BIOLOGY OF REPRODUCTION **70,** 1147–1152 (2004)
Published online before print 26 December 2003.
DOI 10.1095/biolreprod.103.023705

Key Role for Cyclin-Dependent Kinases in the First and Second Meiotic Divisions of Rat Spermatocytes[1]

Murielle Godet,[2,3] Anne Damestoy,[3] Sandrine Mouradian,[4] Brian B. Rudkin,[4] and Philippe Durand[3]

INSERM U418/INRA UMR 1245,[3] Hôpital Debrousse, 69322 Lyon, cedex 05, France
Laboratoire de Biologie Moléculaire de la Cellule,[4] CNRS UMR 5161, Ecole Normale Supérieure de Lyon, 69364 Lyon cedex 07, France

ABSTRACT

In all systems examined so far, the G2/M phase transition is controlled by the M-phase promoting factor (MPF), a complex of cdc2 (CDK1) and cyclin B1. Histone H1 kinase activity and MPF components are present in pachytene spermatocytes (PS). However, it has not been demonstrated yet that direct inhibition of MPF activity prevents the G2/M transition in these cells. When roscovitine, a potent inhibitor of CDK1, CDK2, and CDK5 activities, was added to cocultures of PS with Sertoli cells, the number of both secondary spermatocytes and round spermatids formed were lower than in control cultures, despite similar cell viability. This effect of roscovitine was reversible, did not involve the Sertoli cells, and was dependent on the concentration of the inhibitor. Roscovitine did not modify the amount of MPF in these germ cells but inhibited the CDK1- or CDK2-associated histone H1 kinase activity of PS. Hence a functional relationship between cyclin-dependent kinase activity and the spontaneous processing of the first meiotic division and, for the first time, of the second meiotic division of male germ cells is shown.

gametogenesis, kinases, meiosis, spermatogenesis, testis

INTRODUCTION

Spermatogenesis requires a coordinated series of cycle events involving both mitosis and meiosis. Cyclin-dependent kinases and their regulatory subunits, the cyclins, are key regulators of cell progression. In all systems examined so far, progression to mitotic or meiotic metaphase is controlled by the activity of the M-phase promoting factor (MPF), a complex of the cyclin-dependent kinase cdc2 (CDK1) and cyclin B1 (cycB1) [1]. Histone H1 kinase activity and protein components of MPF are present in pachytene spermatocytes [2, 3], and okadaic acid—a phosphatase inhibitor that leads to an activation of MPF—induces a premature G2/MI transition in short-term cultured pachytene spermatocytes [4]. In addition, disruption of the Hsp70-2 gene that encodes for a chaperone protein of CDK1 results in an inability of spermatocytes to progress to metaphase I, resulting in male infertility [5]. Taken together, these results suggest that the G2/MI transition of pachytene spermatocytes involves the action of MPF. However, it has not been demonstrated yet that direct inhibition of MPF activity prevents the spontaneous (not pharmacologically-induced) G2/MI transition in these cells, and thus alternative pathways cannot be excluded. Moreover, since the above experimental protocols lead to an arrest of the cells in metaphase I, they do not bring any information about the possible involvement of MPF activity in the second meiotic division. Finally, the very low number of secondary spermatocytes in testes [3] has precluded functional studies on these cells, particularly to address the role of CDKs and cyclins in the second meiotic division. We have established a system of coculture of pachytene spermatocytes (PS) with Sertoli cells, which allows these germ cells to complete the two meiotic divisions leading to the in vitro formation of round spermatids (RS) [6]. In the present work, we have used this culture system, together with the pharmacological inhibitor of cyclin-dependent kinases, roscovitine [7, 8], to address the role of MPF activity in both the first and second meiotic divisions of rat spermatocytes.

MATERIALS AND METHODS

Roscovitine (2-[R]-[1-ethyl-2-hydroxyethylamino]-6-benzylamino-9-isopropylpurine; Calbiochem, Euromedex, Mundolsheim, France) was dissolved in dimethyl sulfoxide (DMSO; Sigma, la Verpillière, France) and stored in aliquots at −20°C until use. FSH was obtained through National Hormone and Peptide Program (Dr. A.F. Parlow, Torrance, CA).

Isolation and Coculture of Rat Sertoli Cells and PS

These experiments were conducted according to the Guide for Care and Use of Laboratory Animals. Sertoli cells and PS were isolated and cultured as previously described, and cell viability was assessed by trypan blue exclusion [6]. The purity of the PS fraction was assessed by flow cytometry (see below; 94% ± 3% of cells were 4C cells, 3% ± 2% were 2C cells, and 2% ± 0.5% were 1C cells, n = 5). It was found that 15% of elutriated PS were early PS (stages XIV to IV of the rat seminiferous epithelium [9]), 60% were middle PS (stages V to IX), and 25% were late PS (stages X to XIII; M.H. Perrard, personal communication).

Immunocytochemical Studies on Cultured Cells

Cells were rinsed with phosphate buffer saline (PBS) and fixed directly in the well with Bouin fixative for 20 min at room temperature. After washing with PBS, fixed cells were permeabilized with 0.25% Triton X-100 in PBS containing 1% bovine serum albumin (BSA) for 20 min at room temperature. The immunocytochemical reaction was performed using an antiphosphorylated serine 10 (ser10) of histone H3 (Upstate Biotechnology, Euromedex, Mundolsheim, France) as a primary antibody used at a dilution of 1:300 in blocking buffer: 2% BSA, 10% fetal calf serum (Gibco BRL Life Technologie, Cergy-Pontoise, France) in PBS for 3 h at 4°C and revealed with an LSAB2 kit (DAKO, Trappes, France) using avidin-biotin-peroxidase complex as a staining reaction and amino-3-ethyl-9-carbazole as a chromogen. Phosphorylation of histone H3 on ser10 is correlated with chromosome condensation at G2/M in spermato-

[1]Supported by the Institut National de la Santé et de la Recherche Médicale, the Institut National de la Recherche Agronomique, the Centre National pour la Recherche Scientifique, the Ministry of Research and Technology, and the Université Claude Bernard Lyon I.
[2]Correspondence: Murielle Godet, INSERM U418/INRA UMR 1245, Hôpital Debrousse, 29, Rue Sœur Bouvier, 69322 Lyon cedex 05, France. FAX: 33 4 78256168; e-mail: godet@lyon.inserm.fr

Received: 29 September 2003.
First decision: 10 October 2003.
Accepted: 25 November 2003.

ISSN: 0006-3363. http://www.biolreprod.org

cytes [10, 11], and this antibody staining provides a marker for cells in the division phases.

Immunolabeling of Cells for Flow Cytometry

At selected days of culture, cells were detached from culture wells by trypsinization, washed, then fixed with ice-cold 70% ethanol. Immunolabeling of cultured germ cells and flow cytometric analyses were performed essentially as described by Godet et al. [3]. After washing with PBS, fixed cells were resuspended in 150 μl of permeabilizing buffer: 0.25% Triton X-100 and 1% BSA in PBS for 20 min on ice. The cells were exposed to anti-vimentin antibody (clone V9; DAKO) used at a dilution of 1:500 in blocking buffer for 3 h at 4°C. After three washes in PBS/BSA 1%, the cells were exposed to 70 μl of fluorescein (FITC)-conjugated rabbit anti-mouse immunoglobulins (IgG; DAKO) used at a dilution of 1:60 in blocking buffer for 1 h at 4°C. After washing, the cells were incubated with 100 μl of anti-CDK1 antibody (clone 17; Santa-Cruz, Tébu, Le Perray-en-Yvelines, France) or anti-cycB1 antibody (clone GNS1; Becton Dickinson, Le pont-de-Claix, France), both used at a dilution of 1:200 in PBS/BSA 1% and left overnight at 4°C. After washing, CDK1- or cycB1-labeled cells were incubated with 70 μl of phycoerythrin (PE)-conjugated rabbit anti-mouse IgG (DAKO) used at a dilution of 1:60 in PBS/BSA 1% for 1 h at 4°C. Before analysis, Hoechst 33342 (Sigma, La Verpillière, France) was added to the suspensions of labeled cells at a final concentration of 0.12 μg/ml. Two negative controls were included: cells incubated with mouse IgG1 negative control (DAKO) and FITC- or PE-conjugated secondary antibody.

Flow Cytometric and Computer Analyses

After immunolabeling, cells were analyzed using a FacsStar Plus Cytometer (Becton Dickinson) equipped with a 50-mW argon laser tuned to 448 nm and an INNOVA 300 ion multilined/UV laser tuned to UV. Emission fluorescence was measured with a DF 530/30 filter for FITC, a DF 575/26 filter for PE and DF 424/44 filter for Hoechst. Data acquisition and analysis were performed with "CellQuest" software (Becton Dickinson). Six data parameters were acquired and stored in list mode files: linear forward light scatter (FSC) and linear side angle light scatter (SSC), which roughly represent cell size and cellular granularity, respectively; logarithmic (log) PE (CDK1), log FITC (vimentin), and linear PE (cycB1) to detect the immunolabeling; and linear Hoechst to measure the DNA content of the different population of cells. Contaminating events such as debris and clumped cells were eliminated from the analysis. Each acquisition was performed on 50 000 cells negative for vimentin. Flow cytometry using three different parameters (FSC, SSC, and ploidy) allowed the identification of middle to late PS and RS in pure fractions, whereas secondary spermatocytes (SII) were contaminated by doublets of round spermatids. Then the SII were separated from doublets of RS on the basis of their approximately threefold higher content of CDK1. Each sorted population was further characterized by microscopic examination [3]. Hence the percentage of each category of germ cells was multiplied by the total number of cells per well in order to obtain the absolute number of PS, SII, and RS.

Histone H1 Kinase Assay

Equal amounts of proteins (500 μg) from elutriated PS were precipitated with either anti-CDK1 (clone 17) or anti-CDK2 (clone D-12; Santa Cruz, Tébu); monoclonal antibodies; and 40 μl of a 50% (v/v) suspension of protein-A Sepharose per assay. After 3 h of incubation at 4°C, the beads were collected by centrifugation and washed four times with lysis buffer. Histone H1 kinase activities were performed in the absence or presence of 1 μM roscovitine according to the manufacturer's instructions (SignaTECT cdc2 Kinase Assay System, Promega, Charbonnières-Les-Bains, France).

Statistical Analysis

Analysis of variance followed by Fisher test was used throughout.

RESULTS

In the first series of experiments, purified PS were cocultured with Sertoli cells for 5 days. Cell viability decreased slightly from 86% ± 1% on Day 0 of coculture to 75% ± 1% on Day 5. Over that period the number of PS

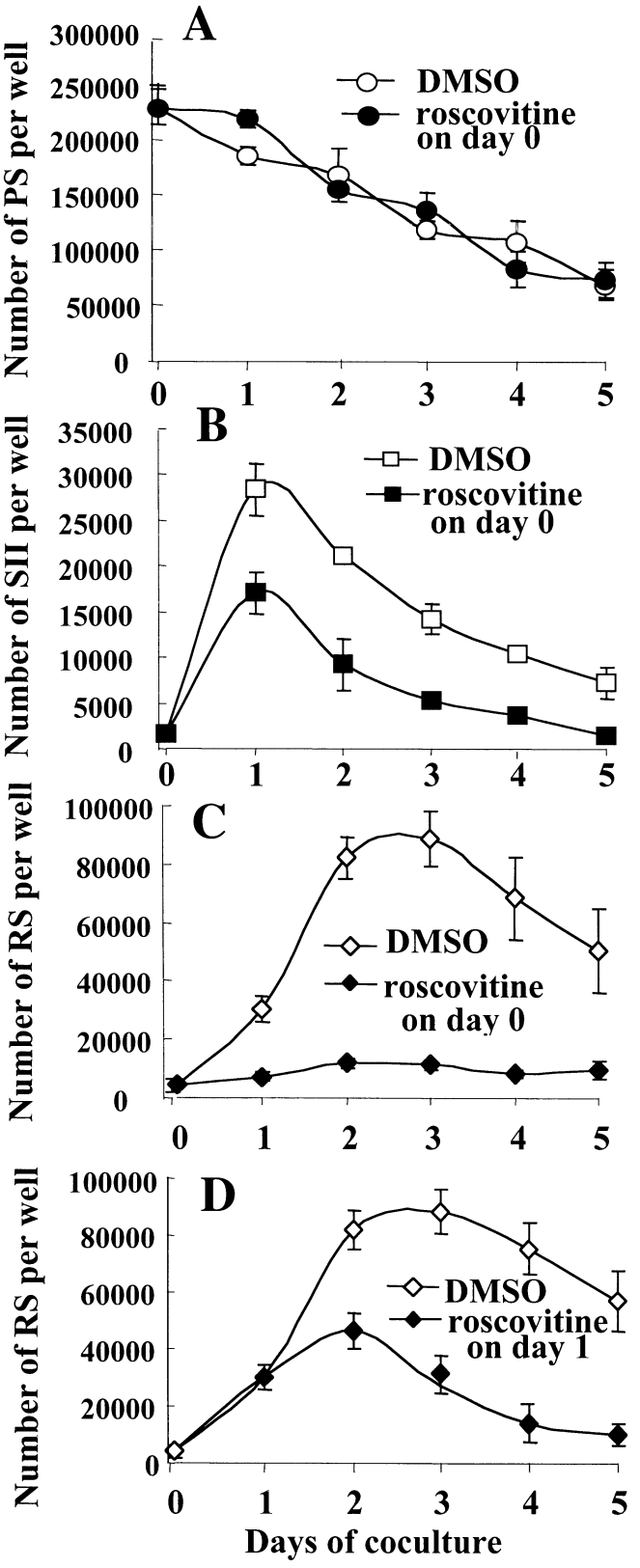

FIG. 1. Kinetics of disappearance of PS (**A**) and of formation of SII (**B**) and RS (**C** and **D**) in cocultures of PS and Sertoli cells in the absence or continuous presence of roscovitine. DMSO (control) or roscovitine (12.5 μM) was added to coculture medium either at the time of seeding of PS on Sertoli cells (Day 0; **A**, **B**, and **C**) or on Day 1 of coculture (**D**). Results are the mean ± SEM of three different experiments.

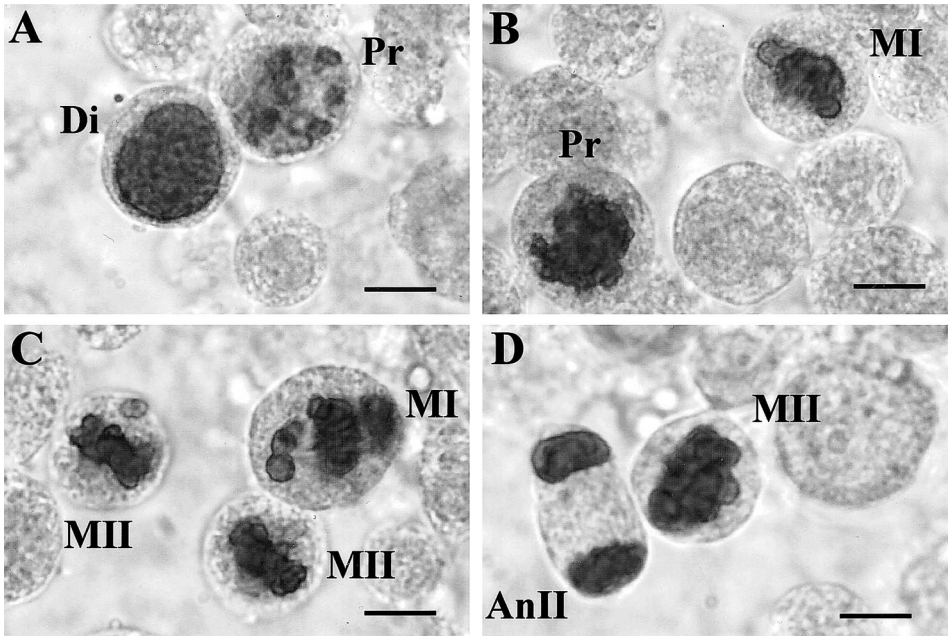

FIG. 2. Meiotic divisions occur in cocultures of PS with Sertoli cells. After 2 days of coculture, cells were fixed and stained with ser10 phosphorylated histone H3 antibody. Diplotene (Di) and prometaphase I (Pr) nuclei (**A** and **B**); metaphase I (MI; **B** and **C**) and metaphase II (MII; **C** and **D**); and anaphase II (AnII; **D**). Preparations were counterstained with hematoxylin. Note the different size of MI and MII figures. Bar = 10 μM.

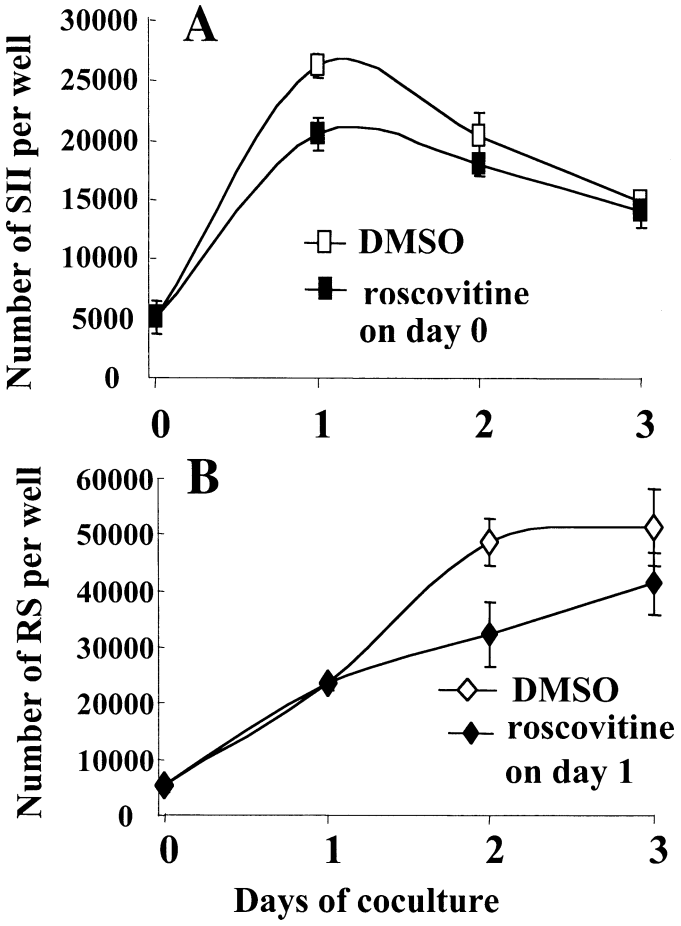

FIG. 3. Changes in the number of SII (**A**) and RS (**B**) in cocultures of PS and Sertoli cells after a 3-h treatment of the cocultures with DMSO or roscovitine. DMSO (control) or roscovitine (12.5 μM) was added to coculture medium for 3 h either on Day 0 (**A**) or on Day 1 (**B**); then the cells were washed and the cocultures were prolonged up to Day 3. Results are the mean ± SEM of three different experiments.

decreased steadily from 229 900 ± 19 200 on Day 0 down to 83 400 ± 7900 on Day 5 (n = 3; $P < 0.001$; Fig. 1A). Some of the PS underwent the two meiotic divisions as illustrated in Fig. 2, which shows cells in division phase as evidenced by labeling of phosphorylated ser10 of histone H3.

Very few SII were present on Day 0 (2200 ± 600) of culture, but their number increased dramatically up to 28 900 ± 3300 on Day 1; thereafter, their number decreased until the end of the culture, but remained always higher than on Day 0 ($P < 0.05$; Fig. 1B). Likewise, only few RS contaminated elutriated PS (5600 ± 1200) at the time of seeding. However, the number of RS increased rapidly, reaching its maximal value on Day 2 (82 000 ± 6900; $P < 0.001$ vs. Day 0; i.e., 1 day later than for SII, which matches the duration of the stage XIV of the seminiferous epithelium of the rat) [9]. Thereafter, it decreased slightly between Day 3 (85 000 ± 4600) and Day 5 (53 500 ± 12 200; $P < 0.02$; Fig. 1C).

When 12.5 μM roscovitine was present in culture media from Day 0 onward, cell viability was identical to that of control cultures on Day 5 (74% ± 2%). Likewise, no effect of roscovitine on the number of PS was ever observed (Fig. 1A). By contrast, the number of SII in roscovitine-treated cultures was only 30%–60% of that of control cultures from Day 1 onward ($P < 0.001$; Fig. 1B), indicating that roscovitine inhibited the first meiotic division of PS. Moreover, very few RS were observed in the presence of the inhibitor (20%–25% of controls; Fig. 1C), which suggests that roscovitine also inhibited the second meiotic division.

In an attempt to confirm this assumption, in the second set of experiments roscovitine was added to culture media only from Day 1 onward (i.e., at the time when the number of SII was maximal). Under such conditions, the number of RS in roscovitine-treated cultures was decreased, when compared with controls, already from Day 2 ($P < 0.02$; Fig. 1D). It should be noted that when roscovitine was added on Day 1, the number of RS on Day 2 was higher than when roscovitine was put on Day 0. This results from the half-life of RS, which is 1.5 days under our culture conditions (M. Vigier, personal communication; see discussion

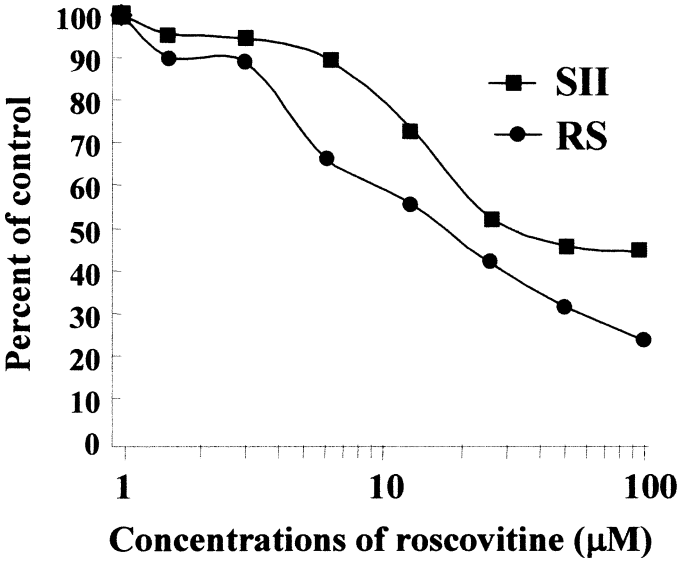

FIG. 4. Representative dose-response curves of the inhibitory effect of roscovitine on the formation of SII and RS. PS were incubated for 3 h in the absence or presence of different concentrations of roscovitine. Results show the number of SII and RS on Day 1 of coculture and are expressed as a percentage of the number of SII (25 406) or RS (28 142) formed in the absence of the inhibitor (control). Similar results were obtained in three additional experiments.

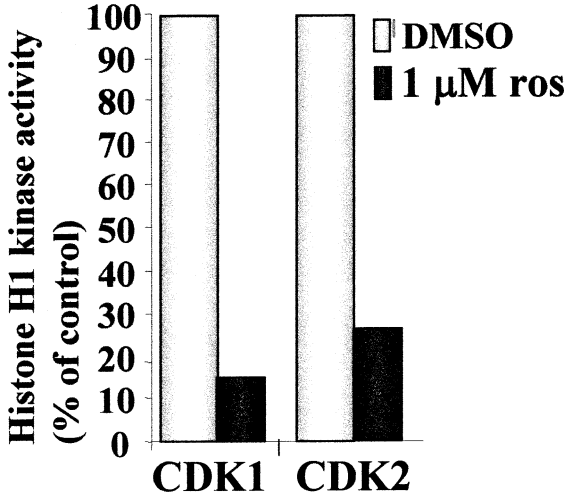

FIG. 5. CDK1- and CDK2-associated histone H1 kinase activities of PS in the absence or presence of roscovitine. CDK1 and CDK2 were immunoprecipitated with specific antibodies from PS lysates; then the histone H1 kinase activity of each fraction was assessed in the presence of either DMSO or 1 μM roscovitine. Results are the mean of two different experiments and are expressed as a percentage of the values obtained in controls.

below). Taken together, these results indicate that roscovitine is inhibiting both the first and the second meiotic divisions of spermatocytes.

In order to determine whether the inhibitory effect of roscovitine was reversible, in the next series of experiments cocultures of PS and Sertoli cells were treated with 12.5 μM roscovitine for 3 h, either on Day 0 or on Day 1, and then the cells were washed to remove the drug and the cultures were prolonged up to Day 3. As expected, roscovitine treatment did not modify the number of PS at any time (not shown). When roscovitine was added to culture media on Day 0, the number of SII on Day 1 was only 78% ± 2% of that of control (n = 3, $P < 0.01$), but thereafter the number of SII in roscovitine-treated wells was similar to that in control wells (Fig. 3A). Likewise, when roscovitine was added on Day 1, the number of RS was lower (65% ± 6%) than in controls ($P < 0.02$) on Day 2, but no longer different on Day 3 (Fig. 3B). These results demonstrate the reversibility of a short treatment with roscovitine on both the first and the second meiotic divisions.

In order to define whether the effect of roscovitine on the meiotic divisions of germ cells involved the Sertoli cells, in the next experiments PS were preincubated for 3 h with 12.5 μM roscovitine, washed, and then seeded on Sertoli cell monolayers. Under such conditions, the number of SII on Day 1 of coculture was 79% ± 4% of that of control (n = 4; $P < 0.02$), which is identical to the percentage observed when the inhibitor was added to PS/Sertoli cell cocultures (see above). Likewise, the number of RS on Day 1 was very close when either PS together with Sertoli cells were treated with roscovitine for 3 h on Day 0 of coculture (13 900 ± 1100) or preincubated for 3 h with roscovitine before seeding on Sertoli cells (12 000 ± 1400). These data indicate that the effect of roscovitine on the meiotic divisions is exerted essentially, if not solely, through the germ cells.

This effect of roscovitine was dependent on the concentration of the inhibitor with an apparent half-maximum effect close to 15 μM, both for the first and the second meiotic divisions, as shown in Fig. 4.

In the next series of experiments, we researched as to whether the effect of roscovitine on male meiotic divisions was due to an inhibition of the histone H1 kinase activity of MPF as in the oocyte [1]. A 3-h treatment with 12.5 μM roscovitine either on Day 0 or on Day 1 did not modify the levels of either cycB1 or CDK1 in PS, SII, or RS at any day of a 3-day culture (Table 1 and data not shown). Since roscovitine acts as a competitive and reversible inhibitor of ATP binding and is removed after washing the cells (see above), histone H1 kinase activity of PS incubated with roscovitine could not be assessed directly. However, roscovitine inhibited the CDK1- and CDK2-associated histone H1 kinase activities of PS (Fig. 5). Taken together, these results suggest strongly that at least part of the inhibitory effect of roscovitine on the male meiosis is exerted through an inhibition of MPF activity of PS.

DISCUSSION

Several teams have now demonstrated that meiosis can proceed in vitro when mammalian spermatogenic cells are cocultured with Sertoli cells [6, 12–16]. In rodents, the kinetics of the meiotic process are similar in vivo and during the first week of culture [6, 12, 15]. Moreover, it has been

TABLE 1. Relative levels of cycB1 and CDK1 proteins in PS, SII, and RS on Day 1 of a 3-day coculture of PS with Sertoli cells.*

Treatment on Day 0	SP	SII	RS
cycB1 control	336 ± 39	201 ± 16	95 ± 4
cycB1 roscovitine	311 ± 15	207 ± 16	96 ± 16
CDK1 control	208 ± 50	140 ± 18	36 ± 8
CDK1 roscovitine	214 ± 29	157 ± 25	42 ± 10

* PS were cocultured with Sertoli cells under the condition described in the legend of Figure 3. The relative levels of cycB1 and CDK1 in different populations were determined by flow cytometry as described in the *Materials and Methods* section. Results are the mean ± SEM for three different experiments. Similar results were also obtained on Days 2 and 3 of cultures.

shown recently that RS developed in vitro can produce normal offspring in the mouse [17]. Taken together, these results support the use of such culture systems to address some aspects of the meiotic process.

The present results have shown that roscovitine, a potent inhibitor of MPF, CDK2-cyclin A, CDK2-cyclin E, CDK5-p25, and, to a lesser extent, MAP kinase activities [7, 8] is able to inhibit in a dose-dependent manner both the first and the second meiotic divisions of spermatocytes. At the concentration of 12.5 μM, which is close to that giving half of the apparent maximum effect (see below) on either the first or the second meiotic division, this effect was not due to cell toxicity. This is ascertained by similar cell viability in both control and roscovitine-treated cultures and the resumption of the meiotic process after removal of the inhibitor. Hence it appears that both the first and the second meiotic divisions of PS are regulated by some cyclin-dependent kinase(s), the activity of which can be inhibited by roscovitine.

Previous studies indicate that CDK1 and CDK2 are present in both PS and SII [2, 3, 18]. It has been reported recently that disruption of the CDK2 gene in the mouse leads to an arrest of spermatogenesis in late prophase of the first meiotic division [19]. Moreover, cyclin A1, which binds both CDK1 and CDK2, is needed for completion of the first meiotic prophase of spermatocytes [20]. However, CDK2 kinase activity is required for entry into mitosis as a positive regulator of MPF activity [19], and cyclin A1 has an essential role in initiating the activation of MPF at the meiotic G2/MI transition of male germ cells [21, 22]. Those data, together with the present results, including those showing similar levels of cycB1 and CDK1 in cultured PS and SII (Table 1), suggest strongly that both CDK1 and CDK2 are operating during the first and second meiotic divisions of spermatocytes. In addition, a possible involvement of CDK5 in this process cannot be excluded, since CDK5 was localized to the microtubules of metaphase spermatocytes [23]. The present data also fit rather well with recent results showing that a knockout of Cks2, a protein that interacts with CDK1, leads to defects in meiosis in males (and females) [24]. Specifically, germ cells do not progress past the first meiotic division as the result of a failure to enter anaphase. Since the defect involves the lack of interaction between Cks2 and a CDK, it gives further support to the requirement of cyclin-dependent kinase activity in male meiotic divisions.

It could seem surprising that a concentration of 12.5 μM roscovitine induced only a partial inhibition of the formation of RS, since it has been reported that the IC_{50} for roscovitine of the in vitro kinase activities of MPF and CDK2 cyclin A and E are 0.45 and 0.7 μM, respectively [7]. However, numerous parameters can influence the effects of an inhibitor on intact cells, such as cell membrane permeability, compartmentalization, and degradation. Indeed, in a variety of cultured cancer cells, IC_{50} values for roscovitine range from 7.9 to 30.2 μM [25]. Moreover, it must be recalled that the number of RS counted, on either day of culture, is the sum of the number of RS formed between this day and the day before and the number of the RS surviving from, but formed during, the preceding days. Since the half-life of RS in culture is 1.5 days (P. Durand, unpublished results), the number of RS formed every day can be easily calculated. Thus, it can be concluded that 12.5 μM roscovitine actually resulted in a complete inhibition of RS formation beyond 24 h of treatment. Finally, the IC_{50} values of 0.45 and 0.7 μM for roscovitine of purified CDK1 and CDK2, respectively, compare quite well with the concentration of 1 μM, which gave more than 80% of inhibition of the CDK1- or CDK2-associated histone H1 kinase activity of PS lysates (Fig. 5). Taken together, these data make it unlikely that the observed effects of roscovitine might be mediated through an inhibition of MAPK activity. Indeed, the IC_{50} of roscovitine on purified ERK1 and ERK2 was reported to be 34 and 14 μM, respectively [8]. This is 30- to 60-fold higher than the value needed for half inhibition of purified MPF, and even higher than the concentration of roscovitine that induced a complete inhibition of RS formation from cultured PS.

Thus a functional relationship has been identified and characterized between roscovitine-sensitive cyclin-dependent kinase activities and the spontaneous processing not only of the first meiotic division, but also, for the first time, of the second meiotic division of spermatocytes. Hence, these results support the importance of MPF for progression of male germ cells to meiotic metaphase I and metaphase II, as found in the oocyte [1].

ACKNOWLEDGMENTS

We are grateful to Dr. Laurent Meijer for helpful discussions and comments regarding the manuscript, and to Michèle Vigier for help with the elutriation of germ cells.

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