Endocrine (1995) 3, 159-167

Effects of phorbol ester and staurosporine on the actions of insulin-like growth factor-I on rat ovarian granulosa cells

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Insulin-like growth factor I (IGF-I) is able to stimulate ovarian granulosa cell steroidogenesis induced by gonadotopins. This gonadotropin-induced potentiation of IGF-I action appears to be due, at least in part, to a gonadotropin-induced increase in membrane-bound IGF-I receptor number and/or decrease in extracellular IGF binding proteins (IGFBPs). Protein kinase C (PKC) has recently been reported to inhibit gonadotropin-induced steroidogenesis in rat ovarian granulosa cells. The role of PKC in the effects of IGF-I on gonadotropin action, however, is unknown. In this study, the effects of phorbol 12-myristate 13-acetate (PMA, a PKC activator) and staurosporine (ST, a PKC inhibitor) on IGF-I action were studied using immature rat ovarian granulosa cells. Activation of PKC by PMA did not affect steroidogenesis or cAMP secretion in cells treated with or without IGF-I. On the other hand, inhibition of PKC by ST alone (10^{-9}–10^{-7} M) led to an increase in progesterone production in a dose- and time-dependent manner without affecting cAMP secretion. In the presence, but not absence, of ST, IGF-I was able to stimulate progesterone production in the absence of any gonadotropin. PMA decreased ST-induced steroidogenesis and essentially abolished ST-potentiated IGF-I stimulation of steroidogenesis, suggesting the effects of ST on IGF-I action involved a PKC-dependent mechanism. Unlike gonadotropin, ST did not change IGF-I receptor binding. However, ST significantly decreased a major IGF binding protein (IGFBP, ~30 kDa) which is likely to be IGFBP-5, whereas it increased a minor IGFBP (~24 kDa) which is likely to be IGFBP-4. Both effects of ST were dose- and time-dependent. Furthermore, ST inhibited the expression of mRNA for IGFBP-5 suggesting that ST decreased IGFBP-5 levels by inhibiting its transcription and/or decreasing the stability of its mRNA. Interestingly, ST also decreased mRNA levels of IGFBP-4 despite a significant increase in secreted IGFBP-4 levels. The mechanisms involved are not known. Activation of PKC by PMA had no acute effect on these IGFBPs. The regulation by ST of IGFBPs was not antagonized by PMA, and was not affected by PKC-down regulation. Thus, it is likely that ST induces granulosa cell steroidogenesis, potentiates the IGF-I stimulation of steroidogenesis and regulates IGFBP via both PKC-dependent and -independent pathways.

Keywords: insulin-like growth factor-I; protein kinase C; steroidogenesis

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Received 5 July 1994; accepted 4 November, 1994

Introduction

Insulin-like growth factor I (IGF-I) is able to amplify ovarian granulosa cell differentiation induced by gonadotropins and stimulators of cAMP (Adashi *et al.*, 1985a,b; 1988a). The amplification by IGF-I is associated with either increased granulosa cell plasma membrane type I IGF receptor number (Adashi *et al.*, 1988b), or decreased IGF binding protein (IGFBP) secretion (Adashi *et al.*, 1991; Grimes *et al.*, 1992; Liu *et al.*, 1993). IGF-I appears to have no effect on most differentiated functions of granulosa cells, including steroidogenesis, in the absence of gonadotropin. Recently, we have shown that activation of protein kinase C (PKC) by phorbol esters inhibits gonadotropin-stimulated steroidogenesis in rat granulosa cells and this inhibitory effect was not present in PKC-down regulated cells (He *et al.*, 1995). Our recent data indicate that a PKC-mediated mechanism is involved in gonadotropin-induced granulosa cell differentiation. The possible role of PKC in the effects of IGF-I on gonadotropin action is currently unknown.

The action of IGF-I in other cell types can be modulated by PKC through various signalling pathways. Phorbol esters, activators of PKC, stimulated the phosphorylation of the β subunits of receptors for insulin and IGF-I in IM-9 lymphocytes (Jacobs *et al.*, 1983) but, in stark contrast, have also been reported to inhibit the autophospyhorylation and kinase activity of these receptors in FRTL5 cells (Condorelli *et al.*, 1992). Phorbol esters also differentially regulated the expression of IGFBPs by human endometrial carcinoma cells, an effect which can be blocked by the PKC inhibitor staurosporine (ST) (Gong *et al.*, 1992). These data suggest a possible role for an involvement of PKC in the regulation of insulin and IGF-I action. Given these links between PKC, gonadotropin-induced steroidogenesis and IGF-I, it is possible that PKC has a role in the effects of IGF-I on ovarian granulosa cell differentiation.

In the present study, the effects of phorbol 12-myristate 13-acetate (PMA, a PKC activator) and ST on IGF-I action were studied using an immature rat granulosa cell model (Hsueh *et al.*, 1984; Amsterdam & Rotmensch, 1987). Evidence is presented that ST induces progesterone production in granulosa cells and permits expression of IGF-I action in stimulating steroidogenesis in the absence of gonadotropin. These effects of ST appear to involve a PKC-mediated mechanism. ST also regulates IGFBP secretion; however, this appears to occur via a PKC-independent pathway.

Results

Effects of PMA on IGF-I action in granulosa cell differentiation

We have shown previously that activation of PKC by PMA (10 ng/ml) inhibited gonadotropin-induced progesterone synthesis, aromatase activity and cAMP production by rat granulosa cells in culture (He *et al.*, 1995). In this study, the effects of PMA on granulosa cell progesterone and cAMP secretion have been studiced in the presence of IGF-I. Cells were cultured with PMA (10 ng/ml) with or without IGF-I (30 ng/ml) for 4 h and 24 h. The extracellular progesterone production and cAMP secretion were determined at the end of each culture period as described in Materials and methods. As shown in Figure 1, PMA alone did not significantly affect basal progesterone production (Figure 1a) or cAMP secretion (Figure 1b) after either 4 h or 24 h of culture. IGF-I alone or IGF-I plus PMA had no significant effect on progesterone production or cAMP secretion at either 4 h or 24 h incubation. These data suggest that activation of PKC by PMA does not affect basal granulosa cell steroidogenesis, cAMP secretion or the action of IGF-I.

Effects of ST on IGF-I action in granulosa cell differentiation

Granulosa cells were cultured with different doses of ST (10^{-9}–10^{-7} M) in the absence or presence of IGF-I (30 ng/ml) for 4 h and 24 h. The extracellular progesterone production and cAMP secretion were determined at the end of culture. By 4 h, neither progesterone production nor cAMP secretion were affected by ST, IGF-I alone or ST plus IGF-I (data not shown). However, by 24 h ST alone increased progesterone production (Figure 2a) in a dose-dependent manner without affecting cAMP secretion (Figure 2b). In the presence of ST, IGF-I was able to stimulate progesterone production despite the absence of gonadotropin. The effectiveness of IGF-I increased with increasing ST dose. The maximum IGF-I effect

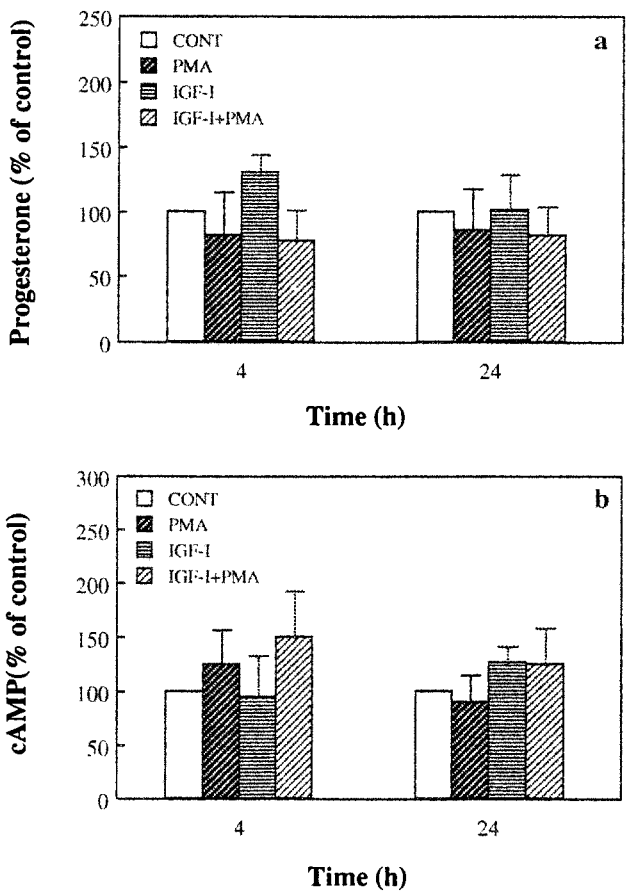

Figure 1 Effects of PMA on IGF-I action on granulosa cell steroidogenesis and cAMP secretion. Immature rat granulosa cells (2×10^5 cells/well) were incubated with PMA (10 ng/ml) in the absence or presence of IGF-I (30 ng/ml) for 4 h and 24 h. Extracellular progesterone production (**a**) and cAMP levels (**b**) were determined at the end of culture as described in Materials and methods. The data were calculated as mean ± SEM for triplicate measurements in three experiments and are expressed as a percentage of control values (100%). The absolute mean values of the controls in the three experiments were: progesterone 0.24 ± 0.05 ng/10^6 cells (4 h) and 0.26 ± 0.11 ng/10^6 cells (24 h); cAMP 0.48 ± 0.15 pmol/10^6 cells (4 h) and 0.98 ± 0.52 pmol/10^6 cells (24 h)

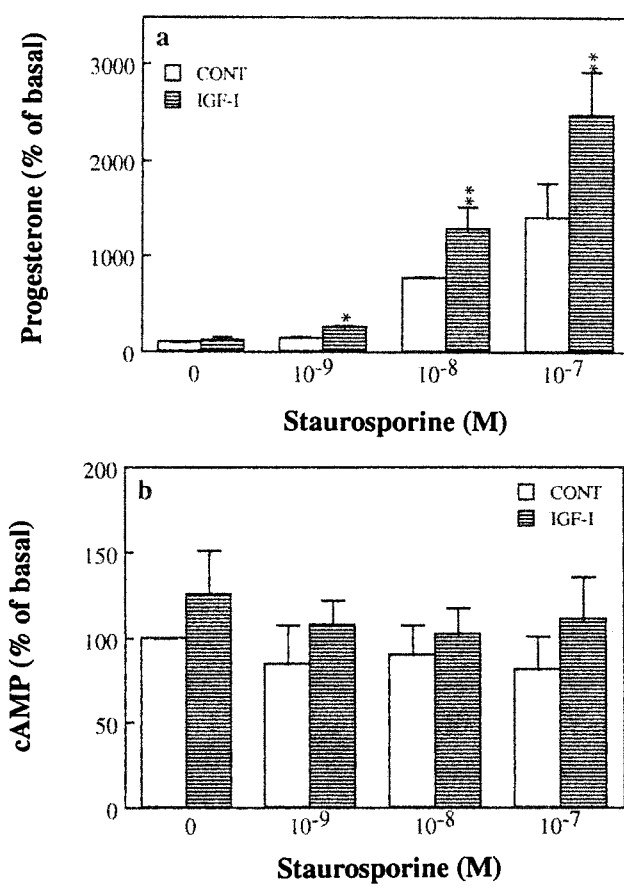

Figure 2 Dose response of the effect of ST on IGF-I action on granulosa cell steroidogenesis and cAMP secretion. Immature rat granulosa cells (2×10^5 cells/well) were incubated with different concentrations of ST (10^{-9}–10^{-7}M) in the absence or presence of IGF-I (30 ng/ml) for 24 h. Extracellular progesterone production (**a**) and cAMP levels (**b**) were assayed at the end of culture as described in Materials and methods. The data were calculated as mean ± SEM for triplicate measurements in two experiments and are expressed as a percentage of control values (100%). The absolute mean values of the controls in the two experiments were: progesterone 0.67 ± 0.16 ng/10^6 cells; cAMP 125 ± 0.56 pmol/10^6 cells. $^*P < 0.05$; $^{**}P < 0.01$; ST *vs* ST plus IGF-I

occurred at 10^{-7} M ST. IGF-I did not affect cAMP secretion in cells treated with or without ST. The time courses of the effects of both ST and IGF-I on progesterone production were also studied by incubating granulosa cells with ST (10^{-7} M) in the presence of IGF-I (30 ng/ml) for 24 h, 48 h and 72 h. As shown in Figure 3, in the presence of ST, progesterone continued to accumulate over the 3 days of culture. By 72 h, ST increased progesterone production by 90-fold. The effect of IGF-I in the presence of ST was maximal at 48 h and then appeared to decrease. These data indicate that in the presence of ST, IGF-I stimulates progesterone production in the absence of gonadotropin.

Regulation of ST-induced steroidogenesis and ST-potentiated IGF-I action by PMA

The effect of PMA on ST-induced steroidogenesis and ST-potentiated IGF-I action on steroidogenesis was determined by culturing granulosa cells for 24 h with different doses of ST (10^{-9}–10^{-7} M) with or without IGF-I (30 ng/ml), in the presence or absence of PMA (10 mg/ml). As shown in Figure 4, PMA decreased ST-induced steroidogenesis and essentially abolished ST-potentiated IGF-I stimulation of steroidogenesis. The antagonism by PMA of ST effects (with and without IGF-I) suggest that a PKC-dependent pathway is involved in both of these events.

Regulation of secreted IGF binding proteins by ST

Previous studies (Liu *et al.*, 1993) have shown that rat granulosa cells secrete two primary IGFBPs, IGFBP-4 and -5. To observe the effects of ST on IGFBPs, granulosa cells were cultured with or without ST (10^{-9}–10^{-7} M) for 24–72 h. The IGFBPs secreted by the cells were detected using Western ligand blot analysis. Figure 5 shows the dose response effect of ST

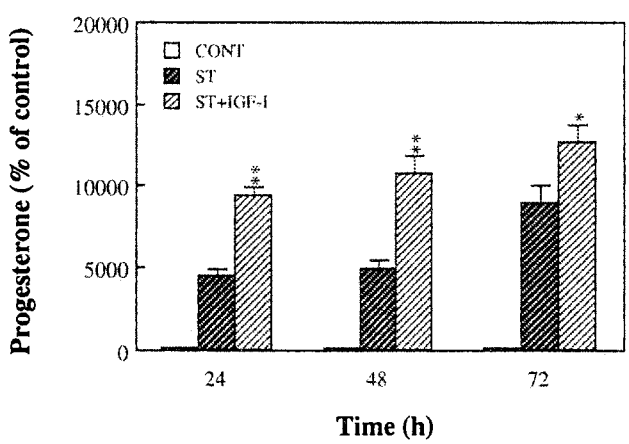

Figure 3 Time course of the effect of ST on IGF-I action on granulosa cell progesterone production. Immature rat granulosa cells (2×10^5 cells/well) were cultured with ST (10^{-7} M) in the absence or presence of IGF-I (30 ng/ml) for 24 h, 48 h and 72 h. Extracellular progesterone production was determined at the end of culture as described in Materials and methods. The data were calculated as mean ± SEM for triplicate measurements in two experiments and are expressed as a percentage of control values (100%). The absolute mean values of the progesterone controls were: 0.15 ± 0.01 ng/10^6 cells (24 h), 0.19 ± 0.01 ng/10^6 cells (48 h) and 0.18 ± 0.03 ng/10^6 cells (72 h). $^{**}P<0.01$, ST *vs* ST plus IGF-I

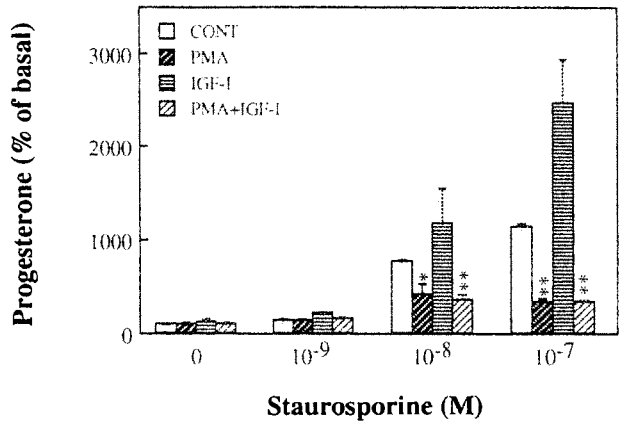

Figure 4 Regulation by PMA of ST-induced progesterone production and IGF-I action. Immature rat granulosa cells (2×10^5 cells/well) were incubated with different concentrations of ST (10^{-9}–10^{-7} M) with or without IGF-I (30 ng/ml) in the presence of PMA (10 ng/ml) for 24 h. At 24 h, extracellular progesterone production was assayed as described in Materials and methods. The data were calculated as mean ± SEM for triplicate measurements in two experiments and are expressed as a percentage of control values (100%). The absolute mean value of the progesterone control was: 0.71 ± 0.16 ng/10^6 cells. $^{**}P<0.01$, ST or ST plus IGF-I in the absence of PMA *vs* ST or ST plus IGF-I in the presence of PMA

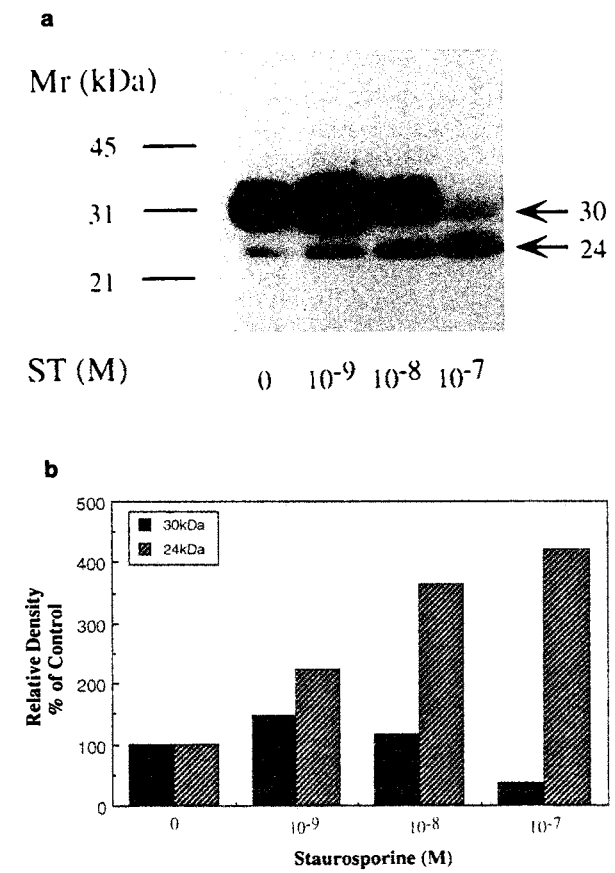

Figure 5 Dose response on the effect of ST on the regulation of IGFBPs. Immature rat granulosa cells (5×10^6 cells/dish) were incubated with different doses of ST (10^{-9}–10^{-7}M) for 72 h. At 72 h culture, the granulosa cell conditioned media were collected and precipitated with 70% ethanol. The levels of IGFBP-4 and -5 secreted by the cells were determined by Western ligand blot analysis (**a**) and scanning densitometry (**b**) as described in Materials and methods

on the two IGFBPs detected. At 72 h, ST, at the highest dose (10^{-7} M), significantly decreased a major IGFBP (~30 kDa) which is likely to be IGFBP-5. All doses of ST increased the minor IGFBP (~24 kDa) which is likely to be IGFBP-4. No other IGFBPs were detected in the present study. Figure 6 shows the time course of the ST effect on IGBPs. ST, at 10^{-7} M, caused a dramatic time-dependent decrease of IGFBP-5 by 48 h, and, as early as 24 h, a marked increase of IGFBP-4. These data raise the possibility that, like gonadotropin, ST may potentiate IGF-I stimulation of steroidogenesis by regulation of IGFBP levels.

Regulation of IGFBP mRNA by ST

To study the mechanisms involved in regulation of IGFBPs by ST, the effects of ST on the mRNAs of IGFBP-4 and IGFBP-5 were measured. Granulosa

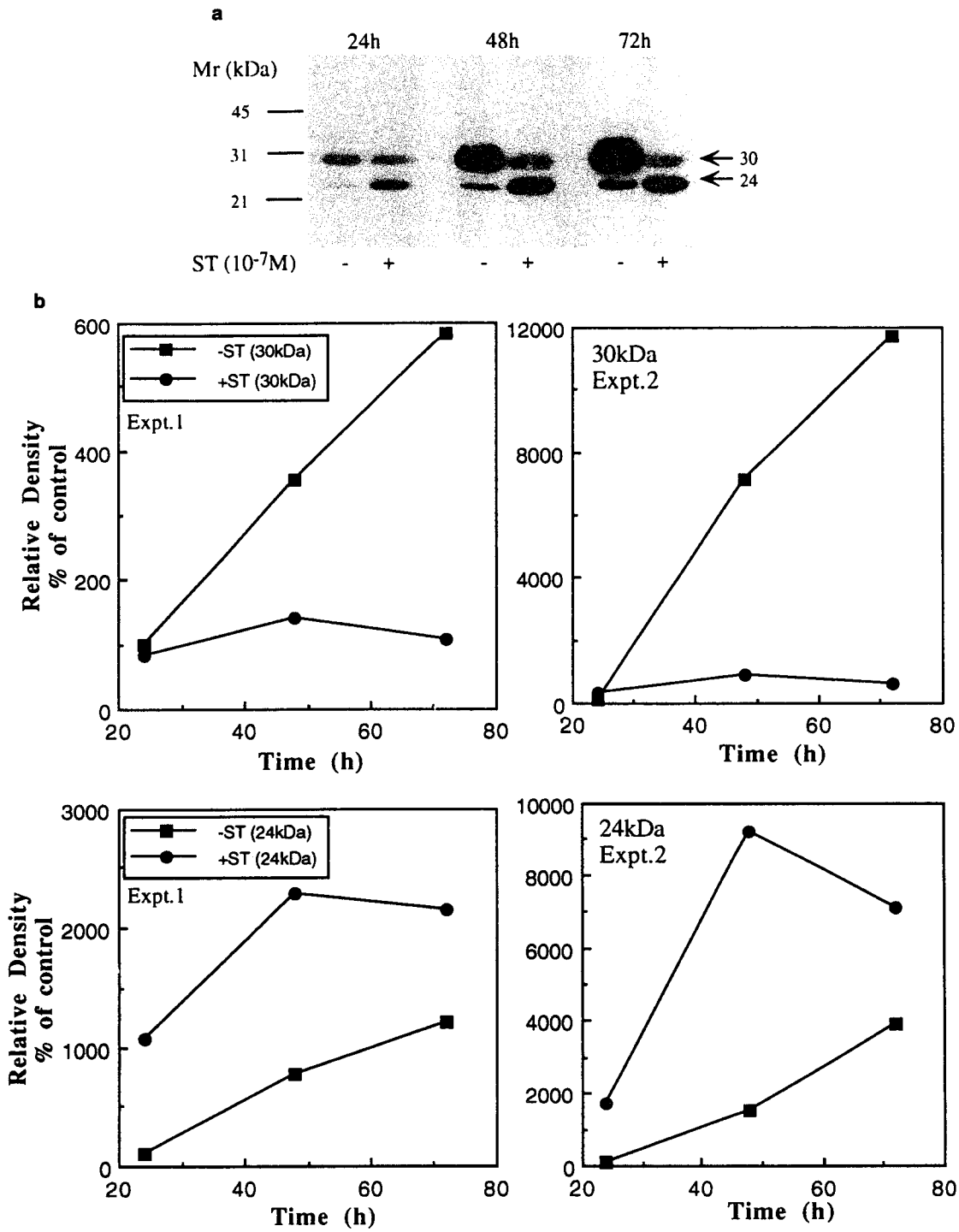

Figure 6 Time course of regulation of IGFBPs by ST. Immature rat granulosa cells (5×10^6 cells/dish) were incubated with ST (10^{-7}M) for 24 h, 48 h and 72 h. At the end of culture, the granulosa cell conditioned media were collected and examined by Western ligand blot analysis and scanning densitometry as described in Materials and methods. A representative Western blot is shown in (**a**). (**b**) presents the individual densitometry results for each IGFBP (30 kDa and 24 kDa) in the two experiments conducted (rather than as a mean result) as the relative density scales were significantly different between the two experiments

cells were cultured with or without ST (10^{-7} M) for 72 h. At the end of culture, total RNAs were isolated and analysed by Northern blots. Figure 7 shows that ST decreased the mRNA of both IGFBP-5 (6.0 Kb) and IGFBP-4 (2.6 Kb) by ~50%. The results suggest that ST decreased IGFBP-5 levels by inhibiting its transcription or decreasing the stability of its mRNA. The mechanism for the marked ST-induced increase in IGFBP-4 levels, despite a 50% drop in mRNA levels is unclear, but suggests a complex array of transcriptional and post-transcriptional events.

Regulation of secreted IGFBPs by PMA

Since a PKC-dependent pathway is involved in both ST-induced steroidogenesis and ST-potentiated IGF-I stimulation of steroidogenesis in granulosa cells, the effect of PMA on IGFBP levels was studied in order to determine whether the regulation of IGFBPs by ST also involved a PKC-mediated mechanism.

The regulation by PMA of ST-induced changes in IGFBP levels was observed at 24 h by culturing granulosa cells with ST (10^{-8} M) in the presence or absence of PMA (100 ng/ml). As in the experiment shown in Figure 6, where a higher dose (10^{-7} M) of ST was used, ST did not affect IGFBP-5 levels at 24 h of culture in the absence of PMA, but increased IGFBP-4 levels (Figure 8). In the presence of PMA, which itself inhibited IGFBP-5 by ~50% whilst having no effect on IGFBP-4, ST further decreased IGFBP-5 by 94% and still increased IGFBP-4 very significantly, as it did in the absence of PMA. The results show that ST regulates IGFBPs in the presence or absence of PMA, indicating an absence of antagonism between ST and PMA, and thereby suggesting a PKC-independent mechanism. This was further tested by examining the effects of ST on IGFBP levels in PKC-down-regulated cells. Granulosa cells were preincubated with a high dose PMA (1000 ng/ml) for 24 h – a situation known to downregulate all isoforms of PKC with the exception of the ζ isoform (Liscovitch & Amsterdam, 1989; Asaoka *et al.*, 1992). The cells were then washed and incubated with ST (10^{-7} M). ST decreased IGFBP-5 and increased IGFBP-4 levels in PMA-induced PKC-down-regulated cells as it did in non-PKC-down-regulated cells (Figure 9). These data suggest that the regulation of IGFBPs by ST does not require phorbol ester-sensitive isoforms of PKC. As all isoforms of PKC identified to date (with the exception of the ζ isoform) are phorbol ester sensitive, it is likely that the regulatory effect of ST on IGFBP does not require PKC.

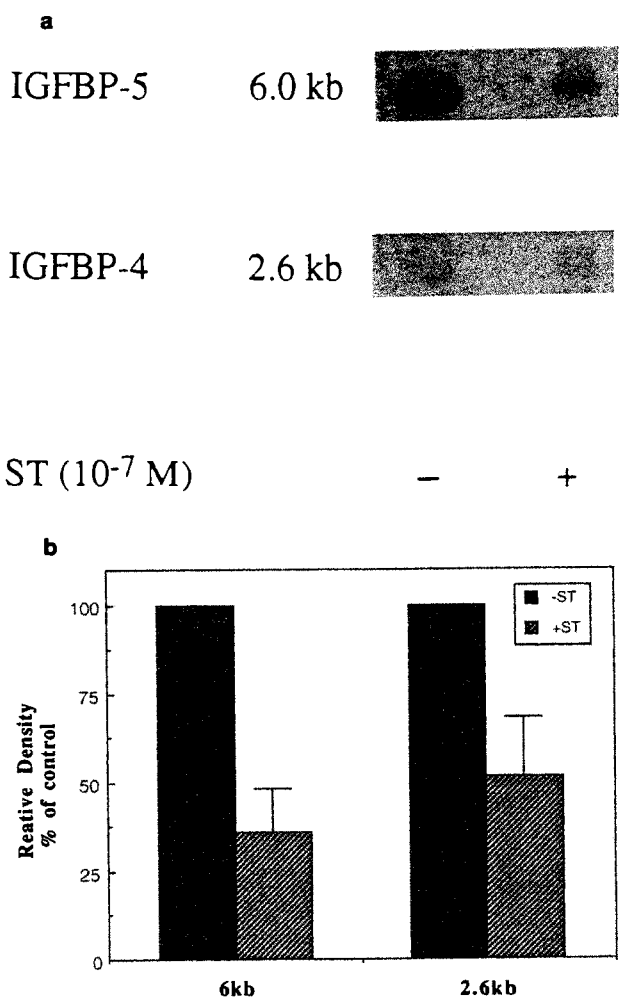

Figure 7 Effect of ST on IGFBP-4 and -5 mRNAs. Immature rat granulosa cells (5×10^6 cells/dish) were incubated with ST (10^{-7} M) for 72 h. At the end of culture, the total RNAs were isolated and the mRNAs of IGFBP-4 and -5 were detected by Northern blot analysis (**a** is a representative blot) and scanning densitometry (**b**), which is presented as the mean value for three individual experiments

Regulation of IGF receptor binding by ST

An additional possibility for ST-induced IGF-I action in granulosa cells is an effect on the number and/or affinity of IGF-I receptors. In this study, this was determined by culturing granulosa cells with or without ST (10^{-9}–10^{-7} M) for different time periods (24–72 h) and measuring IGF-I receptor binding to the cell membrane as described in Materials and methods. By 24 h, the specifically bound [^{125}I]-IGF-I in control cells (ST-untreated) was 35.3 ± 1.62%. ST at various doses (up to 10^{-7} M) had no effect on IGF-I binding. ST, at 10^{-7} M, also had no effect on IGF-I binding following 48 h of treatment. Therefore, it is unlikely that ST potentiated the IGF-I stimulation of granulosa cell steroidogenesis through an increase in IGF-I receptor binding.

Discussion

These data have demonstrated for the first time that ST is able to trigger the stimulation by IGF-I of rat ovarian granulosa cell steroidogenesis without the need for prior, or concurrent, stimulation with gonadotropin. The actions of ST and IGF-I occur through a non-cAMP-dependent pathway since no changes in cAMP were observed. Our previous data (He *et al.*, 1995) has suggested that ST induced steroidogenesis through a PKC-dependent pathway. In the present study we again show that activation of PKC by PMA did not affect progesterone production or cAMP secretion in the absence or presence of IGF-I, but that PMA and ST antagonized each other in both ST-induced steroidogenesis and ST-potentiated IGF-I stimulation of steroidogenesis. This suggests that a PKC-dependent mechanism is also involved in ST

potentiation of IGF-I stimulation of granulosa cell steroidogenesis. These observations also suggest that basal PKC levels in rat granulosa cells are sufficient to prevent IGF-I acting unless gonadotropin is present.

The action of IGF-I on granulosa cells can be affected by regulation of the IGFBPs secreted by the cells, IGF receptor binding and post-receptor signalling mechanisms, including the activation of tyrosine protein kinases. Previous studies have demonstrated that gonadotropin induced the stimulation by IGF-I of granulosa cell differentiation while, at the same time, decreasing IGFBPs (Adashi *et al.*, 1991; Grimes *et al.*, 1992; Liu *et al.*, 1993). ST has had a strikingly similar effect. Two types of IGFBPs have been characterised in rat ovarian granulosa cells, IGFBP-4 and -5 (Liu *et al.*, 1993). IGFBP-5 is the major IGFBP secreted by the cells. In the present study, ST dramatically decreased IGFBP-5 and increased IGFBP-4 levels secreted by the cells. The effect on IGFBP-5 appears to involve transcriptional or mRNA stabilization mechanisms. The effect on IGFBP-4, however, is more complex as IGFBP-4 mRNA was decreased despite an increased secretion of IGFBP-4. Although both exogenous IGFBP-4 and -5 are reported to inhibit gonadotropin-induced ovarian granulosa cell steroidogenesis (Liu *et al.*, 1993), it is unlikely that IGF-I increased granulosa cell steroidogenesis by decreasing endogenous IGFBPs, as it has been reported that IGF-I itself actually increases IGFBP-5 levels in conditioned medium of control and gonadotropin-treated granulosa cells (Fielder *et al.*, 1993). This occurs via the blocking of a protease which specifically cleaves IGFBP-5. IGF-I does not affect IGFBP-4 (Fielder *et al.*, 1993). Furthermore, the expression of IGFBP-5 *in vitro* is enhanced by the addition of IGFs (Adashi *et al.*, 1992). Therefore, it is possible that ST, by decreasing IGFBP-5 levels, increases the level of unbound exogenous IGF-I and hence increases IGF-I bioavailability. Although ST increased IGFBP-4, the net effect of ST on IGFBPs is almost certainly to decrease overall IGFBP production since the amount of IGFBP-5 secreted by the cells is much greater than that of IGFBP-4. Thus, the free IGF-I available to IGF receptors almost certainly increases in the presence of ST. This occurs via a PKC-independent mechanism (ie. it is not blocked by PMA and is still demonstrable in PKC-down-regulated cells). These observations do not exclude, however, a role for the ζ isoform of PKC which is not sensitive to PMA activation or down-regulation.

Having increased available IGF-I levels, ST appears to have a second and perhaps more distinct role in potentiating the action of IGF-I on progesterone synthesis. As the effects of ST on IGFBP-5 and IGF-I-enhanced progesterone production are differentially affected by PMA, it is likely that these increased effects of IGF-I are either independent, or not solely dependent on the changes invoked in IGFBP-5 levels. This second effect of ST occurs via a PKC-dependent

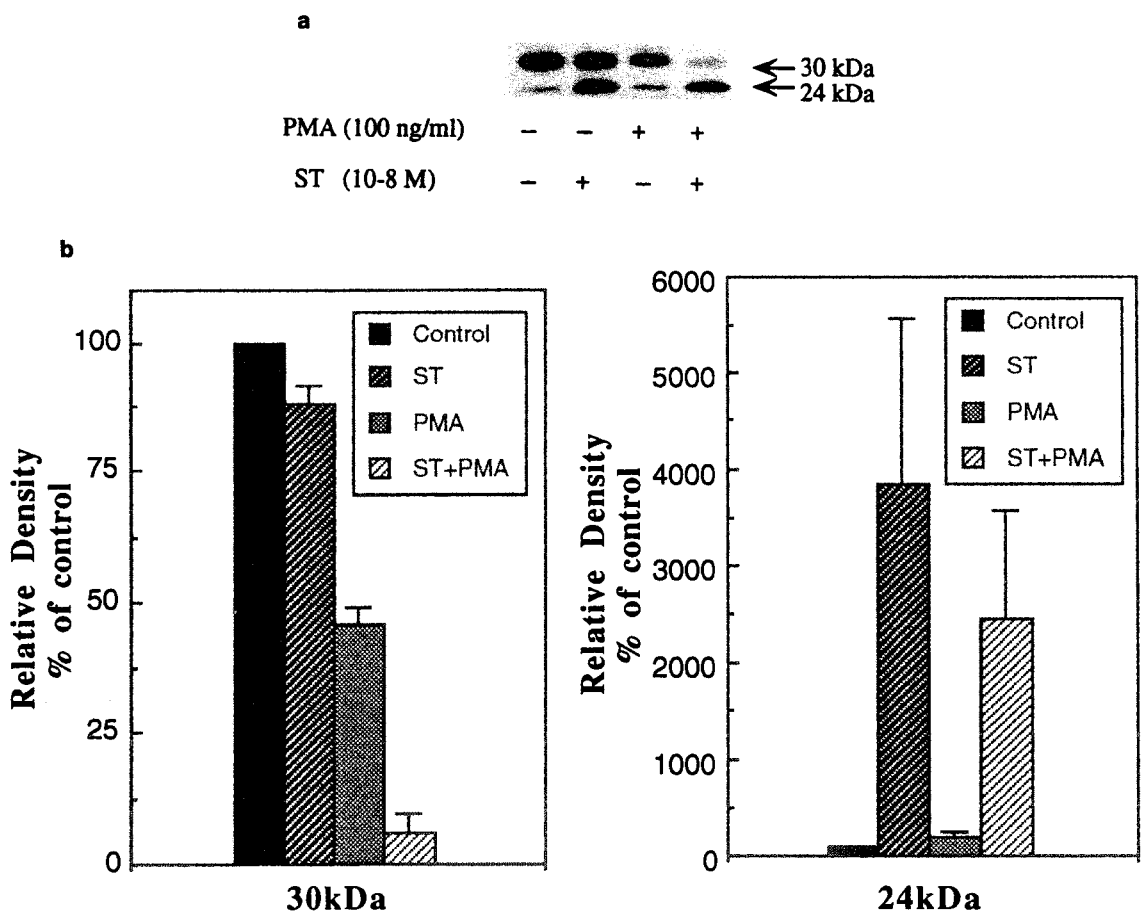

Figure 8 Regulation of effects of ST on IGFBPs by PMA. Immature rat granulosa cells (5×10^6 cells/dish) were incubated with ST (10^{-8}M) in the presence or absence of PMA (100 ng/ml) for 24 h. At 24 h, the conditioned media were collected and examined by Western ligand blot analysis (**a** is a representative blot) and scanning densitometry (**b**) which is presented as the mean values for two individual experiments

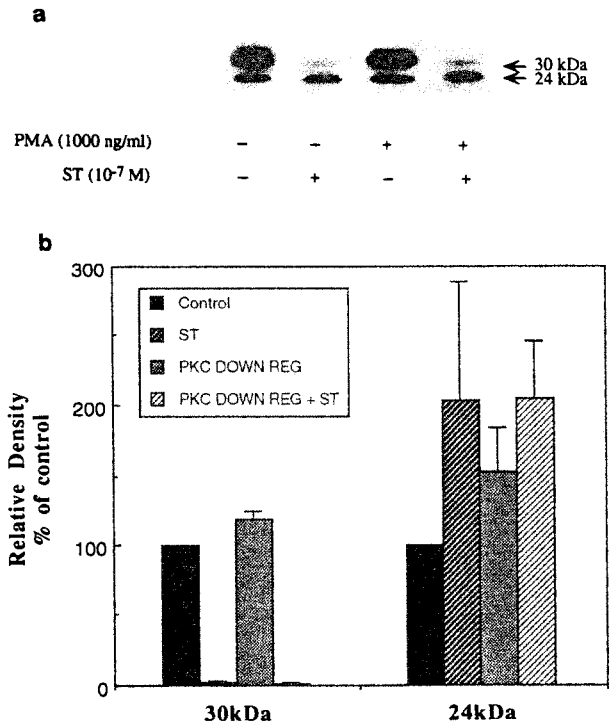

Figure 9 Effects of ST on IGFBPs in PKC-down-regulated cells. Immature rat granulosa cells (5×10^6 cells/dish) were preincubated with a high dose of PMA (1000 ng/ml) for 24 h, then washed with culture medium and incubated with ST (10^{-7}M) for another 48 h (PKC down-regulated cells). At the end of culture, the conditioned media were collected and examined by Western ligand blot analysis (**a** is a representative blot) and scanning densitometry (**b**), which is presented as the mean values for two individual experiments

mechanism (ie. blocked by PMA) and might instead be manifested as increased IGF-I action via an effect of ST on IGF receptor number and/or affinity, receptor autophosphorylation and/or tyrosine kinase activity. It has been reported, for example, that gonadotropin stimulates IGF-I receptor binding by increasing the IGF receptor number on granulosa cells (Adashi *et al.*, 1988b). In the present study, unlike gonadotropin, ST did not affect IGF-I binding to granulosa cells. This is most likely a reflection of no change to receptor number although, since IGFBP-5 is known to associate with cell surface proteoglycans, one cannot definitively exclude a rise in IGF receptors which was coincidentally masked by an equivalent, but opposite, change in net IGFBP levels. It is considered unlikely however, that ST potentiates IGF-I stimulation of steroidogenesis via a direct action at the IGF receptor level. No studies on post-receptor mechanisms have been undertaken.

It should be pointed out that in addition to the inhibitory effect on PKC, ST has also been reported to affect protein kinase A activity (Zhang & Snell, 1993) and tyrosine kinase activity (Fujita-Yamaguchi & Kathiria, 1988). These effects occur, however, at a 10-fold higher concentration than has been used in the present study. Furthermore, the effect of PMA, a PKC activator, to antagonize the action of ST in these experiments clearly implicates PKC at some point as an overriding factor in the progesterone synthetic pathway. IGF-I and insulin both enhance rates of progesterone synthesis by rat and porcine granulosa cells *in vitro* (Adashi *et al.*, 1985c). These steroidogenic actions are exerted, in part, at the level of the rate-limiting mitochondrial cytochrome P450 side-chain cleavage enzyme (P450scc) (Veldhuis *et al.*, 1983). It has been shown in porcine granulosa cells that activation of PKC by phorbol ester suppresses the intracellular accumulation of insulin-stimulated P450scc mRNA, as well as progesterone production (Flores *et al.*, 1993). Thus, activation of PKC by PMA in the present study possibly inhibits the rate-limiting enzyme P450scc for progesterone synthesis, therefore suppressing ST-induced progesterone production as well as ST-potentiated IGF-I stimulation of progesterone production, even though the ST-induced decrease in IGFBP-5 was concurrently unaffected by PMA.

In conclusion, activation of PKC by PMA does not affect either basal or IGF-I treated rat ovarian granulosa cell differentiation. ST induces steroidogenesis and potentiates the IGF-I stimulation of this steroidogenesis by modulation of PKC, though the mechanism involved is not clear. ST also regulates IGFBP levels, probably through a PKC-independent pathway.

Materials and methods

Reagents

Insulin-like growth factor-I (IGF-I) was donated by Eli Lilly (Indianapolis IA, USA) and Kabi Pharmacia (Stockholm, Sweden). McCoy's 5A culture medium (modified, serum-free), trypan blue, L-glutamine, penicillin-streptomycin sulphate, phorbol 12-myristate 13-acetate (PMA), staurosporine and RIA grade bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). RSL anti-progesterone antiserum was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). [^{125}I]IGF-I was purchased from Amersham Australia (Melbourne, Australia). Complementary DNAs for IGFBP-4 and -5 were generously donated by Dr Shunichi Shimasaki (Whittier Institute, La Jolla, CA, USA). Random primed digoxigenin (DIG) DNA labeling kits, DNA molecular weight markers and positively charged nylon membranes were purchased from Boehringer Mannheim (Sydney, Australia). An RNA ladder was purchased from Life Technologies, INC (Gaithersburg, MD, USA). Broad range protein molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Adenosine 3′:5′-cyclic phosphoric acid 2′-0-succinyl ^{125}I-iodotyrosine methyl ester and ^{3}H-thymidine were purchased from DuPont NEN (Boston, MA, USA).

Animals

Immature (21–23 day old) female Sprague Dawley rats were provided by the Animal House, Royal Children's Hospital, Melbourne, and maintained on standard rat chow and tap water *ad libitum*. The animal experiments were carried out according to the code of practice adopted by the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee of the Royal Children's Hospital.

Granulosa cell culture

Immature (21–23 day old) rats were treated with diethylstilbestrol (DES, 10 mg/rat, s.c. implant) for 3 days. Granulosa cells were isolated and collected by standard procedures as previously described (He & Herington, 1991).

Eighty-five percent of cells plated were viable as assessed by trypan blue exclusion. Viable cells were cultured in triplicate, in 48-well Coster tissue culture plates (2×10^5/cells/0.5 ml/well) or in 60×15 mm tissue culture dishes ($5-10 \times 10^6$ cells/5 ml/dish), with McCoy's 5A medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin in the presence or absence of 0.1% BSA. Cells were incubated at 37°C in an atmosphere of 5% CO_2 in air.

Progesterone RIA

Progesterone production was quantitated as the amount of progesterone in the granulosa cell conditioned medium at the end of each culture period. Media samples were directly assayed by radioimmunoassay based on the method described by Hutchinson *et al.* (1988) except that RSL anti-progesterone antiserum was used.

cAMP assay

The extracellular cAMP content of the culture medium was determined by radioimmunoassay using a [^{125}I]-tyrosyl methyl ester of 2'-O-monosuccinyladenosine 3':5'-cyclic monophosphate and adenosine-3':5'-cyclic monophosphate (sodium salt) as standards. Rabbit polyclonal antiserum Ab 5102, raised against 2'-O-monosuccinyladenosine 3':5'-cyclic monophosphate conjugated with 1-ethyl-(3-diethylamino-propyl)-carbodiimide HCl to keyhole limpet haemocyanin was generously donated by Dr Phil Marley (Department of Biochemistry, University of Melbourne, Australia). At the end of culture, aliquots of medium were boiled for 10–15 min to inactivate phosphodiesterase, dried with a vacuum freeze drier and stored at −20°C until assayed. Acetylation of the samples was performed just prior to assay. This was done by adding 10 μl triethylamine and 5 μl acetic anhydride to 0.5 ml sample. The minimum detectable dose significantly different from 0 was 0.0156 pmol/assay tube.

IGF-I receptor binding study

Granulosa cells were inoculated (4×10^5 viable cells/culture) into loosely capped sterile 12×75 mm polystyrene test tubes (Becton Dickinson, Melbourne, Australia) containing 1 ml McCoy's 5A medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin (Adashi *et al.*, 1988b). After culture, for different time periods (24–72 h) with ST ($10^{-9}-10^{-7}$ M), the cells were pelleted by centrifugation (800 *g*, 5 min, 4°C), and the overlying media were removed. The resulting cell pellets were incubated with 0.2 ml assay buffer (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose and 10 mg/ml BSA, pH 8.0) containing [^{125}I]iodo-IGF-I (~10^5 cpm/tube) and the indicated concentration of the specified experimental agents, at 4°C overnight. At the end of the incubation period, the mixtures were washed twice with 3 ml ice-cold assay buffer to remove excess unbound hormone, and the cell-bound radioactivity in the final pellet was determined using a γ-counter. Nonspecific binding was determined in the presence of excess (1 μg) unlabeled recombinant IGF-I, and was normally around 5%.

Western ligand blot analysis

The granulosa cell conditioned media (4 ml) were precipitated in 70% ethanol at −20°C overnight and centrifuged (10 000 *g*, 10 min, 4°C). The resulting pellets were suspended in Laemmli non-reducing sample buffer (200 μl) and boiled for 5 min. The protein content of the samples was determined using the Bradford assay (Read & Northcote, 1981). Equal amounts of protein (15 μg/lane) were loaded onto a 12% SDS–polyacrylamide gel, electrophoresed at a constant voltage of 200 V for 1 h, and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Richmond, CA) at a constant voltage of 100 V (1 h, 4°C).

Ligand blotting was carried out essentially as previously described (Hossenlopp *et al.*, 1986) with the following modifications. After soaking in 3% Nonidet P-40 (NP40) in Tris buffer (0.15 M NaCl, 0.01 M Tris, 0.05% NaN_3, pH 7.4) for 30 min, the nitrocellulose membranes were blocked in 1% BSA-Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were then incubated at 4°C overnight with 10 ml 1% BSA-TBST buffer containing 10^6 cpm [^{125}I]IGF-1. After washing with Tris buffer, the membranes were exposed to Kodak XAR-5 film for up to 20 h at −80°C. Each band was quantitated by scanning densitometry.

RNA isolation and northern blot analysis

Granulosa cells ($5-10 \times 10^6$ cells/dish) were cultured for 72 h in 6×15 mm tissue culture dishes with specified agents, as indicated in the text. At the end of culture, the media was removed from the cells. The total RNA of the cells was isolated according to the method described by Chomczynski and Sacchi (1987). The content of RNA in each sample was quantitated spectrophotometrically at 260 nm. The RNA was denatured by heating to 50°C for 1 h in 1 M glyoxal and 50% DMSO in 10 mM sodium phosphate, pH 7.0. Samples were fractionated on a 1% agarose gel prepared in 10 mM sodium phosphate, pH 7.0 and electrophoresed at a constant current of 90–100 mA for 4–5 h at room temperature. RNA markers were run in parallel lanes. RNAs were transferred overnight to positively charged nylon membranes (Boehringer Mannheim) in $20 \times$ SSC (3 M NaCl, 0.3 M tri-sodium citrate). After UV cross-linking (2.5 J/cm^2), the membranes were prehybridized at 42°C for 4 h in a prehybridization buffer containing 50% formamide, $5 \times$ SSC, 2% (w/v) blocking reagent (Boehringer Mannheim), 20 mM sodium maleate, 0.02% SDS and 0.1% N-laurylsarcosine. The membranes were hybridized overnight at 48°C with rat IGFBP-4 and -5 cDNAs labeled by random primed DIG DNA kit in prehybridization buffer. The membranes were washed twice in $2 \times$ SSC, 0.1% SDS at room temperature for 5 min and twice in $0.1 \times$ SSC, 0.1% SDS at 52°C for 15 min. After washing, the membranes were blocked in blocking solution (2% blocking reagent, 100 mM Tris, 150 mM NaCl, pH 7.5) at room temperature for 1 h and then incubated with anti-DIG-antibody (Boehringer Mannheim) diluted 1:5000 in blocking solution. After washing twice in a solution containing 100 mM Tris, 150 mM NaCl, pH 7.5, the membranes were equilibrated in a buffer containing 100 mM Tris, 100 mM NaCl pH 9.5 for 3 min. The membranes were incubated for 1 min with Lumi-Phos 530 diluted 1/100 in the above buffer. The membranes were exposed to ECL hyperfilm for up to 24 h followed by one dimensional scan on a GS-670 imaging densitometer (Bio-Rad). The membranes were stripped by boiling in 1% SDS for 3 min for further reprobing. The amount of total RNA loaded in each lane was compared by ethidium bromide staining.

Data analysis

The data represent mean ± SEM for triplicate determinations and were assessed by ANOVA. In all cases, comparisons with a $P < 0.05$ were considered to be significantly different.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia (PR, ACH), the Royal Children's Hospital Research Foundation (PR, ACH) and the William Buckland Foundation (PR, ACH). Hong He is a recipient of a University of Melbourne post-graduate scholarship.

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