Involvement of cyclic adenosine monophosphate-dependent protein kinase isozymes in tissue plasminogen activator secretion by rat Sertoli cells stimulated with follicle-stimulating hormone in vitro

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This study was undertaken to investigate, in freshly isolated rat Sertoli cells, the physiological function of the type I and type II cyclic adenosine monophosphate (cAMP)-dependent protein kinase isozymes in tissue-type plasminogen activator secretion and the regulation of this cAMP process by follicle-stimulating hormone (FSH). Follicle-stimulating hormone-induced tissue-type plasminogen activator secretion depends upon intracellular cAMP levels. The changes in cAMP amounts required to activate maximally the tissue-type plasminogen activator secretion are extremely small, a cAMP threshold having to be reached for triggering the tissue-type plasminogen activator output. Intact Sertoli cells were incubated with combinations of cAMP analogs specific for each cAMP-dependent protein kinase type and complementary in their cAMP binding site on the cAMP-dependent protein kinase regulatory subunits: 8-aminohexylamino-cAMP = type I, site 1; 8-thiomethyl-cAMP = type II, site 1 and N'benzoyl-cAMP = types I/II, site 2. This allowed us to activate selectively each cAMP-dependent protein kinase type in a synergistic manner and then to evaluate their respective influence in the specific tissue-type plasminogen activator response. We establish that both of the cAMP-dependent protein kinase types are present and functional; the activity of the type I isozyme is preponderant (60%) in the cAMP-dependent tissue-type plasminogen activator secretion. Likewise, when these cAMP analogs were coupled with endogenously generated cAMP by FSH or forskolin, both of the cAMP-dependent protein kinase types were involved in the tissue-type plasminogen activator production. However, only tissue-type plasminogen activator secretion induced by FSH is mediated predominantly by the type I cAMP-dependent protein kinase, although the type II isozyme sustains an appreciable physiological role in the transmission pathway. We suggest some differences in the pattern of action between FSH and forskolin in Sertoli cells.

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Sertoli cells, which provide the structural and functional environment for the maintenance and progression of mammalian spermatogenesis, are the major site of action for follicle-stimulating hormone (FSH). This pituitary gonadotropin regulates a diversity of Sertoli cell functions through the activation of adenylate cyclase, leading to an elevation of intracellular cAMP (1), which in turn exerts its effects through the activation of cAMP-dependent protein kinases (PKA) (2) and the subsequent phosphorylations of specific protein substrates (3). These phosphorylations provoke diverse processes such as enzyme inductions, gene regulation and secretion of proteins, including tissue-type plasminogen activator (tPA) (4, 5).

Two different forms of PKA, designated type I and type II, are usually present in tissues (6). Differences in the physicochemical properties of their regulatory subunits, including different affinities for cAMP analogs, provide the basis for this classification of isozymes in two types. Each regulatory subunit contains two cAMP-binding sites, designated site 1 and site 2, which differ with respect to both cAMP dissociation rate and cAMP analog selectivity (7). These sites exhibit positive cooperativity (8): selective binding of a cyclic nucleotide at one site favors binding at the other site and, hence, induces in a synergistic manner the dissociation and activation of the catalytic subunits. Furthermore, by the use of cAMP analogs specific of one type of isozyme, the synergistic activation of type I isozyme can be distinguished from that of type II isozyme. Based on these properties, incubation of the cells with a site-selective and type-specific cAMP analog together with a cAMP analog selective for the other site or with cAMP itself leads to a synergistic cellular response mediated by only one type of PKA (9). Rat Sertoli cells contain both types I and II PKA (10) but it is not yet known whether both types of
PKA are involved in the FSH-induced elevation of tPA secretion.

By using, for the first time, different cAMP analogs in combination or co-incubated with low concentrations of FSH, we have determined that both type I and type II PKA isozymes are involved in the FSH-stimulated secretion of tPA in Sertoli cells.

Materials and methods

Materials

Cyclic AMP (cAMP), N°-benzoyl-cAMP (N°-B), 8-amino hexylamino-cAMP (AHA), 8-thiophenyl-cAMP (8-TM) and 3-isobutyl-1-methylxanthine (MIX) were purchased from Sigma Chemical Co. (St Louis, MO). Leibovitz’s L15 medium was obtained from Serva (Heidelberg, Germany). Equine FSH (eFSH) (CY1 368: 864 × NIH-FSH-S1 in homologous equine RRA) was purified in our laboratory using a previously published procedure (11).

Isolation of rat seminiferous tubule cells and incubation

Rat seminiferous tubule cells were prepared from 16–18-day-old rat testes (Wistar INRA 03) according to the method of Dorrington et al. (1), except that collagenase digestion was omitted and one additional mechanical dispersion was performed just after trypsin treatment (12). Tubular cell preparations contained more than 75% Sertoli cells, about 17 ± 5% germ cells and 4 ± 2% myoid cells. Identification of germ cells was achieved by staining with Trioxy Hematein Ferric and that of myoid cells by an alkaline phosphatase reaction (13). Incubations were performed in 300 µl of L15 medium containing 0.1 mmol/l MIX, unless otherwise noted, and 0.5 × 10⁶ cells in 3-ml polystyrene tubes. After the addition of eFSH or forskolin and/or cAMP analogs, the cells were incubated for 4 h at 34°C.

Assay of tissue plasminogen activator activity

The tPA content of the incubation was assayed by the 125I-labelled fibrin digestion method described previously (14). As tPA promotes the conversion of plasminogen to plasmin, its concentration was determined from the release of radioactivity in the medium during fibrinolysis of insoluble radiolabeled fibrin by plasmin. Then, the tPA activity was expressed as human tPA units, as determined with reference to a standard preparation of human tPA (15).

Assay of cAMP

Intra- and extracellular cAMP was assayed using an RIA kit and protocol supplied by the Pasteur Institute (Paris, France). The sensitivity of the cAMP assay is 3 fmol cAMP per tube.

Selectivities of cAMP analogs

The selectivities of the cAMP analogs for the regulatory intrasubunit cAMP-binding sites and for the PKA types are the following: AHA is site 1/type I-selective, 8-TM is site 1/type II-selective and N°-B is site 2/types I and II-selective.

Synergism ratio

To quantitate the resulting effects on tPA secretion, a synergism ratio (SR) was used, determined from the equation

\[ SR = \frac{P_1 + 2 - B}{(P_1 - B) + (P_2 - B)} \]

where B is basal tPA production in the absence of any stimulation; P1 is tPA production stimulated by one cAMP analog or FSH; P2 is tPA production by another cAMP analog; and P1+2 is the tPA production resulting from the combined two agents corresponding to P1 and P2.

A synergism ratio significantly greater than unity indicates a synergistic response to the two agents, whereas a synergism ratio of unity represents a simple additive response to the two agents. The results are presented as the mean ± SEM of four independent experiments. For each experiment, there were between three and five replicates of each treatment.

Statistics

The synergism ratio data were assessed for statistical significance using a single mean Student’s t-test (Statview) and an analysis of variance (SuperANOVA, Abacus Concepts, CA, USA) followed by the Newman–Keuls test, with p < 0.05 considered to be statistically significant.

Results

Characterization of the responses to cAMP analogs and FSH

Figure 1 shows the concentration–response characteristics of each of the three cAMP analogs in the presence and absence of MIX. It can be seen that the presence of MIX in the incubation medium increases slightly the tPA basal level and its production by the cAMP analogs. In the following experiments, the phosphodiesterase inhibitor (MIX) was included in the incubation medium, unless otherwise noted, to obviate the remote possibility that the cAMP analogs could themselves act via phosphodiesterase inhibition.

The cAMP analogs used in these experiments are PKA site- and type-selective. Their site selectivity and their ability to discriminate between the two isozymes can be observed only at relatively low concentrations, which led us to work in the subsequent experiments with levels
Fig. 1. Tissue-type plasminogen activator (tPA) secretion in response to cAMP-dependent protein kinase site/type-selective cAMP analogs. Rat Sertoli cells were incubated for 4 h in the absence (○) or presence (●) of 0.1 mmol/l 3-isobutyl-1-methylxanthine, with increasing concentrations of N^6^-benzoyl-cAMP (N^6^-B), 8-thiomethyl-cAMP (8-TM) or 8-aminohexylamino-cAMP (AHA). The tPA levels were determined in the Materials and methods section. Each value represents the mean ± SEM of two experiments, each done with five replicates.
of cAMP analogs in the range 50–100 μmol/l to elicit a slight measureable tPA production.

Figure 2 shows the dose–response relationship for FSH-stimulated tPA secretion and cAMP production in the absence (Fig. 2A) and presence (Fig. 2B) of MIX. In the absence of MIX, the steep increase in tPA production occurred with FSH concentrations between 5 and 100 μg/l. In the same range of FSH concentrations, cAMP levels rose from 20 up to 25 nmol/l (for 0.5 × 10^6 cells in 4 h). In the presence of MIX, FSH concentrations between 0.5 and 5 μg/l provoked the steep increase in tPA secretion. The rise in cAMP levels induced by this range of FSH concentrations was also from 20 up to 25 nmol/l (for 0.5 × 10^6 cells in 4 h).

The presence of MIX in the incubation medium slightly affected the ED_{50} for FSH in the stimulation of cAMP formation but the basal cAMP level was increased twofold (9 vs 18 nmol/l) and the maximum production was also increased (25 vs 70 nmol/l).

In the presence of MIX, the ED_{50} for FSH-stimulated tPA secretion was strongly reduced from 50 to 1 μg/l while the maximum tPA production was increased from 2 to 4.5 equivalent human tPA.

These results show that, whether MIX is present or absent, the stimulation of tPA secretion was triggered in Sertoli cells when the cAMP formation was increased.

Table 1. Effect of site/type-selective cAMP analogs, alone or in combination, on tissue-type plasminogen activator (tPA) production by rat Sertoli cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Human tPA (mUnits)</th>
</tr>
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<tbody>
<tr>
<td>Basal</td>
<td>195 ± 25</td>
</tr>
<tr>
<td>8-TM</td>
<td>278 ± 29</td>
</tr>
<tr>
<td>AHA</td>
<td>248 ± 26</td>
</tr>
<tr>
<td>N^B</td>
<td>285 ± 25</td>
</tr>
<tr>
<td>8-TM + N^B</td>
<td>961 ± 112</td>
</tr>
<tr>
<td>AHA + N^B</td>
<td>1167 ± 145</td>
</tr>
<tr>
<td>8-TM + AHA</td>
<td>420 ± 55</td>
</tr>
</tbody>
</table>

* Values are means ± ssm of four experiments. 8-TM (100 μmol/l): 8-thiomyethyl-cAMP. AHA (100 μmol/l): 8-aminohexylamino-cAMP. N^B (50 μmol/l): N^B-benzoyle-cAMP.
from the threshold level concentration of 20 nmol/l up to 25 nmol/l (for 0.5 × 10⁶ cells in 4 h).

Effect of isozyme-directed cAMP analog pairs on tPA production

For synergism experiments, combinations of varying concentrations of cAMP analogs were assayed in the presence of MIX. The doses eliciting optimal synergism in tPA secretion were chosen: 50 μmol/l for N⁶-B and 100 μmol/l for 8-TM and AHA (Table 1).

Sertoli cells were co-incubated with the site I/type II-selective analog 8-TM and the site 2-selective type-independent analog N⁶-B. A significant (p ≤ 0.01) synergistic increase (SR = 4.9 ± 1.6) in tPA secretion was observed (Fig. 3). Likewise, incubation of Sertoli cells with the site 1/type I-selective analog AHA in combination with the analog N⁶-B caused a significant (p ≤ 0.01) synergistic (SR = 8.2 ± 2.4) increase in tPA secretion. Co-exposure of Sertoli cells to AHA and 8-TM, which both bind to site 1 of each isoenzyme, did not elicit (p = 0.1) any synergistic response (SR = 1.2 ± 0.3). When Sertoli cells were incubated with the type I-directed analog pair (AHA + N⁶-B), the synergism ratio was significantly (p ≤ 0.05) higher than with the type II-directed analog pair (8-TM + N⁶-B), indicating that type PKA is preponderant in cAMP-dependent tPA secretion by Sertoli cells.
Interaction of type-selective analogs with endogenously generated cAMP

Figure 4 shows the dose–response curves for FSH (Fig. 4A) and for the diterpene forskolin (Fig. 4B) in the presence of MIX. Both of these treatments were capable of eliciting, through activation of adenylate cyclase, a cAMP-mediated increase in tPA production.

In the synergism experiments reported below (Fig. 5), performed in the presence of MIX, FSH was used at a concentration of 125 ng/l, a concentration eliciting an extremely low stimulation of tPA secretion. Likewise, forskolin, which produces a steep response at concentrations ranging from 1 to 10 μmol/l, was used at a concentration of 1 μmol/l corresponding to the threshold of stimulation of tPA secretion.

Each of the PKA type-specific cAMP analogs combined with FSH (Fig. 5A) or forskolin (Fig. 5B) was shown to increase the potency of endogenous cAMP generated as potential activator of the corresponding type of PKA. Coincubation with FSH provoked a synergistic response in tPA secretion by Sertoli cells, predominantly through the type I isozyme with an SR of 3.2 ± 0.5 (p < 0.01), although significant interaction via the type II isozyme was also evident (SR = 1.9 ± 0.1 p < 0.01).

The addition of forskolin with either of the PKA type-specific cAMP analogs also elicited a slight but still significant synergistic increase in tPA production. Forskolin, paired with the type I PKA specific analog AHA and with the type II PKA specific analog 8-TM, presented a synergistic effect of 1.70 ± 0.14 (p < 0.01) and 1.40 ± 0.07 (p < 0.01) respectively.

Discussion

The activation of protein kinases by cAMP is the main well-demonstrated intracellular step occurring in the stimulation of the Sertoli cell by FSH (1). Recent works involving complementary DNA cloning have revealed great heterogeneity of the PKA. In mammals, four closely related but distinct isoforms of type I and II regulatory subunits (R) have been demonstrated: R1α, R1β, R1δ, and R1γβ. Furthermore, three isoforms of C subunits have been characterized: Ca, Cβ, and Cγ (16, 17). In rat testis, Sertoli cells (10, 18–21) and Leydig cells (19, 22) contain the mRNAs and proteins of both types I and II PKA. Sertoli cells from immature rats contain approximately 75% R1α and 25% R1δ and very low levels of R1β. One of the interesting aspects of this PKA holoenzyme system is the cell’s ability to maintain, within itself, two types of PKA that might play distinct roles in the cellular responses to hormone stimulation.

To elucidate specific functions of type I and type II PKA, it is necessary to assess separately their activities. Therefore, the use of cAMP analogs, which preferentially activate one of the two isozymes, provides an in vitro approach to this system in intact cells. The efficacy of an analog such as selective protein kinase agonist in the cell is dependent upon its concentration at its site of action. At low concentrations each analog binds selectively to a single site, while at higher concentrations the analog will bind to both sites and then no or a low synergism extent will be observed. Therefore, the concentrations of analogs used in this study were determined in order to obtain optimal synergism and to preserve their relative site and type selectivities. In keeping with this, the effects of the two site-1-specific analogs (AHA and 8-TM) were additive.

In the present study performed on intact Sertoli cells, the tPA secretion was chosen as the biological end-point reflecting kinase activation. The results presented here indicate that selective activation of either type I or type II PKA with pairs of site- and type-selective cAMP analogs was able to induce tPA secretion in Sertoli cells. The extent to which the Sertoli cells respond to the analog pairs is not merely the sum of the effects achieved with either of the two analogs individually but is far greater than expected if the effects were simply additive. This synergistic increase in tPA secretion was found to be greater with the type I-directed analogs (8-TM and N6-B: SR = 8.2) than with the type II-directed analogs (8-TM and N6-B: SR = 4.9) (Fig. 2). This indicates that both types of PKA isozymes are present and functionally active in the Sertoli cells. The significant (p < 0.05) difference between the effect observed with the combination of AHA and N6-B on the one hand and 8-TM and N6-B on the other hand might reflect a difference in the levels of type I and type II PKA in Sertoli cells (23).

Simultaneous changes in cAMP levels and tPA secretion as functions of FSH concentration can be compared in the absence or presence of MIX. One of the essential features that is different in FSH-stimulated Sertoli cells treated with MIX compared with untreated cells is a prominent increase in the levels of tPA secretion and cAMP production. The marked potentiation of the hormone action by a phosphodiesterase inhibitor confirms that phosphodiesterases are very active in Sertoli cells (24). In spite of the differences in the magnitude of the response and the sensitivity to FSH, it can be seen that identical cAMP thresholds must be reached for triggering tPA secretion. Indeed, the increase in tPA production occurs in all cases in the range 20–25 mmol/l cAMP expressed for 0.5 × 10⁶ cells in 4 h. Thus, the increase in intracellular cAMP levels required to activate maximally the responding system occurs over a very narrow range under the conditions used. This indicates that, upon FSH stimulation, the tPA response of Sertoli cells is switched on at a very precise threshold of cAMP concentration (and consequently over a narrow range of FSH concentrations). It might be that other responses to FSH are also triggered at precise second messenger concentrations that might be similar or different from that stimulating tPA secretion. Another interesting point is that after FSH addition in the absence of MIX a significant increase in intra- and extracellular cAMP levels occurs, although the increase in tPA secretion was
not detectable. Whereas in the presence of MIX, tPA production is strongly increased without an appreciable increase in cAMP level. This can be explained by the fact that the cAMP basal level is strongly increased from 9 to 18 nmol/l in the presence of MIX and is then very close to the cAMP threshold over which tPA production is triggered. Thus, in the presence of a phosphodiesterase inhibitor, the slightest increase in cAMP levels induced by very low FSH concentrations leads to tPA secretion by Sertoli cells. The blockade of the essential role of phosphodiesterase activity in Sertoli cells allows us to observe that FSH at doses below 0.05 μg/l (1.5 × 10^{-12} mol/l) is already able to bind to its receptor and to trigger tPA secretion by Sertoli cells.

Characterization and FSH regulation of Sertoli cell PKA was studied first by Fakunding et al. (23). They employed the Sertoli cell-enriched testis system (SCE) obtained from prenatally irradiated rats and measured directly the PKA activity in a cell-free preparation. By using DEAE chromatography and [32P]-cAMP, they demonstrated the presence of two distinct forms of PKA in Sertoli cells with the ratio of type I to type II PKA activity evolving as a function of Sertoli cell maturation. The application of the PKA activity assay on cytosol obtained from SCE previously incubated with FSH suggests that both types of PKA isozymes are activated by FSH. On the other hand, recent studies on the PKA activity have been performed by Nistico et al. (25) on rat Sertoli cell-enriched cultures. Using the same procedure, they demonstrated that the percentage of specific type I PKA activity varied from 70 to 90%, the small percentage of PKA activity left corresponding to the type II isozyme. The type I isozyme was strongly stimulated by cAMP (9–15 fold), whereas the type II isozyme was slightly cAMP-dependent (stimulation of 20% of the activity). In our experiments, we determined for the first time the respective PKA activities and their modulation by FSH in freshly isolated Sertoli cells owing to the measurement of the specific Sertoli secretion of tPA. We demonstrated that both types of PKA isozymes play a physiological role supported by synergistic interaction via both type I and type II PKA with low stimulatory levels of FSH. A predominance of the type I isozyme was always present.

It is interesting to note that the synergistic interaction with the analogs was much more prevalent with cAMP generated endogenously by FSH than that achieved upon co-exposure with forskolin. Moreover, no significant preponderance of the type I isozyme in the forskolin-induced tPA production could be found. The lack of consistent synergism between forskolin and the type I- or II-specific analog compared to that noted with FSH is difficult to explain, as all treatment paradigms seemingly act via the same cAMP messenger cascade. In the cell, FSH may exert specific effects on other systems, such as inhibition of the Ca^{2+} - phospholipid-dependent pathway (26) and/or the cytosolic calcium rise via calcium channels (27), which might be involved in cooperative activation of the cAMP-dependent kinases. Forskolin may involve intracellular changes independent of adenylate cyclase activation (28–31) that are not optimal for cooperative activation of the PKA. Similar discrepancies were observed between the action of LH and forskolin in rat and mouse Leydig cells (32, 33). The authors suggested that forskolin may instigate other cellular events in addition to cAMP synthesis. Further investigation of the activities of forskolin in tPA production and cAMP-dependent kinases in the Sertoli cell are certainly warranted.

In conclusion, the secretion of tPA in Sertoli cells is triggered when the cAMP concentration reaches a precise threshold and the two types of PKA are present and participate in the stimulatory control of tPA production by FSH in these cells.

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