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Extracellular calcium protects against verapamil-induced metaphase-II arrest and initiation of apoptosis in aged rat eggs

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Abstract

Non-specific L-type calcium channel blockers, such as verapamil (≥50 μM), induce metaphase-II (M-II) arrest and apoptosis in aged rat eggs cultured in Ca^{2+}-deficient medium. However, the effects of extracellular Ca^{2+} on verapamil-induced M-II arrest and apoptosis have not yet been reported. We have demonstrated that postovulatory aging induced exit from M-II arrest by extruding a second polar body, a morphological sign of spontaneous egg activation (SEA). Verapamil inhibited SEA and induced egg apoptosis in a dose-dependent manner in Ca^{2+}-deficient medium. The initiation of apoptotic features was observed at 50 μM of verapamil. Extracellular Ca^{2+} (1.80 mM) reduced intracellular H_2O_2 level, bax protein expression, caspase-3 activity, DNA fragmentation and protected against 50 μM, but not higher concentrations of ≥100 μM in verapamil-induced egg apoptosis. These results suggest that extracellular Ca^{2+} ions have a role during SEA and protect against verapamil-induced apoptosis in aged rat eggs.

Keywords: Apoptosis; Calcium; Caspase-3 activation; DNA fragmentation; Hydrogen peroxide; Metaphase-II arrest; Verapamil

1. Introduction

Intracellular calcium homeostasis is very important in maintaining the normal functions of a cell (Whitaker and Patel, 1990). The transition from one meiotic phase to the next is regulated by cell cycle control checkpoints, which are in turn modulated by a transient increase of intracellular calcium ion $[Ca^{2+}]_i$ (Tosti, 2006; Boni et al., 2007). A transient increase of $[Ca^{2+}]_i$ triggers several events that activate eggs, including cortical granule exocytosis, pronuclear formation, exit from metaphase-II (M-II) arrest and extrusion of a second polar body at the time of fertilization (Kline and Kline, 1992; Xu et al., 1997; Raz et al., 1998; Whitaker, 2006). In the absence of fertilization, postovulatory aging mimics the action of fertilizing spermatozoa, increases $[Ca^{2+}]_i$ (Vincent et al., 1992; Xu et al., 1997; Raz et al., 1998) and induces spontaneous egg activation (SEA) only in a few mammalian species such as mouse (Xu et al., 1997; Vincent et al., 1992), hamster (Austin, 1956), and rat (Zernika-Goetz, 1991; Raz et al., 1998; Ross et al., 2006; Galat et al., 2007; Chaube et al., 2007; Tamura et al., 2008). The SEA limits somatic cell nuclear transfer during cloning in rat (Ross et al., 2006).

Changes in intracellular calcium $[Ca^{2+}]_i$ level modulate various cell functions such as meiotic cell cycle, apoptosis and/or cell death (Homa et al., 1993; Homa, 1995; McConkey and Orrenius, 1997; Tosti, 2006; Whitaker, 2006). For instance, a transient increase of $[Ca^{2+}]_i$ induces egg activation (Vincent et al., 1992), while high sustained Ca^{2+} level leads to apoptosis (McConkey and Orrenius, 1997; Berridge et al., 1998; Gordo et al., 2002). In contrast, abnormally high $[Ca^{2+}]_i$ level results in cell death (Gordo et al., 2000). The calcium rise in an egg occurs by means of two principal mechanisms: the efflux from the stores via ligand-gated channels or organelle membrane and entry through ion channels in the plasma membrane (Tosti, 2006).

A significant change in L-type calcium channel activity from diplotene arrest to M-II stage has been identified in

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mammalian oocytes (Tosti et al., 2000; Tosti, 2006). The L-type calcium channels have a role in resumption of meiosis (Tosti, 2006) since verapamil, a known non-specific L-type calcium channel blocker, inhibits calcium current activity (Tosti et al., 2000), and the resumption of meiosis in rat, pig and bovine eggs cultured in vitro (Paleos and Powers, 1981; Bae and Channing, 1985; Kaufman and Homa, 1993; Tosti et al., 2000). Recently, we reported that verapamil ($\geq$50 μM) inhibits resumption of meiosis and induces apoptosis in aged rat eggs cultured in Ca^{2+}-free medium (Chaube et al., 2007). Furthermore, the calcium ionophore A23187, which is known to increase $[Ca^{2+}]_i$ generates hydrogen peroxide (H_2O_2) and thereby induces apoptosis in aged rat eggs cultured in vitro (Chaube et al., 2008). It is possible that the release of Ca^{2+}, mainly from endoplasmic reticulum (Boni et al., 2007), and inhibition of calcium channels by verapamil may induce the accumulation of $[Ca^{2+}]_i$ in aged eggs (Rozinek et al., 2003), which may generate intracellular hydrogen peroxide (H_2O_2). This in turn may inhibit the meiotic cell cycle and induce apoptosis in aged rat eggs cultured in vitro. Hence, the objectives of the present study examined whether the generation of intracellular H_2O_2, changes in bax/bcl_2 expression and caspase-3 activation are associated with verapamil-induced egg apoptosis in Ca^{2+}-deficient medium. If so, we tested whether extracellular Ca^{2+} protects against verapamil-induced initiation of apoptosis in aged eggs of rat cultured in vitro.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of verapamil working concentrations

The culture media used in the present study were either serum-free and Ca^{2+}-free medium-199 or serum-free and Ca^{2+}-free medium-199 containing 1.80 mM Ca^{2+} (HiMedia Laboratories, Mumbai, India). This concentration of calcium ion is generally used to prepare medium-199 (Ca^{2+}-supplemented medium) for the culture of various cell types. Working concentrations of verapamil (25, 50, 100, 200 and 300 μM) were prepared separately in Ca^{2+}-deficient or Ca^{2+}-supplemented medium from its stock solution (10 mg/ml) and kept at 37 °C for 5 min before use. Addition of verapamil at its final concentration did not alter the osmolarity (295 ± 5 m Osmol) or pH (7.2 ± 0.1) of the culture medium used in the present study. Since absolute alcohol was used as a solvent in the verapamil stock solution, an equivalent dilution of the highest concentration (0.01% absolute alcohol) was used in the control group.

2.3. Rats and the collection of their eggs

Holtzman rats were housed in air-conditioned, light controlled room, with food and water, *ad libitum*. Twenty-three to 25-day-old female rats were primed with a single subcutaneous injection of (20 IU) pregnant mare serum gonadotropin (PMSG) followed 48 h later by (20 IU) hCG. Eggs were collected 17 h after hCG injection. Ovulated cumulus-enclosed eggs were isolated in Ca^{2+}-deficient medium under a dissecting microscope (Carl Zeiss, Oberkochen, Germany) and denuded using 0.01% hyaluronidase at 37 °C. Denuded eggs were washed 3 times with Ca^{2+}-deficient medium. The eggs used in this study were collected 17 h post-hCG treatment to obtain aged eggs susceptible for partial SEA (Ross et al., 2006). All procedures conformed to the regulations of the University Animal Ethical Committee of Banaras Hindu University, Varanasi and were in keeping with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication).

2.4. Effect of verapamil on morphological changes in apoptosis

Fifteen to 20 eggs were cultured in a 35 mm petri dish containing 2 ml of either Ca^{2+}-deficient medium, or Ca^{2+}-supplemented medium, or either one of these media containing various concentrations (25, 50, 100, 200 and 300 μM) of verapamil. Petri dishes were maintained at 37 °C for 3 h. At the end of incubation period, oocytes were removed, washed 3 times with Ca^{2+}-deficient medium, transferred onto a grooved slide with 100 μL of medium and examined for morphological changes characteristic of SEA, apoptosis and degeneration using a phase-contrast microscope (Nikon, Eclipse; E600, Japan) at 400 × magnification.

Since extracellular calcium protected against 50 μM verapamil-induced initiation of morphological apoptotic features such as shrinkage, membrane blebbing and cytoplasmic granulation, 50 μM verapamil was selected for further biochemical analysis.

2.5. Quantitative estimation of intracellular H_2O_2 level

The intracellular H_2O_2 level was measured using an H_2O_2 assay kit purchased from R&D Systems, Inc. (MN, USA). Seventy-five eggs collected from 2 animals after superovulation induction were randomly divided into 3 groups of 25. The first was cultured in Ca^{2+}-deficient medium (control group). The second and third were cultured separately in Ca^{2+}-deficient or Ca^{2+}-supplemented media for 3 h with both media containing 50 μM verapamil. Thereafter, eggs were washed 3 times with 10 mM phosphate buffer saline (PBS) and lysed in 100 μL hypotonic lysis buffer (5 mM Tris, 20 mM ethylenediamine tetraacitic acid (EDTA), 0.5% Triton X-100; pH 6.0). After 1 h of incubation on ice, lysates were centrifuged at 10,000 rpm at 4 °C for 15 min and the clear supernatant was immediately stored at −30 °C until use. Hydrogen peroxide concentrations of the egg lysates were measured by calorimetric assay as described in our previous publication (Chaube et al., 2008). All samples were run in triplicate in one assay to avoid inter-assay variations. Intra-assay variation was ∼3%.