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Journal of Steroid Biochemistry & Molecular Biology 85 (2003) 167–173

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Progesterone as a regulator of granulosa cell viability☆

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Abstract

Progesterone (P4) prevents numerous cells, including uterine, mammary and ovarian cells, from undergoing apoptosis. Interestingly, P4 prevents apoptosis of ovarian granulosa cells (GCs), which do not express the classic nuclear P4 receptor. This review presents data that support a non-genomic action of P4 in granulosa cells. These studies were conducted using both primary rat granulosa cells and rat spontaneously immortalized granulosa cells (SIGCs). Specifically, these studies reveal that (1) ^{3}H-P4 specifically binds to SIGCs; (2) an antibody directed against the ligand binding domain of the nuclear P4 receptor (C-262) detects a 60 kDa protein, which localizes to the plasma membrane and binds P4; and (3) treatment with C-262 blocks P4's ability to maintain granulosa cell viability. Additional studies demonstrate that a protein kinase G (PKG) activator, 8-br-cGMP, mimics and PKG antagonists, Rp-8-pcCPT-GMP and KT5823, attenuate P4's action. These studies support the concept that the 60 kDa P4 binding protein functions as membrane receptor for P4 which activates a PKG-dependent mechanism to regulate granulosa cell survival.
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Keywords: Granulosa cell; Apoptosis; Progesterone; Protein kinase G

1. Cell specific responses to progesterone

Progesterone (P4) promotes the viability of several different cells (Table 1), although in breast and ovarian cell lines P4 induces apoptosis [1,2]. In breast and ovarian cells, P4 blocks mitosis with the apoptotic effects of P4 not being observed until 48 or 72 h after treatment [1,2]. This delayed response suggests that the ability of P4 to induce apoptosis is a result of its anti-mitotic actions.

Regardless of its biological effect, it is generally assumed that P4 binds to its nuclear receptor (PR) and subsequently modulates gene transcription and cellular responses. A casual review of the literature supports the concept. For example, lymphoma-derived cells express the classic nuclear PR and P4 acts directly on these cells to prevent tumor necrosis factor alpha (TNFα)-induced apoptosis [3]. However, not all of P4's anti-apoptotic actions can be explained by this mechanism. P4 maintains the viability of mammary epithelial cells of lactating rats [4,5] but these cells do not express the PR [6,7]. Corticosterone also prevents mammary epithelial cell apoptosis [4,5] and these cells express the glucocorticoid receptor (GR) [6,7]. Since P4 can bind and activate the GR [8,9], it is likely that in mammary epithelial cells P4 mediates its anti-apoptotic action through the GR and not the PR. A similar GR-mediated mechanism could account for P4's anti-apoptotic effect in rat luteal cells, which express GR and not PR [10].

Another way P4 can prevent apoptosis is through a paracrine action. Several studies have shown that P4 prevents uterine epithelial cells from undergoing apoptosis [11]. Since these cells express PR [11], it is logical to assume that P4 controls the viability of uterine epithelium via the PR. To directly test this assumption, tissue recombinants were made in which uterine epithelium from PR knock-out mice were combined with PR positive uterine stroma of wild-type mice. In these recombinants, P4 maintained uterine epithelial cell viability. In contrast, P4 did not maintain the uterine epithelium of recombinants made with PR positive uterine epithelium and PR negative uterine stroma. These observations indicate that P4 preserves uterine epithelial cells indirectly by acting on stromal cells even through the epithelial cells express functional PRs.

Interestingly, there are two cells in which P4 prevents apoptosis by a mechanism that cannot be explained by an interaction with either the PR, the GR or a paracrine mechanism. In both thymocytes and granulosa cells (GCs) isolated from immature rat ovaries, P4 prevents apoptosis in vitro even through these cells do not express the PR [12,13]. Further, activation of the GR can be ruled, since glucocoidocoids induce apoptosis of thymocytes [12] and do not

☆ Presented at the 11th International Congress on Hormonal Steroids and Hormones and Cancer, ICHS & ICHC, Fukuoka, Japan, 21–25 October 2002.

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doi:10.1016/S0960-0760(03)00192-4

Table 1
The effect of progesterone on the viability of various cells

Cell type	PR status	Site of action
Lymphoma-derived (U937) cells)	Present [3]	P4 blocks TNFα-induced apoptosis in vitro, indicating a direct effect of P4 [3].
Mammary epithelium of lactating rats	Absent [5,6]	P4 and corticosterone block apoptosis in ovariectomized/adrenalectomized lactating rats [5,6]. This action could be mediated by the GR receptor, since GRs but not PRs are expressed in these cells [5,6].
Uterine epithelium	Present [11]	Tissue recombinant studies from PRKO and wild-type mice suggest a paracrine action [11].
Decidual (stromal-derived) cells	Present [43]	RU 486 induces apoptosis of these cells in vivo but a site of action cannot be determined from these in vivo studies [44].
Neurons within the hippocampus, dorsolateral caudate nucleus and substantia nigra	Present [45]	In vivo studies show that P4 reduces neuronal damage after ischemia [46]. P4 also protects hippocampal neurons in vitro from glutamate toxicity. P4 activates Erk pathway and Bcl-2 expression with the Erk response occurring within 30 min [47]. This could indicate that P4 promotes cell survival by a non-genomic mechanism.
Thymocytes	Absent [13]	P4 not only inhibited spontaneous thymocyte apoptosis in vitro, but also prevented in vitro glucocorticoid-induced apoptosis [12]. This suggests that P4 has a direct effect of thymocytes in the absence of PR.
Granulosa cells: pre-gonadotropin (LH) surge	Absent [20,21]	P4 inhibits apoptosis in a dose-dependent manner in vitro. This action is not mimicked by dexamethasone or other steroids [26,27,48].
Granulosa cells: post-gonadotropin (LH) surge	Present [21,25]	PR antagonists, RU 486 and Org 31710, induce apoptosis in hCG-exposed rat and human GCs [25,49].
Luteal cell	Present in bovine luteal cells [50], but absent in rat luteal cells [10]	In bovine luteal cells, aminoglutethimide (AG) blocks P4 secretion and induces apoptosis. AG-induced apoptosis can be prevented by P4 [50]. In rat luteal cells RU 486 induced apoptosis of rat luteal cells [51]. Further, P4 blocked Fas-induced apoptosis in rat luteal cells in vitro [52].

effect the viability of immature granulosa cells [14]. In this light, immature rat granulosa cells provide a very interesting model to assess P4's ability to maintain cell viability in the absence of its nuclear receptor.

2. Granulosa cells as a model system to study P4's anti-apoptotic action

P4 is one of the major steroids synthesized and secreted by the ovary [15]. P4 is synthesized by preantral follicles with just two to four layers of granulosa cells [16] with the rate of P4 secretion increasing as follicular development proceeds [16,17]. Although the concentration of P4 within the antral follicles fluctuates throughout the estrous cycle, the concentration of P4 within antral follicles is always in the micromolar range [17]. The importance of these high P4 levels in regulating ovulation has been emphasized by several studies [18,19]. These investigations have shown that the nuclear PR is expressed by granulosa cells just prior to ovulation [20,21]. Further treatment with PR antagonists or genetic ablation of the nuclear PR interferes with gonadotropin-induced ovulation [18,19,22–24].

Based on the expression pattern of the PR, it is predictable that P4 inhibits apoptosis of granulosa cells isolated during the periovulatory period [25]. Surprisingly, P4 also prevents apoptosis of granulosa cells isolated from immature rats prior to the gonadotropin (LH) surge (Fig. 1A) [26,27]. P4 does so in a steroid-specific, dose-dependent manner [26,27]. Similarly, P4 inhibits apoptosis of spontaneously immortalized granulosa cells (SIGCs) (Fig. 1B) [27]. These observations are controversial due to the fact that granulosa cells isolated prior to the ovulatory gonadotropin surge do not express the classic nuclear PR [20,21].

3. Possible mediators of P4's anti-apoptotic action

The mechanism through which P4 regulates the viability of granulosa cells of immature rats is not well defined. This is due in part to the failure to conclusively identify a receptor that mediates P4's actions. Over the years several different types of receptors have been proposed to account for P4's effects on immature granulosa cells.

For example, $GABA_A$ receptors have been suggested as a mediator of P4's actions since P4 can modulate $GABA_A$ receptor activity [28,29]. However this does not appear to be the case given that a $GABA_A$ receptor-activating metabolite of P4 neither displaces ^{3}H-P4 binding nor inhibits granulosa cell and SIGC function as effectively as P4 [30].

It is possible that P4 transduces its survival signal by activating the GR. The GR is expressed by granulosa cells [8,9] and P4 can activate this receptor [8,9]. This possibility is also unlikely, since dexamethasone, a GR agonist, does not displace ^{3}H-P4 binding [30] or mimic P4's anti-apoptotic [14].

Recent studies have shown that the nuclear PR can localize to the plasma membrane and function as a membrane receptor for P4 [31–33]. As seen in Fig. 2, PR mRNA is not be detected in RNA isolated from SIGCs. This failure to detect PR mRNA is not due to the quality of SIGC RNA,

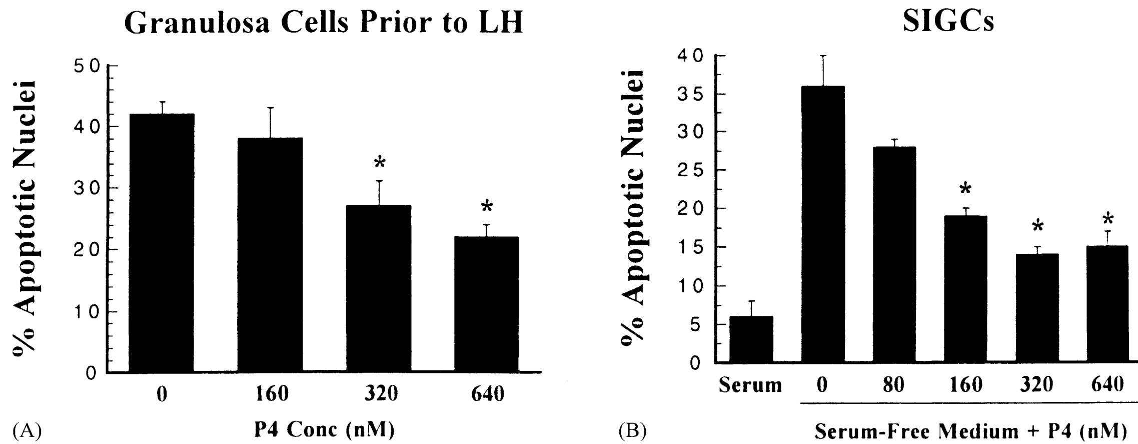

Fig. 1. The effect of P4 on the percentage of apoptotic primary granulosa cells (A); SIGCs (B) (taken from Peluso et al. [27]). The primary granulosa cells were isolated from immature rat ovaries as outlined by Peluso et al. [30]. SIGCs were cultured in serum-supplemented medium prior to treatment. To assess the effect of P4, primary granulosa cells and SIGCs were placed in serum-free medium with increasing concentrations of P4. Primary granulosa cells were cultured for 24 h while the SIGCs were cultured for 5 h. After culture, the cells were stained with YOPRO-1 to identify apoptotic nuclei; (∗) indicates a value that is significantly different from serum-free treatment group ($P < 0.05$).

since moesin is readily detected. These findings are consistent with published reports that indicated that PR mRNA is not present within rat granulosa cells until after the gonadotropin surge [20,21]. These findings suggest that the nuclear PR is not functioning as a membrane P4 receptor in either primary granulosa cells or SIGCs.

Although its identity is not known, both granulosa cells and SIGCs express a protein that binds P4. Ligand binding studies indicate that ^{3}H-P4 binds to a single binding site within SIGCs with an apparent K_d of ≈300 nM (Fig. 3) [27]. P4's binding capacity is also revealed by studies using FITC-BSA-conjugated-P4 as a ligand. In these studies, FITC-BSA-conjugated-P4 specifically binds to the plasma membrane of granulosa cells [14]. These studies show that FITC-BSA-conjugated P4 is not detected within the nucleus even after 45 min of incubation. Moreover, FITC-BSA-P4 binding could be attenuated with an antibody (C-262) directed against the ligand binding domain of the nuclear PR [14]. By using this antibody in a ligand blot assay, it has been demonstrated that this antibody detects a 60 kDa protein, which is localized to the plasma membrane and binds P4 (Fig. 4) [27]. This C-262 antibody also blocks P4's anti-apoptotic action [26]. Taken together, these studies are consistent with but do not prove the concept that this 60 kDa protein functions as a membrane receptor for P4.

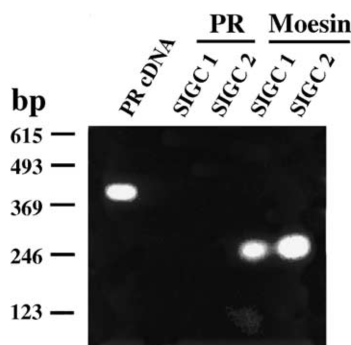

Fig. 2. RT-PCR analysis of progesterone receptor (PR) and moesin expression in RNA isolated from two different SIGC cultures. The PR cDNA was run as a positive control for PR RNA. Figure modified from Peluso et al. [30].

4. P4 anti-apoptotic action and membrane-initiated events

The previously cited studies suggest that P4 transduces its anti-apoptotic signal through a 60 kDa protein. It is possible that P4's action is mediated via a non-genomic, membrane-initiated mechanism. This hypothesis is supported by several observations. First, the 60 kDa P4 binding protein localizes to the plasma membrane as determined by both biochemical and immunocytochemical analyses [27,30]. Second, P4 that is conjugated to a large molecule (i.e. horseradish peroxidase or BSA) to prevent entry into the cells, mimics P4's anti-apoptotic actions [14]. These P4-conjugates do so at essentially the same concentrations as P4. Third, P4 is required to be present continuously to maintain $[Ca^{2+}]_i$ and can act to rapidly suppress $[Ca^{2+}]_i$ (Fig. 5) [27]. Fourth, P4 suppresses the phosphorylation of Erk within 5 min of treatment [30]. Finally, deprivation of P4 for just 10–20 min is sufficient to irreversibly initiate the apoptotic cascade [34]. These rapid effects cannot be explained by a genomic mechanism.

Collectively, these findings support the hypothesis that P4, acting via membrane-initiated events, regulates granulosa cell apoptosis. However, very little is known about the mechanism of P4's action. Recent evidence demonstrate that P4 controls apoptosis by maintaining low basal $[Ca^{2+}]_i$

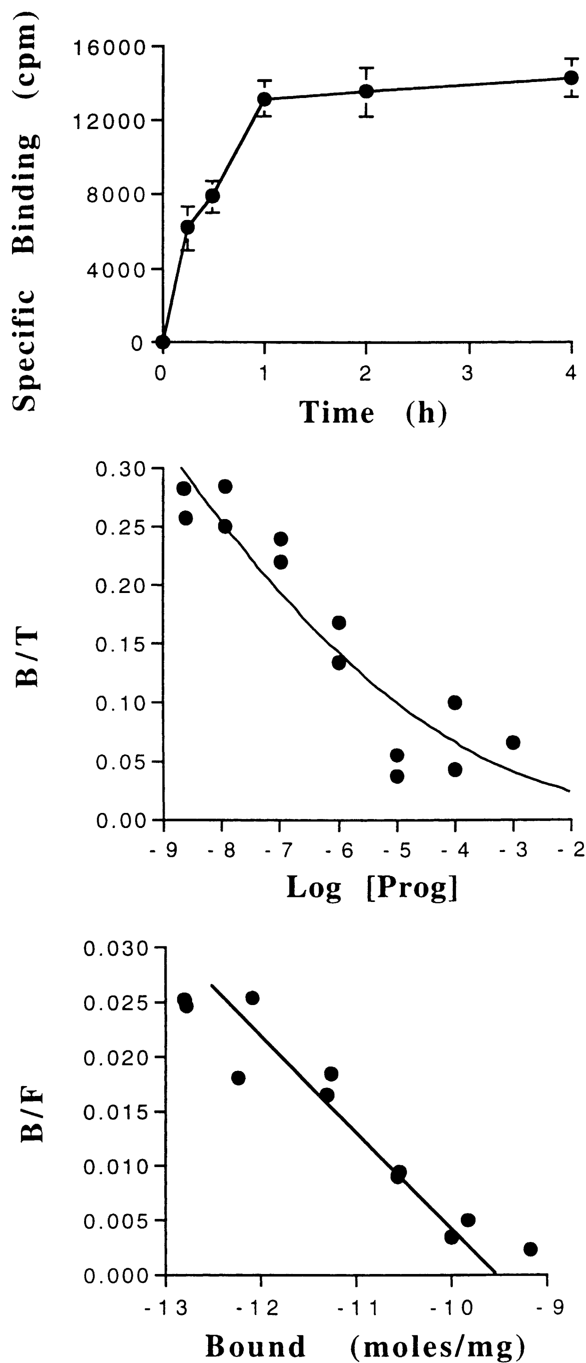

Fig. 3. Ligand binding analysis of ^{3}H-progesterone binding to SIGCs. In the upper panel, ^{3}H-progesterone (1 nM) was incubated with SIGCs for various time intervals in the presence or absence of 1 mM non-labeled progesterone. Specific binding was determined by subtracting the total cpm bound from the cpm bound in the presence of 1 mM progesterone. Values are mean ± 1S.E. In the middle panel, the effect of increasing concentrations of non-labeled progesterone on ^{3}H-progesterone binding is shown. Values are expressed as a ratio of specifically bound ^{3}H-progesterone (B) to the total counts (T) added. A Scatchard plot analysis is shown in the lower panel. Figure taken from Peluso et al. [27].

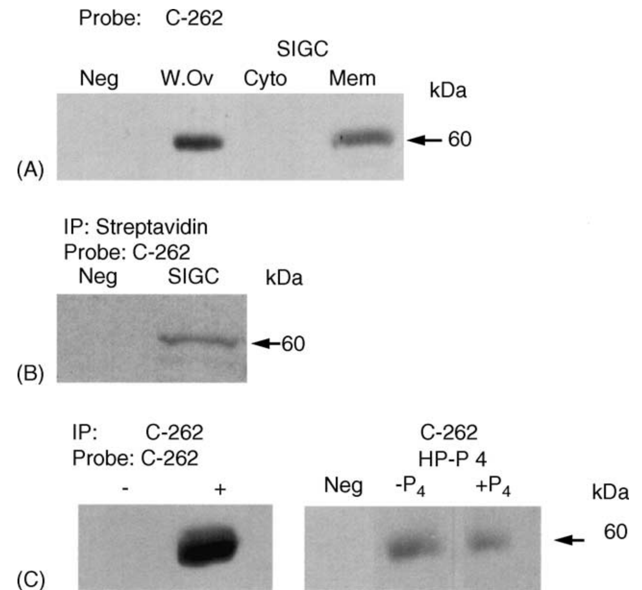

Fig. 4. Western blot and ligand blot analyses of a 60 kDa P4 binding protein in SIGCs. Panel (A) is a Western blot of SIGC cytoplasm (Cyto) and membrane (Mem) preparations. A whole ovarian lysate (W.Ov) is shown as a positive control. Panel (B) is a Western blot of biotinylated membrane proteins of SIGCs after affinity purification with streptavidin beads. A ligand blot analysis of SIGCs is shown in panel (C). On the left side of panel (C) a C-262 immunoprecipitate is shown. On the right side of panel (C) a C-262 immunoprecipitate was probed with 0.5 μM horseradish perioxidase conjugated-P4 (HP-P4) in the presence (+P4, 10 μM) or absence (−P4) of non-labeled P4. Figure taken from Peluso et al. [27].

levels [27,30] (Fig. 5). This implies that understanding how P4 regulates $[Ca^{2+}]_i$ could reveal P4's signal transduction pathway and ultimately the identity of P4's putative membrane receptor.

5. P4, protein kinase G and intracellular calcium

P4 maintains low basal levels of $[Ca^{2+}]_i$ not only in granulosa cells but also cardiac muscle cells, smooth muscle cells and endothelial cells [35–39]. Although the mechanism of P4's actions in these cells has not been elucidated, cGMP-dependent protein kinase 1, also known as protein kinase G (PKG), plays an essential role in controlling $[Ca^{2+}]_i$ in these non-ovarian cells [40]. Specifically, this enzyme acts to (1) stimulate calcium efflux by activating the plasma membrane calcium ATPase; (2) inhibit calcium influx by closing calcium channels; and (3) block IP_3 receptor mediated calcium release from cellular stores [40]. As evidenced by its broad range of targets, PKG could represent a pivotal regulatory point through which P4 influences $[Ca^{2+}]_i$ and ultimately granulosa cell viability.

Pharmacological data indicate that PKG is involved in P4's anti-apoptotic action. For example, a PKG activator, 8-br-cGMP, prevents SIGC apoptosis. Conversely, Rp-8-pCPT-cGMP and KT5823, PKG antagonists, attenuate

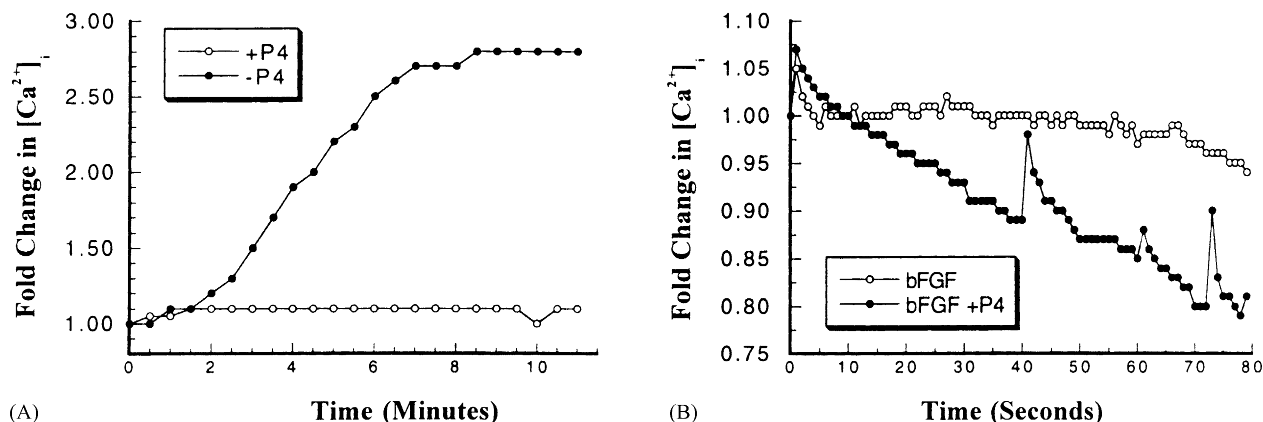

Fig. 5. The effect of P4 (640 nM) on $[Ca^{2+}]_i$ levels. SIGCs were loaded with Fluo-4 AM, a calcium dye indicator, in the presence of bFGF (10 ng/ml), washed and observed under FITC filter set. After a 3.5 min stabilization period, bFGF-supplemented buffer was replaced with either vehicle or P4-supplemented buffer (A). For the studies shown in panel (B), bFGF was present throughout the test period and either vehicle or P4 added after the 3.5 min stabilization period. $[Ca^{2+}]_i$ levels were expressed as an increase from the 3.5 min value. In panel (A) the average standard deviation was 0.58 and 0.09 for $[Ca^{2+}]_i$ levels in the absence or presence of P4, respectively. The average standard deviation in panel (B) was 0.10 for both groups. Figure taken from Peluso et al. [27].

P4's anti-apoptotic action (Peluso, unpublished observations). Preliminary studies also suggest that KT5823 reduce P4's ability to maintain calcium homeostasis (Peluso, unpublished observations). Finally, studies conducted in the 1980s reveal an inverse correlation between follicular fluid concentrations of cGMP and granulosa cell apoptosis [41,42]. These observations imply that PKG mediates P4's anti-apoptotic action.

6. Conclusions and future directions

The studies conducted to date suggest that P4's anti-apoptotic action is mediated through a 60 kDa P4 binding protein. P4 binding to this protein appears to activate a series of membrane-initiated events that regulate calcium homeostasis and ultimately granulosa cell viability. Based on pharmacological studies, it appears that P4 promotes calcium homeostasis by a PKG-dependent mechanism. While these studies constitute a framework, considerably more work is needed to more completely elucidate P4's non-genomic actions. Specifically, the identity of the 60 kDa protein must be determined. In addition, more detailed studies that utilize biochemical and genetic approaches must be conducted in order to establish a cause and effect relationship between P4, PKG, the regulation of intracellular calcium levels and granulosa cell survival.

Acknowledgements

The author would like to thank Ms. Anna Pappalardo, Dr. Gus Fernandez and Dr. Terri Bremner for their assistance in conducting the experiments outlined in this review. The author would also like to acknowledge the thoughtful insights provided by Dr. Bruce White of the Department of Physiology, University of CT Health Centre. This work was supported by NIH grant HD 34383.

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