Can intracellular signalling pathways predict developmental abnormalities?

Sensitivity of the adenylyl cyclase/c-*fos* protooncogene cascade to β-adrenergic agonists and glucocorticoids in foetal rat

T. A. Slotkin, C. Lau, S. E. Lappi and F. J. Seidler

We examined whether measurements of adenylyl cyclase and its control of c-*fos* protooncogene mRNA expression in mid-gestation foetal rat could be used to detect developmental effects of apparently unrelated compounds: terbutaline, a β-adrenergic receptor stimulant, and dexamethasone, a glucocorticoid hormone. On gestational day 14, acute administration of terbutaline to pregnant rats resulted in six-fold induction of c-*fos* mRNA within 1 h; the same increase was obtained when a membrane-permeable analogue of cAMP was given. Treatment with dexamethasone on gestational days 11, 12 and 13 produced the same increase in c-*fos* mRNA on gestational day 14 as had been seen with acute terbutaline or cAMP; no further increase could be obtained with acute cAMP treatment in the dexamethasone-pretreated animals. Adenylyl cyclase activity was evaluated on gestational day 14. Animals treated with dexamethasone showed a 50% enhancement of basal enzyme activity that reflected a parallel increase in total catalytic activity as measured with forskolin–Mn^{2+}. Dexamethasone also increased adenylyl cyclase activity in the presence of a β-agonist but to a lesser extent than the increase in total catalytic activity. These results indicate that cell signalling pathways mediating the expression of the genes that control cell differentiation are a likely target for structurally and mechanistically unrelated drugs and chemicals and may therefore be useful as early biomarkers of abnormal development.

Keywords: *adenylyl cyclase, β-adrenergic receptors, c-fos, dexamethasone, terbutaline, foetal development.*

Abbreviations: 8-Br-cAMP, 8-Bromo-cAMP; ANOVA, analysis of variance; cAMP, cyclic adenosine 3′:5′-monophosphate; SDS, sodium dodecylsulphate.

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Introduction

A major goal in developmental toxicology and teratology is to establish early detection of the sequence of events that will eventually produce adverse effects on cell structure and function. Among the limitations in connecting the immediate insult to a developing cell and the eventual outcome in the intact organism, is the fact that outright teratogenesis and lethality are an extreme of the spectrum of possible developmental perturbations, often observed only with very high doses of exposure. In addition, malformations may not be uniform even within a litter and functional impairment, rather than morphologic change, may represent the true liability. A strategy to approach this problem is to focus on biomarkers of perturbed functional and structural development that may be applicable to a wide variety of developmental insults. Indeed, the observation that various classes of known toxicants with little resemblance in structure, physical and chemical properties, and modes of action can all produce similar developmental perturbations, supports the idea that there may be a set of common mechanistic links to connect the immediate cellular insult during a critical phase of development with an eventual adverse structural/functional outcome.

One way to achieve this is to focus on the intracellular responses, typically involving second and third messenger signalling cascades, that are potentially common to all such primary perturbations, that could lead to alterations in cell replication and differentiation and ultimately to aberrant structure and function. The best candidate for an intracellular cascade, essential to normal cell development and a likely target for disparate types of developmental toxicants, is that involving cAMP, a second messenger that is obligatory in regulation of cell proliferation and differentiation in organisms ranging from bacteria to mammalian cells (Guidotti 1972, Van Wijk *et al.* 1973, Claycomb 1976, Bhat *et al.* 1983, Hultgårdh-Nilsson *et al.* 1994). The underlying difficulty is that cAMP is involved in functions other than the control of cell proliferation and differentiation; accordingly, it is necessary to examine the events downstream from cAMP that specifically connect this second messenger to developmental processes as distinct from general metabolic events.

In the current study, we took advantage of the fact that initiation of differentiation across a wide variety of cells involves a common pathway mediated through the family of 'early immediate genes', typified by c-*fos* (Hesketh and Whitelaw 1992, Matiuck and Swain 1992, Gatherer 1993, Yamada *et al.* 1994). Proteins expressed by these genes are nuclear transcription factors that, in turn increase the expression of enzymes that participate in the differentiation process, including ornithine decarboxylase, whose activity is known to be an obligatory regulator of cell differentiation (Slotkin 1979, Heby and Emanuelsson 1981, Galliani *et al.* 1983, Slotkin and Bartolome 1986, Lau and Kavlock 1994). In the case of c-*fos*, the formation of its protein product, Fos, leads to the activation of the promoter sequence, AP-1, that is found in the regulatory region of the ODC gene and other genes related to cell proliferation and differentiation. We have recently shown that, during critical periods of cell

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development, c-*fos* is controlled through receptors, such as β-adrenergic receptors, that initiate the cAMP cascade (Wagner *et al.* 1991, 1994a, b, 1995, Slotkin *et al.* 1995). Thus, developmental toxicants acting either directly on adenylyl cyclase, on the G-proteins that regulate the coupling of receptors to cyclase activity, or on the receptors that act through the cyclase pathway, will all have convergent intermediary mechanisms, acting eventually on the cascade from cAMP to c-*fos* to ODC to cell replication and differentiation.

Accordingly, we have addressed this question by examining the response of c-*fos* mRNA expression to β-adrenergic stimulation in the mid-gestation rat embryo, using terbutaline, a β-agonist known to cross the placenta and to produce widespread structural and functional alterations (Bergman *et al.* 1984, Hou and Slotkin 1989, Kudlacz *et al.* 1989, Slotkin *et al.* 1989, 1990a, Fletcher *et al.* 1991, Bey *et al.* 1992, Lenselink *et al.* 1994). During this developmental period the embryo is transiently enriched in β-adrenergic receptors that are distributed ubiquitously and that are linked to adenylyl cyclase (Slotkin *et al.* 1994a). The effects of terbutaline have been compared to those of 8-Br-cAMP, a membrane-permeable cAMP analogue. In addition, we have explored the potential for interaction of terbutaline with dexamethasone, a synthetic glucocorticoid. Foetal exposure to glucocorticoids produces overexpression of adenylyl cyclase (Slotkin *et al.* 1994a), and thus although these compounds are otherwise unrelated, their convergence on the cAMP signalling pathway could lead to similar effects on differentiation markers.

METHODS

Animals and treatments

Timed pregnant Sprague–Dawley rats (Charles River, Raleigh, NC) were shipped by climate-controlled truck and housed individually in breeding cages with free access to food and water. Each determination represents embryos from a single dam; because of the small amounts of tissue, approximately half the embryos from a given dam were pooled to make a single determination.

Beginning on gestational day 11, animals received daily s.c. injections of either 0.2 mg kg^{-1} of dexamethasone phosphate or an equivalent volume (1 ml kg^{-1}) of isotonic saline; injections were repeated on days 12 and 13. On gestational day 14, acute stimulation of c-*fos* was evaluated by administering to the dams 10 mg kg^{-1} s.c. of terbutaline sulphate, a β-adrenergic agonist that readily passes the placental barrier (Bergman *et al.* 1984), to elicit induction of genes involved in cell differentiation (Morris and Slotkin 1985, Kudlacz *et al.* 1990a, b); additional animals were given either 18 mg kg^{-1} s.c. of the membrane-permeable cAMP analogue, 8-Br-cAMP, or equivalent volumes (1 ml kg^{-1}) of vehicle. One hour later, animals were decapitated and the foetuses rapidly dissected, immediately frozen on dry ice, and stored at –45 °C until analysed. The dose and duration of dexamethasone treatment used here have been shown previously to cause maximal promotion of adenylyl cyclase activity in the mid-gestation embryo (Slotkin *et al.* 1994a). Similarly, the doses of terbutaline and 8-Br-cAMP elicit robust stimulation of c-*fos* and other cAMP-dependent signals in developing rat tissues (Lau and Slotkin 1979, Kudlacz *et al.* 1990a, Pracyk and Slotkin 1991, Slotkin *et al.* 1995); stimulation of c-*fos* expression is optimal at 1 h post-treatment (Slotkin *et al.* 1995).

The effects of dexamethasone pretreatment on adenylyl cyclase activity were also determined in an additional cohort. Embryos were harvested and frozen on gestational day 14 without the acute terbutaline or 8-Br-cAMP treatment.

RNA isolation and quantitation

Total cellular RNA was isolated using the guanidinium thiocyanate–CsCl method (Chirgwin *et al.* 1979). In brief, tissues were homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in at least five volumes of 4 M guanidine thiocyanate, 100 mM Tris–HCl, 1% β-mercaptoethanol, 0.5% Antifoam-A (pH 7.5). Sodium lauryl sarcosine was then added to a final concentration of 0.5% and the homogenate was layered onto a cushion of 5.7 M CsCl, 10 mM EDTA (pH 7.5) and sedimented at 260000 × *g* for 4 h. The resultant pellet was resuspended in 1X TES (10 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.5), precipitated in 95% ethanol, and the RNA stored at –80 °C until use.

As described previously (Wagner *et al.* 1994a), the c-*fos* transcript was detected using the 2.1 kb EcoRI pcfos(rat)-1 cDNA insert (Curran 1987), labelled with a random-primer extension reaction using α-[^{32}P]dCTP, with resultant specific activity of 1–2 × 10^9 DPM μg^{-1}. The specific amount of c-*fos* mRNA was quantitated using both Northern blot and slot blot techniques. Northern blotting was performed by loading approximately 20 μg of total cellular RNA, dissolved in a buffer consisting of 20% formaldehyde, 20 mM 3-(*N*-morpholino)propane sulphonic acid, 50 mM sodium acetate, 10 mM EDTA, and 3 μg of ethidium bromide, onto a 1.2% agarose gel. The samples were then subjected to electrophoresis for 16 h at 20 V. The resultant gel was photographed under ultraviolet illumination and transferred to a high-performance nylon membrane (Hybond-N) by capillary blotting. RNA was then linked to the membrane by exposure to high frequency ultraviolet light for 5 min.

For slot blotting, an aliquot of the ethanol precipitate was lyophilized and resuspended in a denaturing solution consisting of 500 μl of 20X SSC (3 M sodium chloride, 0.3 M sodium citrate), 300 μl of 1X TE (10 mM Tris, 1 mM EDTA, pH 7.5), and 200 μl of 37% formaldehyde. A range of total cellular RNA of up to 256-fold was vacuum-blotted and linked onto a nylon membrane (Hybond-N) as already described.

Blots were prehybridized for 30 min at 65 °C in a commercially available buffer (RapidHyb). After prehybridization, 2 ng ml^{-1} of labelled probe was added to the reaction, and the blots were hybridized for 2.5 h at the same temperature. After hybridization, blots were first washed in 2X SSC, 0.1% SDS for 15 min at room temperature, then washed for an additional 15 min in 0.2X SSC, 0.1% SDS at 65 °C. Membranes were exposed to film (Hyperfilm) for 1–4 days at –80 °C with an intensifying screen, or were placed on phosphorimaging plates. After development, the radiograms were analysed using a Howtek Scanmaster 3 and NIH Image software (version 1.44; Public Domain, available on the Internet by anonymous FTP at zippy.nimh.gov); phosphorimages were analysed using a Molecular Dynamics Phosphorimager (Sunnyvale, CA) and ImageQuant 3.3 software. The amount of c-*fos* mRNA in samples run on Northern blots was estimated by measuring the density of the single 2.2 kb band appearing on the autoradiogram. To correct for differences in potential RNA load applied to each lane, the amount of RNA was determined from the density of ethidium bromide staining of the 28S band of ribosomal RNA. In addition, results were taken only from undegraded samples having a ratio of 28S:18S ribosomal RNA staining of at least 2.

For slot blots, the amount of c-*fos* mRNA in the experimental sample was determined by comparing the density of the sample on the radiogram to a standard curve of densities produced by blotting serial dilutions of predetermined amounts of unlabelled authentic c-*fos* onto the same membrane as the experimental samples. Using the standards to determine the relationship between amount of c-*fos* and density of the exposed film, the actual amount of c-*fos* mRNA present in the experimental samples could then be determined. This value was standardized to the amount of RNA actually present on the blot by reading the

absorbance of the denatured RNA solutions at 260 nm, which agreed with loads estimated from ethidium bromide staining of the same samples run on Northern blots; the purity of each sample also was assessed by the ratio of absorbance at 260 nm to absorbance at 280 nm, which was always above 1.8. Multiple exposures were performed to bring the density of the experimental spots to within the linear range of the standards. As a final check on the densitometric procedure, several slot blots were cut apart and the radioactivity determined directly by liquid scintillation spectrometry. Under these conditions, the two standardization procedures were in agreement with each other, as were results obtained with either blotting technique (Northern blots vs slot blots). A detailed description of the procedure for slot blot quantitation, including the methods of internal standardization and verification of optimal hybridization efficiency, has appeared previously (Slotkin *et al.* 1995).

Membrane preparation and adenylyl cyclase activity

These were carried out essentially as described previously (Slotkin et al. 1994a). Tissues were thawed and homogenized (Polytron) in at least 39 volumes of ice-cold buffer containing 145 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris (pH 7.5) and sedimented at 40000 × g for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer followed by resedimentation. The final pellet was dispersed with a Teflon-to-smooth-glass homogenizer in a buffer consisting of 250 mM sucrose, 10 mM Tris (pH 7.4), 1 mM EGTA, to achieve a final protein concentration of approximately 0.8–1.6 mg ml^{-1} (Lowry *et al.* 1951). Aliquots of membrane preparation containing 40–80 µg of protein were incubated for 30 min at 30 °C with final concentrations of 100 mM Tris HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5'-triphosphate, 10 mM $MgCl_2$, 1 mg bovine serum albumin and a creatine phosphokinase–ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU phosphocreatine kinase and 10 µM GTP, in a total volume of 250 µl. The enzymatic reaction was stopped by placing the samples in a 90–100 °C water bath for 5 min, followed by sedimentation at 3000 × g for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits. Preliminary experiments showed that the enzymatic reaction was linear well beyond the 30 min time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and in particular, addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 100 µM forskolin + 10 mM $MnCl_2$ (Seamon and Daly 1981, Chaudhry and Granneman 1991), in the presence of GTP; β-adrenergic stimulation of activity was determined with 100 µM isoproterenol in the presence of GTP. The concentrations of all the agents used here have been found previously to be maximal for stimulating adenylyl cyclase (Slotkin *et al.* 1990b, Chaudhry and Granneman 1991, Navarro *et al.* 1991).

Data analysis

Data are presented as means and standard errors. Differences among groups were evaluated by ANOVA (data log-transformed whenever variance was heterogeneous), with individual comparisons to the saline control group determined *post-hoc* with Fisher's Protected Least Significant Difference.

Materials

The cDNA probe used in these studies was synthesized commercially (BioServe Laboratories, MD). α-[^{32}P]dCTP, Hybond-N nylon membranes, RapidHyb buffer, Hyperfilm, MegaPrime labelling kits and cAMP radioimmunoassay kits were obtained from Amersham Products (Chicago, IL). All other compounds were obtained from Sigma Chemical Co. (St Louis, MO).

Results

At 14 days of gestation, basal expression of c-*fos* mRNA (animals receiving acute saline treatment) was low as quantitated on slot blots (about 50 fmol mg^{-1} total RNA), but acute treatment with terbutaline or 8-Br-cAMP produced a robust, six-fold stimulation (Figure 1). Pretreatment with dexamethasone for the 3 days preceding the experiment also raised c-*fos* expression levels (five-fold); after dexamethasone pretreatment, acute challenge with 8-Br-cAMP failed to stimulate further.

These results were confirmed by examination of Northern blots; representative lanes are shown in Figure 2. The cDNA probe localized to a single band at the 2.2 kilobase locus of the gel. The band was barely detectable in saline-treated animals but was much darker in animals challenged acutely with terbutaline or 8-Br-cAMP, in animals pretreated with dexamethasone, and in animals pretreated with dexamethasone and challenged acutely with 8-Br-cAMP. Ethidium bromide staining of the gel confirmed the purity and load of each lane.

To explore the potential mechanism for stimulation of c-*fos* by dexamethasone pretreatment and the inability of 8-BR-cAMP to cause further stimulation in the dexamethasone-pretreated animals, we examined effects of the pretreatment on adenylyl cyclase activity (Table 1). Dexamethasone increased basal enzyme activity by about 50%; the increase could be completely accounted for by a change in total enzymatic activity, as the same increase (46%) was obtained when

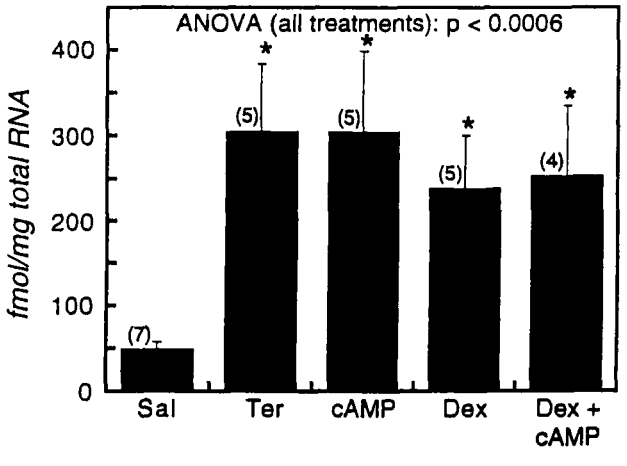

Figure 1. Slot-blot quantitation of c-*fos* mRNA in whole embryos on gestational day 14. Abbreviations: Sal = animals pretreated with saline on gestational days 11, 12 and 13, and challenged acutely with saline on gestational day 14; Ter = animals pretreated with saline on gestational days 11, 12 and 13, and challenged acutely with terbutaline on gestational day 14; cAMP = animals pretreated with saline on gestational days 11, 12 and 13, and challenged acutely with 8-Br-cAMP on gestational day 14; Dex = animals pretreated with dexamethasone on gestational days 11, 12 and 13, and challenged acutely with saline on gestational day 14; Dex + cAMP = animals pretreated with dexamethasone on gestational days 11, 12 and 13, and challenged acutely with 8-Br-cAMP on gestational day 14. Data represent means and standard errors obtained from the number of determinations in parentheses. Asterisks denote values that are individually distinguishable from the saline control group.

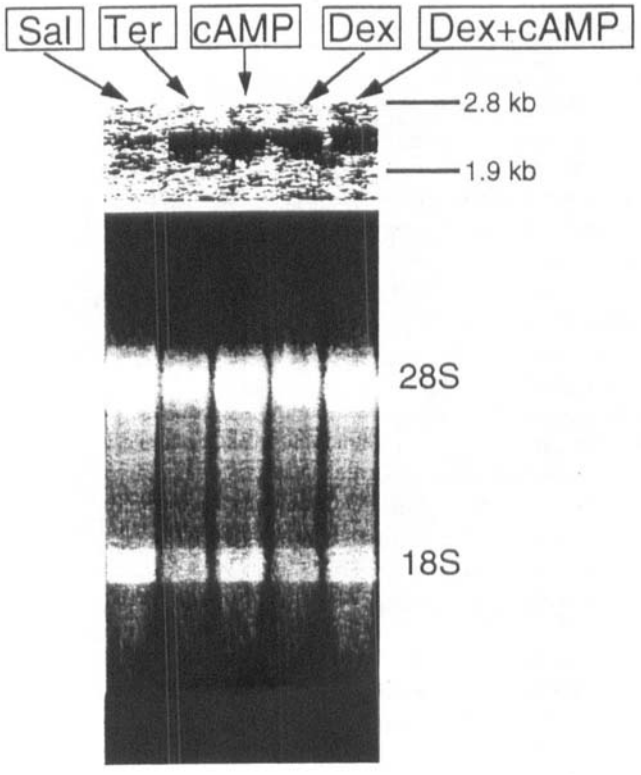

Figure 2. Representative Northern blot analysis of c-*fos* mRNA expression in whole embryos on gestational day 14. Abbreviations are the same as in Figure 1. Phosphorimage of the c-fos cDNA probe in the region corresponding to c-*fos* (2.2 kb) is shown at the top and ethidium bromide staining of the entire gel is on the bottom. Location of DNA ladder standards closest to the c-*fos* band are indicated, as are the 28S and 18S ribosomal RNA bands.

measuring activity in the presence of forskolin–Mn^{2+}. Activity stimulated by the β-adrenergic agonist, isoproterenol, was also elevated in the dexamethasone-pretreated group but the effect was significantly less than that on basal activity. Thus, the percentage of stimulation over basal activity achieved with isoproterenol was over 30% in the control group, but half that value in the dexamethasone–pretreated animals. These results are in agreement with earlier findings for effects of glucocorticoids on adenylyl cyclase activity (Slotkin *et al.* 1994a).

Discussion

Catecholamine neurotransmitters have long been hypothesized to play a role in the differentiation of their target cells (Vernadakis and Gibson 1974, Claycomb 1976, Lauder and Krebs 1978, Slotkin *et al.* 1987, 1988, Navarro *et al.* 1989). If these relationships hold true in embryonic development, we are faced with an apparent paradox because the appearance of functional synaptic connections is a relatively late event in nervous system development (Coyle and Axelrod 1971, Reinis and Goldman 1980, Slotkin 1986). Three conditions would have to be met for neurotrophic relationships to operate prior to the onset of synaptogenesis: cells would have to be able to make norepinephrine, adjacent cells would have to possess the appropriate adrenergic receptors, and the receptors would have to be coupled to a signalling cascade that initiates changes in the expression of genes controlling differentiation. Earlier work has shown that numerous cells transiently express the ability to manufacture norepinephrine and then differentiate to non-adrenergic phenotypes or, as with extra-adrenal chromaffin tissue, disappear altogether (Cochard *et al.* 1978, Jonakait *et al.* 1979, Coupland 1980). We have recently shown that β-adrenergic receptors are transiently overexpressed during mid-gestation, reaching concentrations higher than those seen in most adrenergically innervated tissues in the adult (Slotkin *et al.* 1994b); the receptors are effectively coupled to adenylyl cyclase and thus if stimulated, can generate the cAMP necessary to elicit changes in gene expression. The current study completes the demonstration that all three conditions are met in the embryo by showing that the receptors are linked, through generation of cAMP, to induction of the protooncogene c-*fos*, a nuclear transcription factor that has been shown to control expression of gene products known to affect differentiation.

The presence of an adrenergic receptor linked to cell differentiation also implies that these receptors are likely targets for abnormalities of development caused by β-adrenergic agonists such as terbutaline. Indeed, terbutaline was able to elicit as big a stimulation of c-*fos* expression as was cAMP itself, implying that activating the β-receptor can actually drive this endpoint of the signalling cascade maximally. Previous work has shown that the limiting factor in transducing the β-adrenergic signal into the induction of c-*fos*

Measure	Control (fmol min^{-1} mg^{-1} tissue)	Dexamethasone (fmol min^{-1} mg^{-1} tissue)	Significance
Basal activity	135 ± 13	206 ± 10	$p < 0.0006$
Forskolin–Mn^{2+}-stimulated activity	2306 ± 249	3370 ± 158	$p < 0.003$
Isoproterenol-stimulated activity	175 ± 17	237 ± 9	$p < 0.007$
Percent stimulation over basal	32 ± 6	16 ± 2	$p < 0.03$

Table 1. Effects of dexamethasone pretreatment on adenylyl cyclase activity.
Data represent means and standard errors of nine determinations in each group. Significance values compare control and dexamethasone groups for each variable. Membrane protein recovery was unchanged by dexamethasone: control, 15 ± 1 mg^{-1} g tissue; dexamethasone 13 ± 1 (NS).

mRNA lies in the steps in between cAMP generation and activation of gene expression, probably involving protein kinase A and the cAMP Response Element Binding Protein (Boutillier *et al.* 1992, Ginty *et al.* 1994, Wagner *et al.* 1995); our findings are thus consonant with the idea of a ceiling limitation on the stimulation of c-*fos* by cAMP, whether cAMP is given directly or produced inside the cell by β-adrenergic stimulation. In any case, the ability of terbutaline to elicit maximal changes in c-*fos* have important implications in evaluating the potential critical period for terbutaline-induced abnormalities. Whereas many studies with this drug have concentrated on late gestational exposure and specific actions at known adrenergic target tissues such as heart, lung, kidney and brain regions enriched in noradrenergic projections (Bergman *et al.* 1984, Hou and Slotkin 1989, Kudlacz *et al.* 1989, Slotkin *et al.* 1989, 1990a, Fletcher *et al.* 1991, Bey *et al.* 1992, Lenselink *et al.* 1994), our results predict that terbutaline may cause more widespread disruption of cell development when administered in mid-gestation during the ubiquitous expression of β-receptors. Because the events downstream from c-*fos* have not yet been elucidated in their entirety, it is not suitable to project whether the alterations will be expressed as structural or functional changes but certainly this supposition needs to be examined further. A suitable starting point would be to show whether enhanced c-*fos* transcription produces increased expression of target genes that are known to control differentiation and to determine whether the changes are ubiquitous or are targeted to specific embryonic tissues. In older embryos or neonates, where β-receptors are more selectively distributed, and where limitations in the amount of tissue available for analysis are not an issue as they are here, targeted receptor-mediated induction of enzymes linked to c-*fos* expression has already been demonstrated (Morris and Slotkin 1985, Kudlacz *et al.* 1990c, Pracyk *et al.* 1991, Wagner *et al.* 1991, 1994a, Slotkin *et al.* 1995).

Elucidating the cAMP-c-*fos* link in developing cells also has specific importance in understanding how apparently unrelated compounds can result in the same eventual morphological or functional alteration. In the current study, we found that pretreatment of mid-gestation embryos with dexamethasone for 3 days elevated c-*fos* expression to the same extent as did acute cAMP or terbutaline treatment. Although dexamethasone could conceivably influence gene expression through a multitude of mechanisms, our data suggest a specific relationship to cAMP in that the animals pretreated with dexamethasone showed no further increase when cAMP was given acutely. Thus, in raising the c-*fos* expression level to that achieved with acute stimulants, dexamethasone achieved the same ceiling effect that was seen with terbutaline or cAMP. A definitive test of a mechanistic connection between the glucocorticoid and β-adrenergic stimulation was provided by measurement of adenylyl cyclase activity. Dexamethasone pretreatment stimulated adenylyl cyclase by 50%, an effect mediated at the level of total catalytic activity as demonstrated by parallel changes in basal and forskolin–Mn^{2+}-stimulated activity; by implication, the glucocorticoid either evokes an increase in expression of the catalytic subunit (more enzyme protein) or switches the cell to production of a different isoform with higher catalytic efficiency. By acting at a point distal to the β-receptor linkage to adenylyl cyclase, dexamethasone activates the same cascade, resulting in comparable changes in c-*fos* gene expression. Although effects on adenylyl cyclase were measured *ex vivo* (*in vivo* treatment of animals, *in vitro* enzyme assay), the results clearly imply a cAMP-mediated effect in the intact tissue, as evidenced by stimulation of c-*fos*; in older animals, we have also shown that elevated adenylyl cyclase activity results in increased expression of other cAMP-linked gene products (Morris and Slotkin 1985, Kudlacz *et al.* 1990c, Pracyk *et al.* 1991, Wagner *et al.* 1991, 1994a, Slotkin *et al.* 1995).

Accordingly, we propose the following model to demonstrate, in this case, how unrelated compounds can elicit the same cell signalling endpoint that in turn affects cell differentiation (Figure 3). Terbutaline stimulates the β-adrenergic receptors that are known to be present in mid-gestation and are linked through G-proteins to the activation of adenylyl cyclase. From that point, generation of cAMP leads through protein kinase A and the cAMP Response Element Binding Protein to transcription of c-*fos* mRNA and production of Fos protein. After Fos forms heterodimers with Jun, they activate the transcription of genes possessing the AP-1 promoter sequence, including ornithine decarboxylase

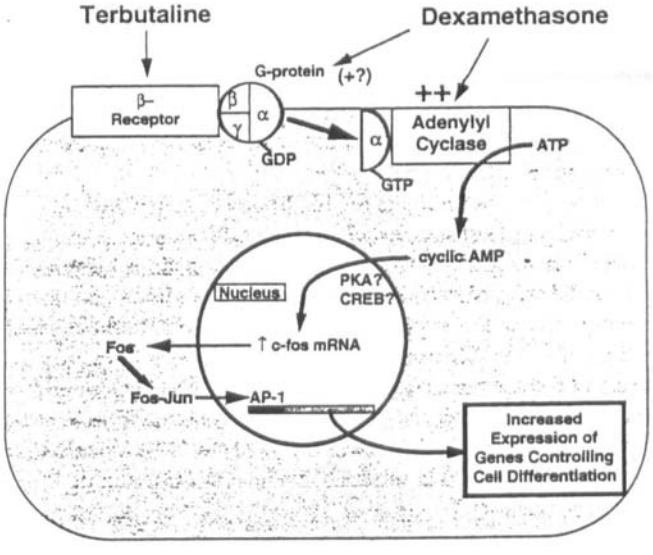

Figure 3. Schematic representation of the convergent actions of terbutaline and dexamethasone on the cAMP-c-*fos* signalling pathway in the mid-gestation embryo. Stimulation of the β-receptor by terbutaline leads to replacement of GDP with GTP on the G-protein, G_s, followed by dissociation of the βγ-subunit from the α-subunit. The α-subunit-GTP interaction with adenylyl cyclase leads to cAMP formation, which through the likely participation of protein kinase A (PKA) and the cAMP Response Element Binding Protein (CREB), leads to increased transcription of the c-*fos* gene. Production of Fos protein leads to formation of Fos-Jun heterodimers which activate the transcription of genes containing the AP-1 promoter sequence, including ornithine decarboxylase and numerous other genes involved in cell differentiation. Dexamethasone achieves the same final result, but only after repeated administration increases the expression or catalytic activity of adenylyl cyclase. Smaller effects of dexamethasone on G-protein expression have also been postulated.

and other genes involved in cell differentiation (Chiu *et al.* 1988, Vendrell *et al.* 1991, Matiuck and Swain 1992, Chien *et al.* 1993, Gatherer 1993, Wrighton and Busslinger 1993, Collaço-Moraes and De Belleroche 1994, Miao and Curran 1994, Wagner *et al.* 1994a, b, 1995, Yamada *et al.* 1994). Dexamethasone produces the same net effect but by a different action on the signalling pathway, requiring repeated administration to upregulate adenylyl cyclase expression and hence to achieve an elevation of cAMP. Effects of dexamethasone on G-protein expression have also been postulated but are likely to be of smaller magnitude than is the massive effect on cyclase activity itself (Saito *et al.* 1989, McLellan *et al.* 1992, Kawai and Arinze 1993, Slotkin *et al.* 1994a). In either case, it is important to note that the effects of dexamethasone to promote functioning of this signalling cascade are unique to development; in mature tissues, glucocorticoids tend to reduce the expression of c-*fos* and AP-1-dependent genes (Kerppola *et al.* 1993, Collaço-Moraes and De Belleroche 1994, Heck *et al.* 1994, Unlap and Jope 1994). This scheme does not incorporate other mechanisms by which terbutaline or dexamethasone can perturb development, including but not limited to effects on protein synthesis and degradation, expression of differentiation genes other than those regulated by the AP-1 site or even generalized cell toxicity. In fact, because cAMP is obligatory to cell differentiation (Guidotti 1972, Van Wijk *et al.* 1973, Claycomb 1976, Bhat *et al.* 1983, Hultgårdh-Nilsson *et al.* 1994) this cascade should ultimately be affected regardless of whether a developmental toxicant acts directly upon its components. We therefore suggest that evaluation of the cascade components in the adenylyl cyclase/c-*fos* signalling pathway will serve to detect developmental toxicity of a wide variety of apparently unrelated insults.

Our results also indicate the likelihood of sensitization to a wide variety of substances when corticosteroids are present, either from exogenous sources or from endogenous hormones released during maternal stress, such as when teratogen testing is conducted at doses evoking maternal distress. In the current setting, the dose of dexamethasone chosen was sufficiently high to produce maximal activation of adenylyl cyclase and maximal stimulation of c-*fos* expression. Sensitization to other agents is likely to involve lower levels of steroid exposure than those used here, which produce maximal stimulation of adenylyl cyclase (Slotkin *et al.* 1994a). A further limitation may be imposed by the level of β-receptor expression and its reactivity to glucocorticoids. In the mid-gestation embryo, dexamethasone does not induce the formation of β-receptors (Slotkin *et al.* 1994a) and the ability of β-agonists to stimulate the cAMP-c-*fos* cascade may be limited under these circumstances. In the current study, we found that the ability of isoproterenol to stimulate cAMP production *in vitro* was enhanced much less than was cyclase catalytic activity, implying a limitation in the ability of receptor stimulation to elicit increased enzyme function. In contrast, later in development, dexamethasone turns on both cyclase catalytic activity and β-receptor formation and a synergistic interaction is much more likely to occur under those circumstances. The ability of glucocorticoids to lower the threshold for effects of disparate compounds that converge on the adenylyl cyclase signalling pathway is thus a crucial area for future study.

Finally, our results have implications for future directions in the establishment of cell signalling cascades as biomarkers for abnormal development. It is highly improbable that the cAMP-c-*fos* pathway is the only intracellular mechanism involved in transduction of stimuli controlling cell differentiation; signals operating through calcium, inositol phosphates, tyrosine kinases, etc., are all likely candidates for similar convergent actions of disparate drugs and chemicals, and represent fruitful areas of pursuit. The understanding of the intracellular signals operating in normal and abnormal cell differentiation, and particularly the identification of the critical periods in which different cell populations exhibit coupling of signalling intermediates to expression of the genes operating the 'master control points' of differentiation, may provide the framework for early detection of developmental toxicity at the molecular level.

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