulated T 47D and MCF 7 cells is that, in the latter cell line, the amount of isoenzyme II consistently exceeds that of isoenzyme I. Partially purified MCF 7 isoenzymes I and II obtained from cells were indistinguishable kinetically from those obtained from T 47D cells (Ng *et al.*, 1983). All isoenzymes showed a similar responsiveness to cyclic AMP [$K_{0.5}$ of $(5-8) \times 10^{-8}$ M].

Response of cyclic AMP-dependent protein kinase to salmon calcitonin in T 47D and MCF 7 cells

When T 47D and MCF 7 cells in culture were treated for 10 min with increasing concentrations of salmon calcitonin, dose-dependent activation of isoenzyme II occurred (Figs. 1 and 2). This is reflected in the loss of intact holoenzyme eluted from the DE-52 column, consistent with dissociation and hence activation. In these and in the

Fig. 1. Dose-dependent activation of cyclic AMP-dependent protein kinase isoenzymes by salmon calcitonin in T 47D cells. Extracts of T 47D cells were prepared as described in the Materials and methods section for control cells (a) or after exposure of intact cells to increasing concentrations of salmon calcitonin for 10 min. Concentrations of salmon calcitonin: (b) 10^{-12} M, (c) 10^{-11} M, (d) 10^{-10} M, (e) 10^{-9} M. After application to DE-52 columns, each column, representing a single hormone dose, was washed with column equilibrium buffer and then eluted with a simultaneous linear gradient (0–0.6 M) of NaCl (40 ml/column). Kinase activity for each column was assayed in the presence of $6.25 \mu\text{M}$ -cyclic AMP. The inset shows the percentage activation of isoenzyme I (○) and isoenzyme II (Δ) relative to unstimulated cells, determined by integration of isoenzyme peaks.

Fig. 2. Dose-dependent activation of cyclic AMP-dependent protein kinase isoenzymes by salmon calcitonin in MCF 7 cells. Extracts of MCF 7 cells were prepared after treatment with increasing concentrations of salmon calcitonin, and processed as described in the legend to Fig. 1. (a) Control; (b-e) concentrations of salmon calcitonin: (b) 10^{-13} M, (c) 10^{-12} M, (d) 10^{-11} M, (e) 10^{-10} M. The inset shows the percentage activation of isoenzyme I (○) and isoenzyme II (△) relative to unstimulated cells, determined by integration of isoenzyme peaks.

other experiments reported in this paper, it was confirmed that disappearance of intact holoenzyme from DE52 profiles was accompanied by appearance of free catalytic unit activity after CM-Sephadex chromatography (Ng *et al.*, 1983).

Control experiments to assess post-extraction activation

In any such work, and for each cell type and set of experimental conditions, it is essential that

certain control experiments be carried out. Two types of control experiments were carried out to assess whether the activation of the cyclic AMP-dependent protein kinase isoenzymes detected using this method represented intracellular activation or resulted from the artefact of post-cell rupture activation. First, partially purified isoenzyme II from the same cell types was used as an external control (Table 1). Isoenzyme II was added to cell extraction buffer. Hormone activation experiments were then carried out with treated cells being processed in buffer alone or buffer with added isoenzyme. By comparing experimental and predicted results it can be determined whether exogenous (and therefore cellular) isoenzymes are activated during cell processing. At low hormone concentrations little post-cell rupture activation was demonstrated. At supramaximal hormone concentrations however (3×10^{-8} M and greater), activation of added isoenzymes did occur, indicating that the dilution step employed in the assay system is critical in overcoming the artefact of post-cell rupture activation. Identical results were obtained if partially purified isoenzyme I was used as the exogenous enzyme (results not shown). This approach was used previously to determine the extent of post-extraction activation (Livesey *et al.*, 1982).

In the second type of control experiment, stimulated cells were processed together with an equivalent number of unstimulated cells. Activation taking place after cell rupture would appear as artefactual stimulation of the isoenzymes in this second group of control cells. Results obtained were consistent with those using exogenous partially purified isoenzymes as control (Table 2).

Response of cyclic AMP-dependent protein kinase to prostaglandin E₂ in T 47D cells

Prostaglandin E₂ is the only significant alternative agonist of adenylate cyclase in T 47D cells (Lamp *et al.*, 1981). It was of interest to compare its effects on the isoenzymes of cyclic AMP-dependent protein kinase. In contrast to calcitonin, which specifically activated only isoenzyme II, prostaglandin E₂ activated both isoenzymes I and II in a dose-dependent manner (Fig. 3). Although cyclic AMP levels generated in response to prostaglandin E₂ were not measured in the protein kinase experiments, the maximum amount of cyclic AMP generated by T 47D cells in response to prostaglandin E₂ is considerably less than that to calcitonin (Michelangeli *et al.*, 1984). Thus the different pattern of responsiveness of protein kinase to prostaglandin E₂ cannot be due to greater amounts of cyclic AMP being generated. Furthermore, although the control experiments of Tables 1 and 2 exclude significant post-cell rupture activation, they do not exclude the possibility that reassociation of isoenzyme subunits might occur. The response of both isoenzyme I and II to prostaglandin E₂ might favour the view that reassociation of isoenzyme I subunits is not an explanation of the lack of responsiveness of that isoenzyme to calcitonin stimulation.

Time course of isoenzyme II response to salmon calcitonin in T 47D and MCF 7 cells

In these experiments the duration of the isoenzyme response was determined by incubating cells with 3×10^{-10} M salmon calcitonin in complete culture medium for various periods of time.

Table 1. *Assessment of post-extraction activation by use of exogenous purified isoenzyme II*

Isoenzyme II was partially purified from T 47D cells by DE52 chromatography. Partially purified isoenzyme was present in or absent from buffer sample used to dilute calcitonin-treated or non-treated cells. Predicted isoenzyme II activity was determined by the sum of added exogenous isoenzyme II alone and activity of cells alone. If significant post-extraction activation of exogenous and cellular isoenzyme II occurs, the predicted value is significantly greater than that determined experimentally.

[Calcitonin] (M)	Added isoenzyme II	Added cells	Isoenzyme II activity (pmol/min)	
			Determined	Predicted
0	—	+	5.8	
0	+	—	28.6	
0	+	+	36.2	33.4
1.5×10^{-10}	—	+	4.2	
1.5×10^{-10}	+	+	25.4	32.8
3×10^{-10}	—	+	2.6	
3×10^{-10}	+	+	21.6	31.2
3×10^{-8}	—	+	0.7	
3×10^{-8}	+	+	13.2	29.5
3×10^{-6}	—	+	0	
3×10^{-6}	+	+	1.6	28.6

Fig. 3. Dose-dependent activation of cyclic AMP-dependent protein kinase isoenzymes by prostaglandin E_2 in T 47D cells. After incubation with increasing concentration of PGE_2 for 10 min, control (a) and treated cells (b-e) were processed as described previously. Concentrations of PGE_2 : (b) 10^{-9} M, (c) 10^{-8} M, (d) 10^{-7} M, (e) 10^{-6} M. The inset shows calculated percentage activation of each isoenzyme relative to the control; $\bullet$, type I isoenzyme; $\circ$, type II isoenzyme.

We had previously shown that T 47D cells respond rapidly, with virtually full activation of cyclic AMP-dependent protein kinase isoenzyme II occurring within 30 s and persisting for at least 24 h (Ng *et al.*, 1983). A comparison of the duration of isoenzyme II activation by calcitonin in MCF 7

and T 47D cells as expressed by percentage activation is shown in Fig. 4. In MCF 7 cells the response to calcitonin was again rapid, but unlike T 47D cells, the activation of isoenzyme II was transient in MCF 7 cells, with a return to basal profile within 6 h. This contrasting time-course was a

Table 2. Assessment of post-extraction activation by use of control nonstimulated cells

As an external control of post-cell rupture activation, an equivalent number of untreated cells was present in or absent from buffer used to dilute hormone-treated cells. Predicted kinase activity was determined by the sum of added control cells and hormone-treated cells. Significant post-extraction activation occurs if the predicted value is greater than the determined value.

[Calcitonin] (M)	Added control cells	Added test cells	Kinase activity (pmol/min)		
			Determined	Predicted	
0	+	-	I 2.6	2.6	
			II 4.7	4.7	
0	-	+	I 2.6		
			II 4.6		
0	+	+	I 5.4	5.2	
			II 10.8	10.9	
3×10^{-10}	-	+	I 2.6		
			II 2.9		
3×10^{-10}	+	+	I 4.6	5.2	
			II 9.2	9.1	
3×10^{-6}	-	+	I 0		
			II 0		
3×10^{-6}	+	+	I 0	2.8	
			II 0	6.2

Fig. 4. Comparison of duration of type II isoenzyme activation by salmon calcitonin in MCF 7 and T 47D cells

Salmon calcitonin (3×10^{-10} M) was added to separate flasks of cells (Δ , T 47D cells; $\blacktriangle$, MCF 7 cells) which were incubated for the indicated times before being solubilized and processed as described for Figs. 1 and 2. Results have been expressed as percentage activation relative to untreated cells by integration of isoenzyme II peaks for each time point and expressing as:

$$\frac{\text{isoenzyme II activity at time 0} - \text{isoenzyme II activity at time } x}{\text{isoenzyme II activity at time 0}} \times 100$$

consistent difference between T 47D and MCF 7 cells, the latter frequently returning to basal profile within 4 h.

Control experiments were carried out to deter-

mine if the transient activation of isoenzyme II in MCF 7 cells was the result of rapid degradation of salmon calcitonin by these cells (Findlay *et al.*, 1981). Where a fresh dose of salmon calcitonin was added to cells which had been incubated in salmon calcitonin (10^{-10} M) for 4 h, little further stimulation of isoenzyme II resulted (Fig. 5). However, when medium from these 4 h incubations was added to resting cells, significant stimulation of isoenzyme II occurred, indicating that active calcitonin was not limiting.

Discussion

Cyclic AMP-dependent protein kinase isoenzyme activation was determined in these experiments by a modification of a method which we had used to show that selective isoenzyme activation can occur in response to hormone stimulation in osteoblast-like cells (Livesey *et al.*, 1982). The modified method, as outlined by Ng *et al.* (1983) and validated in the present paper in further detail, overcomes certain limitations in the earlier approach. First, the effects of trypsinization on some cell types in modifying the cyclic AMP response system precludes the use of suspended cells. Secondly, the gradient elution method obviates the need, arising from slight variations in elution characteristics of the isoenzymes from different cells, to characterize the batch elution criteria for each new cell type. The control experiments involving addition of partially purified isoenzymes or of control cells with the extraction buffer at the time of cell rupture were essential to ensure that

Fig. 5. *Refractoriness of calcitonin-pretreated MCF 7 cells*

Time course of salmon calcitonin stimulation and recovery of isoenzyme II activity were determined as described, and is shown in (a), (b) and (c). In (d), fresh calcitonin (10^{-10} M) was added to cells previously exposed to calcitonin for 4 h (i.e. the equivalent of c); in (e), conditioned medium from cells incubated for 4 h with calcitonin was added to untreated cells. Incubation times for (d) and (e) were 10 min.

post-cell rupture activation of enzyme activity did not contribute significantly to the measured intracellular state of activation. Using two types of control experiment, it is concluded that the measured isoenzyme activation at calcitonin concentrations less than $3 \times 10^{-10} \text{ M}$ reflects intracellular activation. If cell incubations are not diluted sufficiently at time of cell rupture, the problem of post-cell rupture activation occurs at lower hormone concentrations. We have drawn attention to this previously (Livesey *et al.*, 1982). Thus the selectivity of calcitonin stimulation of isoenzyme II of cyclic AMP-dependent protein kinase in T 47D cells is confirmed in T 47D cells and demonstrated in the MCF 7 line. It is a sensitive and dose-dependent response, and the lack of responsiveness of isoenzyme I cannot be explained by any kinetic abnormality of that isoenzyme, nor is it likely that re-association of the isoenzyme I occurs under experimental conditions.

These results and others (Podesta *et al.*, 1978; Byus *et al.*, 1977; Hunzicker Dunn, 1981) lead to the conclusion that the pattern of isoenzyme activation in response to hormonal stimulation of cyclic AMP formation in the intact cell is a function both of hormone and of cell type. It is of particular note that in the present experiments the only known alternative agonist of adenylate cyclase in T 47D breast cancer cells, prostaglandin E_2 , stimulated both isoenzymes I and II of cyclic AMP-dependent protein kinase. In cultured rat osteoblasts we found previously that prostaglandin E_2 exclusively activated isoenzyme II, whereas parathyroid hormone stimulated both I and II. Thus selectivity of isoenzyme activation is cell- as well as hormone-specific. It is not clear how hormones can elevate cellular cyclic AMP levels but nevertheless have a selective action upon the isoenzymes of cyclic AMP-dependent protein kinase. The molecular basis of this process is being investigated, and the different patterns of response to calcitonin and prostaglandin E_2 might prove a useful model in such studies.

MCF 7 and T 47D breast cancer cells are very similar in their calcitonin responsive properties, including the characteristics of the calcitonin receptor in each cell line (Findlay *et al.*, 1980), and of the kinetics of calcitonin-responsive adenylate cyclase (Findlay *et al.*, 1981). The cyclic AMP-dependent protein kinase isoenzyme response in the two cells is similar in its selectivity, but the kinetics differ. T 47D cells show persistent activation of isoenzyme II for at least 24 h, whereas in the MCF 7 cells the response is transient over 4–6 h, by which time the activity returns to control levels and the cells become resistant to re-stimulation of the isoenzyme by addition of further calcitonin. Our experiments showed that this

difference was not due to differences in the rate of calcitonin degradation in incubation media, and it seems likely that the explanation involves a post-receptor event. The differences in persistence of activation of isoenzyme II might be reflected in different later responses in the cell. Hormones which activate cyclic AMP are known to have both immediate metabolic effects (Khoo *et al.*, 1978) and delayed actions at the level of gene transcription (Firestone & Heath, 1981). Autophosphorylation of the type II regulatory subunit of the cyclic AMP-dependent protein kinase is known to inhibit reassociation of the isoenzyme (Erlichman *et al.*, 1974) and may be important in the nuclear translocation of the isoenzyme (Cho Chung *et al.*, 1981). It will be of interest to learn if the persistence of the isoenzyme activation is a further component of the hormone signal relevant to these responses.

In view of the postulated different functions of the cyclic AMP-dependent protein kinase isoenzymes and the diverse range of cellular events modulated by the nucleotide, selective activation of the kinase isoenzymes may represent the first step in diversification of the effector-specific cyclic AMP signal.

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