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Bovine serum albumin enhances calcium currents in chicken granulosa cells

Meicheng Chiang [a], Judith A. Strong [b] and Elikplimi K. Asem [a]

[a] *Department of Physiology and Pharmacology, School of Veterinary Medicine and*
[b] *Department of Biological Sciences, Purdue University,*
West Lafayette, Indiana, USA

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Summary

The effect of bovine serum albumin (BSA) on Ca^{2+} currents in chicken granulosa cells was examined using both the nystatin-perforated and the conventional whole cell patch clamp techniques. Under voltage-clamp conditions, depolarizing voltage steps evoked inward Ca^{2+} currents with both methods. The time- and voltage-dependence of Ca^{2+} currents measured with the perforated patch technique was similar to those obtained with conventional whole cell recording. Commercially prepared BSA and essentially fatty acid free BSA both rapidly enhanced the amplitude of Ca^{2+} currents. However, the fatty acid free BSA was more potent, and its potency was greatly reduced by incubation with saturating concentrations of oleic acid. These data show that BSA, a common constituent of incubation media, can influence ion channels in the plasma membrane of granulosa cells.

Introduction

Hormonal regulation of ovarian granulosa cell growth, differentiation and function (e.g. steroid hormone biosynthesis) is the subject of intensive investigation. Several laboratories conduct their studies in vitro using chemically defined media that are supplemented with whole serum (e.g. Erickson et al., 1979; Savion and Gospodarowicz, 1980; May and Schomberg, 1981; Veldhuis et al., 1986, 1987; Kurten and Richards, 1989). Others supplement a chemically defined incubation medium with purified serum albumin (e.g. Ciancio and LaBarbera, 1984; Baranao and Hammond, 1985; Davoren et al., 1986; Asem et al., 1992; Novero and Asem, 1993), especially if the inclusion of serum in the system would make the interpretation of data difficult.

Our long-term goal is to elucidate signal transduction mechanisms of the gonadotropic hormones and growth factors that regulate granulosa cell function. We are particularly interested in the possible roles of ion channels in these processes. The patch clamp technique provides a powerful approach for studying these questions. In order to relate results from patch clamp studies to previous biochemical and physiological studies, it is desirable to conduct the electrophysiological studies in media with compositions similar to those used previously.

Much of the available information on gonadotropin regulation of chicken granulosa cell function (notably steroidogenesis) stems from experiments conducted in chemically defined media supplemented with bovine serum albumin (BSA) fraction V (e.g., Hammond and Hertelendy, 1981; Calvo and Bahr, 1982; Marrone and Hertelendy, 1983; Asem and Hertelendy, 1983, 1985; Robinson and Etches, 1986; Tilly and Johnson, 1987; Lee and Bahr, 1989; Etches et al., 1990; Tilly et al., 1991). During our initial patch clamp studies, we found that voltage-activated Ca^{2+} currents were rapidly enhanced when BSA was added to the bath solution.

The aim of this communication is to document the observation that the addition of BSA enhances Ca^{2+} currents in chicken granulosa cells. Because serum albumin binds fatty acids with high affinity (Spector et al., 1969) and is a reservoir and carrier of these lipophilic molecules (Spector, 1975), the possible role of the BSA fatty acid binding site(s) in enhancing Ca^{2+} currents was also examined.

Correspondence to: Dr. E.K. Asem, Dept. of Physiology and Pharmacology, School of Veterinary Medicine, Purdue University, 1246 Lynn Hall, West Lafayette, IN 47907-1246, USA. Tel.: (317) 494-6447; Fax: (317) 494-0781.

Materials and methods

Chemicals

N-methyl-glucamine, nystatin, ethyleneglycol tetraacetic acid (EGTA), triethylammonium chloride (TEA), dimethylsulfoxide (DMSO), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), collagenase type 1A, soybean trypsin inhibitor and bovine serum albumin (BSA; Fraction V; catalog no. A-6793; lot no. 39F-0451), essentially fatty acid free BSA (FAF-BSA; catalog no. A-6003; lot no. 127F-9312) and bovine serum gamma globulin, were from Sigma Chemical Co. (St. Louis, MO, USA). Medium 199 (M199) containing Hank's salts was from Gibco (Grand Island, NY, USA). Bovine serum albumin (True Cohn crystalline powder; catalog no. 81-001), was purchased from ICN Immuno-Biologicals, Lisle, IL.

Animals and granulosa cell culture

White Leghorn hens in their first year of reproductive activity were obtained from Purdue Baker Farms, West Lafayette, IN, and caged individually in a win-

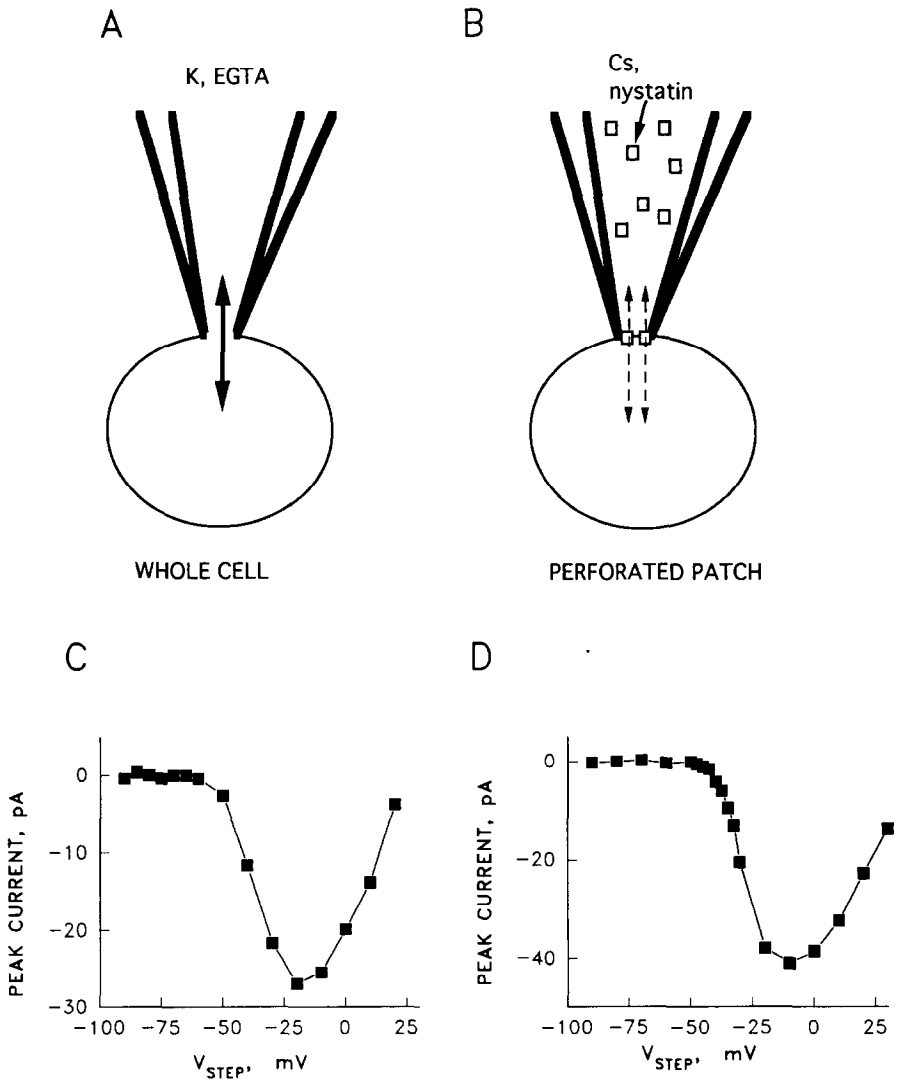

Fig. 1. Schematic diagram of the two different methods of whole cell voltage clamp. Panel A: conventional whole cell recording (Hammill et al., 1981): the recording pipette is placed on the surface of the cell, and an electrically tight seal is formed between the membrane and the glass. Electrical access to the cell is achieved by disrupting the membrane beneath the recording pipette. Small molecules in the pipette exchange rapidly with the cytoplasm. This method was used in experiments in which it was necessary to chelate intracellular Ca^{2+}. Panel B: perforated patch recording (Horn and Marty, 1988; Korn and Horn, 1989): the pipette solution contains the pore-forming antibiotic nystatin. Electrical access is obtained when nystatin inserts into the patch of membrane beneath the pipette. Only monovalent ions in the pipette exchange with the cytoplasm. This method does not disrupt intracellular Ca^{2+} handling, and was used when longer, more stable recordings were desired. In both methods, the inside of the recording pipette is connected to the headstage of a patch clamp amplifier, which allows the intracellular voltage to be controlled, and the resulting membrane currents to be measured. Panel C: an example of the current voltage relationship recorded with the whole cell recording method, with K^+ and EGTA inside the pipette. Peak inward current during depolarizing steps is plotted against the size of the depolarization. In this and all subsequent experiments the extracellular solution included 7.4 mM Ca^{2+} and 5 mM TEA. Panel D: an example, from a different cell, of the current voltage relationship recorded with the perforated patch recording method, with Cs^+ inside the pipette.

dowless, air-conditioned room with a 14 h light:10 h darkness cycle. They had free access to a layer ration and tap water.

The animals were killed by cervical dislocation, and granulosa cells were isolated from the largest preovulatory follicle (F_1). The expected time of ovulation was noted, but this variable had no systematic effect on the responsiveness of the cells to BSA. The granulosa cell layer was separated from the theca layer as described by Gilbert et al. (1977) and the cells were dissociated in Medium 199 containing $NaHCO_3$ (350 mg/l), HEPES (10 mM) pH 7.4, collagenase (500 U/ml) and trypsin inhibitor (200 μg/ml) (Asem et al., 1984). Cell viability, determined by the trypan blue exclusion method, was routinely greater than 95%. The cells were kept at 4°C until use. Keeping the cells up to 12 h at 4°C had no influence on the BSA effect. A small aliquot of cells was plated in bath solution (see below) in 35 mm tissue culture dishes (Falcon Primaria 3801, Fisher, PA) 10 to 15 min before each experiment. All recordings were made at 23°C from single cells which had adhered to the dish.

Voltage clamp

Two different methods of whole cell voltage clamp were used (Fig. 1). For most experiments, we used the perforated patch recording method, because it provided more stable, long-lasting recordings. The conventional whole cell recording method was used for experiments in which experimental control of intracellular Ca^{2+} levels was necessary. In both methods, recording pipettes were fabricated either from borosilicate filament glass (Warner Instrument Co., Hamden, CT) or from a softer glass, 100 μl micropipettes (Drummond Scientific, Broomall, PA). Electrode resistances were 3 to 9 megaohm in bath solution. In conventional whole cell recording (Hamill et al., 1981), the pipette solution included the Ca^{2+}-chelator EGTA. Electrical access to the inside of the cell was obtained by applying a pulse of suction to the inside of the pipette so as to rupture the piece of membrane just beneath the pipette tip. In perforated patch recording (Horn and Marty, 1988; Korn and Horn, 1989), the pipette included the pore-forming antibiotic, nystatin. Electrical access to the inside of the cell was obtained by waiting until the nystatin diffused down to the tip of the electrode and inserted into the piece of membrane just beneath the pipette tip. In the conventional whole cell recording method, electrolytes as well as small organic molecules inside the cell exchange rapidly with the contents of the pipette solution. By comparison, in the perforated patch method, only monovalent ions in the cell cytoplasm exchange with the pipette solution (Horn and Marty, 1988; Korn and Horn, 1989). This recording method is less invasive than conventional whole cell recording technique; in particular, intracellular Ca^{2+} handling remains intact. The observed Ca^{2+} currents had similar properties whether monitored with the perforated patch or conventional whole cell recording method (Fig. 1C and D). However, in perforated patch recordings, there was some variability in the absolute value of the voltage at which the currents first activated. This is presumably due to the small junction potentials which develop across the nystatin-perforated membrane patch (for discussion see Horn and Marty, 1988).

Membrane currents were measured with an Axopatch 1-D patch clamp (Axon Instruments, Foster City, CA) and filtered at 1 kHz. The currents were digitized, stored, and analyzed with a C-Lab interface system (Indec Systems, Sunnyvale, CA) under the control of dedicated programs written in the laboratory. Unless otherwise noted, the linear (leak) component of the total membrane current was subtracted by extrapolating the linear currents obtained during voltage steps in more negative potential regions (-90 to -60 mV), where no voltage-activated currents were seen. Current amplitudes were small enough that the series resistance error was less than 5 mV. Membrane capacitance was estimated by integration of the current transient observed during small voltage steps in the hyperpolarized region (-90 to -60 mV), measured at 5 kHz. BSA did not cause significant changes in membrane capacitance. Unless otherwise indicated the cells were held at -80 mV between voltage steps.

Solutions

Bath (extracellular) solution contained NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.1 mM, glucose 5.6 mM, HEPES 10 mM (pH 7.4). In order to enhance Ca^{2+} currents and block K^+ currents, 5 mM TEA (a blocker of K^+ channels) and an additional 5 mM Ca^{2+} (for a final concentration of 7.5 mM) were added to this solution before the currents were recorded. Where noted, Na^+-free solutions were made by substituting equimolar concentrations of *N*-methyl-glucamine$^+$ for Na^+. The intracellular solution for conventional whole cell recording contained 150 mM KOH, 130 mM HCl, 3 mM ATP (disodium salt), 3 mM $MgCl_2$, 0.2 mM GTP (sodium salt), 5 mM EGTA, 10 mM HEPES (pH 7.2 with HCl). We did not use Cs^+ in this solution because this made it difficult to obtain good seals between the pipette and cell membrane. A sizeable portion of the K^+ currents in these cells is activated by intracellular Ca^{2+} (Asem et al., 1988), so the extracellular TEA and intracellular EGTA blocked K^+ currents to a great extent, allowing isolation of inward Ca^{2+} currents (e.g. see Fig. 1). The intracellular solution for perforated patch recording contained 125 mM CsOH, 14.25 mM HCl, 5.7 mM $MgCl_2$, 14.3 mM HEPES (pH 7.2 with methanesulfonic acid). Just before use, 4 μl/ml of a nystatin stock solution (50

mg/ml DMSO) was added, and the solution was sonicated for 30 sec in a cylindrical bath sonicator (Laboratory Supplies, Hicksville, NY). The tip of the pipette (300 to 400 μm) was filled with a nystatin free solution in which the Cs^+ was replaced by K^+.

Initial experiments were conducted with fraction V bovine serum albumin (Sigma catalog no. A-6793; BSA), referred to in the text as "commercially prepared BSA". The vendor does not specify the fatty acid content of this preparation. Essentially fatty acid free BSA (Sigma catalog no. A-6003), referred to in the text as "FAF-BSA", was also used where indicated. These BSA preparations were used without further purification since our goal was to investigate the effect of commercial BSA preparations in the forms that have been used in biochemical experiments with avian granulosa cells (Hammond and Hertelendy, 1981; Calvo and Bahr, 1982; Marrone and Hertelendy, 1983; Asem and Hertelendy, 1983, 1985; Robinson and Etches, 1986; Tilly and Johnson, 1987; Lee and Bahr, 1989; Etches et al., 1990; Tilly et al., 1991). These commercial preparations of BSA may be contaminated by proteins or polypep-

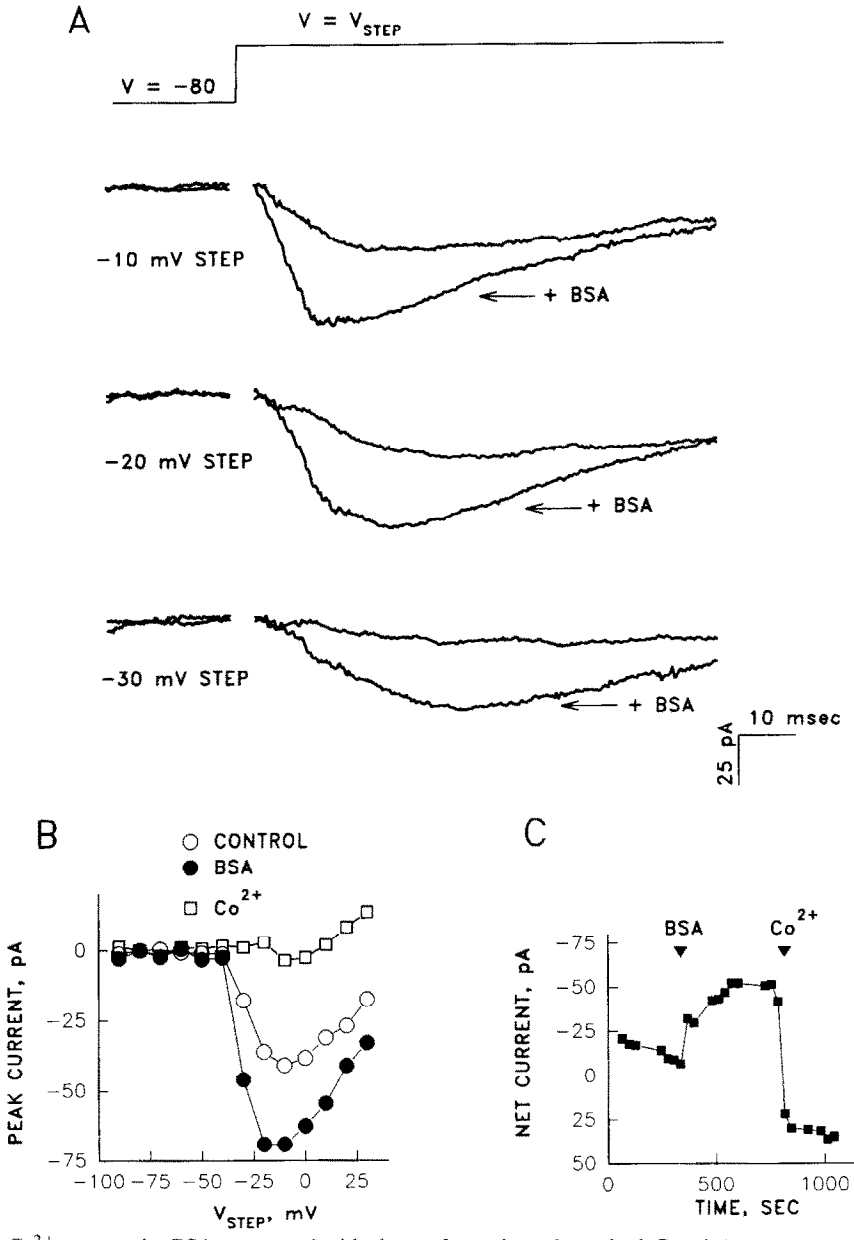

Fig. 2. Enhancement of Ca^{2+} current by BSA, measured with the perforated patch method. Panel A: current traces observed in a single cell before and 1–2 min after addition of 0.06% commercially prepared, fatty acid free BSA. Currents were evoked by step depolarizations to the indicated potential (top trace shows the voltage protocol). Panel B: peak inward current vs. step potential, from the same experiment as described for panel A except that 6 mM cobalt (Co^+) was added after the BSA. Panel C: time course of the BSA and cobalt effects. Net inward current during steps to −20 mV was measured every 30 seconds. Gaps in the time course appear whenever the full current-voltage relation (panel B) was measured. Linear leak subtraction was used in data shown in panel B but not in panel C.

tides that are capable of influencing granulosa cell function (Kane and Keadon, 1980). Therefore, 0.1–3% solutions (1–30 mg/ml) of the BSA preparations used in the present study were screened for possible luteinizing hormone (LH) contamination by a validated specific radioimmunoassay (RIA) as described by Malven and Hudgens (1987) in the laboratory of Dr. P.V. Malven, Purdue University, West Lafayette, IN. However, LH was not detected in any of the BSA preparations (up to 3%; 30 mg/ml) with this RIA, which has a sensitivity of 0.2 ng LH/ml (data not shown). A more highly purified BSA, from a different supplier, was also tested in some experiments ("True Cohn crystalline powder", catalog no. 81-001, from ICN ImmunoBiologicals, Lisle, IL).

In order to test the potential role of the fatty acid binding sites of the BSA, we saturated FAF-BSA with oleic acid using the method of Spector et al. (1969). Briefly, FAF-BSA was dissolved in buffer (116 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 15 mM NaH_2PO_4, pH 7.4) at 250 mg/ml. In 12×75 mm round bottom polystyrene tubes (Falcon 2054), 1 ml of the FAF-BSA buffer solution was layered beneath 1 ml of heptane containing 130 mM oleic acid. According to the partition coefficients measured by Spector et al. (1969), this concentration of oleic acid in the heptane phase yields, after equilibration, a concentration of free oleic acid in the aqueous phase in the order of 10^{-4} M, which is more than adequate to saturate both the high (approx. 1 μM Kd) and medium affinity (approx. 10 μM Kd) fatty acid binding sites on the albumin molecule. The BSA/buffer solution was equilibrated with the heptane phase for 40 hours at room temperature on an orbital shaker. The BSA/buffer was then carefully removed with a hypodermic syringe through a punctured hole in the bottom of the tube, and filter sterilized (Nalgene cellulose acetate 0.2 μm filters). Aliquots were stored at 4°C for up to four weeks, and diluted just before use. This preparation is referred to as oleic acid-saturated BSA. As noted above, this solution also contained free oleic acid; hence addition of 0.025% of the oleic acid-saturated BSA resulted in a final concentration of 0.1 μM free oleic acid. Control preparations were, (1) unsaturated FAF-BSA (prepared in identical fashion, except that oleic acid was omitted); (2) buffer with oleic acid (BSA omitted); and (3) buffer (both oleic acid and BSA omitted).

Statistical analysis

Significant differences between the data presented in Fig. 4 were determined by the Student's "*t*" test.

Results

Schwartz et al. (1988) using conventional whole cell recording techniques, demonstrated that granulosa cells obtained from mature preovulatory (F_1) chicken ovarian follicles express Ca^{2+} currents activated by depolarization. We recorded very similar calcium currents in freshly isolated chicken granulosa cells with the perforated patch method (Fig. 2). At more negative potentials (below -50 mV), the Ca^{2+} channels are closed. When the potential across the membrane is stepped to more positive values (above -50 mV), the Ca^{2+} channels begin to open (activate). The resulting movement of Ca^{2+} into the cell is recorded electrically as a downward deflection in the current trace (Fig. 2A). These currents are known to be carried by Ca^{2+} because they are increased when extracellular Ca^{2+} is increased, and are blocked by Ca^{2+} channel blockers such as Co^{2+} (Schwartz et al., 1988). In addition, we could observe similar currents in bath solutions in which extracellular Na^+ had been replaced by the impermeant cation *N*-methyl-glucamine$^+$. Stable recordings of the Ca^{2+} current could often be obtained for up to 120 min with the perforated patch technique. In contrast, it was difficult to make conventional whole cell recordings for more than 10 min because, as in many cell types, the Ca^{2+} current tended to "rundown" with this recording method.

Addition of BSA to the bath solution rapidly enhanced the inward currents. An example, using FAF-BSA, is shown in Fig. 2. The primary effect of the BSA was to enhance the amplitude of the peak inward current at all potentials, without much change in the voltage dependence of activation. Decay of the inward current during the depolarizing pulse (inactivation) also tended to be more marked in BSA-treated cells, as shown in Fig. 2A. The BSA-enhanced current, like the control inward current, was carried by Ca^{2+}, since it was blocked by extracellular Co^{2+}, a nonspecific inhibitor of Ca^{2+} currents (Fig. 2B and C; $n = 2$). In addition, in a cell bathed in Na^+-free solution, BSA enhancement of the inward current was readily observed (2.5 fold increase in peak current at -40 mV). The BSA preparations used in these experiments are 98–99% pure with the remaining 1–2% made up primarily of globulins. To determine if the action of BSA preparations on Ca^{2+} current was caused by globulin, the effect of bovine serum gamma globulin on Ca^{2+} currents was examined. Addition of the bovine gamma globulin (0.03%) did not have any effect on the Ca^{2+} current ($n = 2$, data not shown). The concentration of gamma globulin tested was 10-fold greater than the amount of globulin that would have been associated with the highest dose of BSA tested in the experiments described herein. We also tested the effects of a more highly purified BSA preparation, from a different source (ICN ImmunoBiologicals, Lisle, IL). This BSA preparation, when added at a concentration of 0.06 to 0.09% increased the peak Ca^{2+} current by 50 to 54% ($n = 2$).

As in most cells, the amplitude of the Ca^{2+} current depends on the holding potential. As the holding potential is made more positive, more Ca^{2+} channels inactivate and hence cannot open in response to a depolarizing voltage step. At holding potentials above −40 mV, the current inactivates completely and depolarizing steps evoke essentially no inward current. However, the effect of BSA was not due to a change in the steady state voltage dependence of this inactivation process, which was essentially identical before and after BSA addition (Fig. 3). The fact that little inward current was observed at holding potentials above −40 mV suggests that the majority of the channels responsible for the Ca^{2+} current in the present study were of the low-threshold type (see Discussion).

In some cells, the enhanced inward currents seen after BSA addition seemed to decay spontaneously, after cells had been exposed to BSA for anywhere from 17 to 50 min. However, this decay was not observed in all cells. It was difficult to tell if this decay represented a reversal of the BSA effect or simply a nonspecific loss of the Ca^{2+} current; often, the currents decayed to smaller levels than observed prior to BSA addition. It was difficult to reverse the BSA effect by washing with bath solution; often, a slight transient increase in current was observed when the cell was first washed with BSA-free solution.

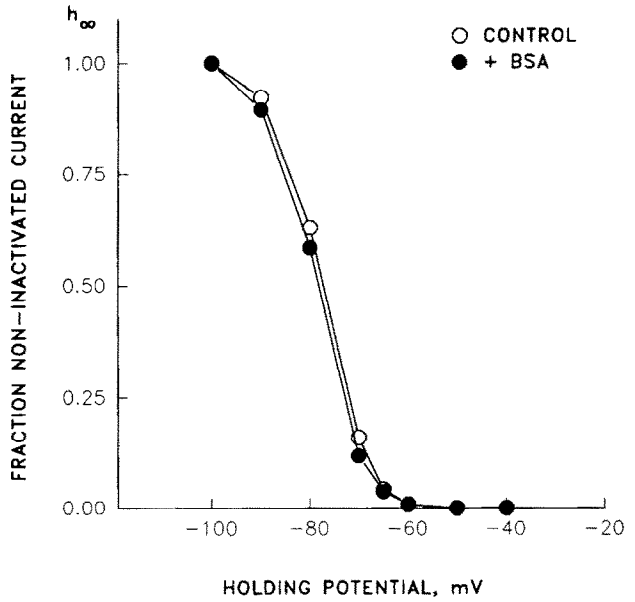

Fig. 3. Voltage dependence of steady-state inactivation is unaffected by BSA. Peak current during voltage steps to −30 mV was measured at the indicated holding potential, using the nystatin perforated patch clamp technique, and normalized to the maximum current (obtained at a holding potential of −100 mV). Hence the value of the inactivation parameter, *h*-infinity (h_∞), goes from 1.0 at negative holding potentials where no inactivation is seen, to a value of 0.0 at more positive potentials, where inactivation is complete. Open circle, control; closed circle, after addition of 0.03% commercially prepared BSA. BSA enhanced the peak inward current by a factor of 1.4 in this cell. Similar results were obtained in two other experiments.

Initially, we observed enhancement of the Ca^{2+} current using commercially prepared BSA. Seven out of seven cells responded to BSA concentrations ranging from 0.03% to 0.15% (higher doses were not examined). For this type of BSA, 0.03% seemed to represent the lowest effective dose, since three out of three cells failed to respond to a dose of 0.003%. Because this type of BSA can also contain fatty acids (as contaminants), we next tested the effect of commercially prepared fatty acid free BSA (FAF-BSA) on Ca^{2+} currents. The FAF-BSA proved to be even more effective at enhancing the Ca^{2+} currents. Seven out of 10 cells responded to doses of 0.003%. Overall, 21 out of 24 cells responded to concentrations of FAF-BSA ranging from 0.003% to 0.09%; the three unresponsive cells were tested with the lowest dose only (0.003%). We observed that the absolute value of amplitude of the BSA-induced current tended to be greater in cells which had larger control (basal) Ca^{2+} currents initially. On average, FAF-BSA enhanced the amplitude of the current by a factor of 1.64 ± 0.13. There was no obvious effect of increasing the BSA concentration above that needed to elicit the response. For example, 0.003% FAF-BSA gave essentially the same increase in current (1.81-fold) as did 0.06% FAF-BSA (1.75-fold).

The observation that FAF-BSA was more effective suggested the hypothesis that BSA may act by removing some lipophilic molecule(s) from the plasma membrane. If this hypothesis is correct, then BSA's effect on Ca^{2+} current should be diminished or abolished when all its binding sites are saturated with fatty acid. In order to test this idea, FAF-BSA was completely saturated with oleic acid, as described in the methods section, so that the effects of FAF-BSA and oleic acid-saturated BSA could be compared.

As shown in Fig. 4, unsaturated FAF-BSA effectively increased Ca^{2+} currents measured with the conventional whole cell recording technique. This effect was similar to that seen with the perforated patch technique. In contrast, the BSA which had been saturated with oleic acid failed to increase the Ca^{2+} current under under these conditions. The apparent decrease of the inward current with oleic-saturated BSA shown in Fig. 4 was probably due to the "rundown" of Ca^{2+} current which is seen during conventional whole cell recording; indistinguishable results were obtained during control experiments with simple addition of bath solution ($n = 4$), or with addition of oleic acid alone ($n = 4$), as shown in Fig. 4C.

The comparison of FAF-BSA and oleic acid-saturated BSA was done with the conventional whole cell recording technique for the following reason; the stock solutions of oleic acid-saturated BSA prepared in the laboratory necessarily contained 10^{-4} M free oleic

acid as well (see methods section). In preliminary experiments using the perforated patch recording method, we found that oleic acid alone, at final concentrations similar to those which would result from additions of the stock solution of oleic acid-saturated BSA could reduce the Ca^{2+} currents. We suspected that oleic acid might be decreasing Ca^{2+} currents by increasing the internal free Ca^{2+} concentration because oleic acid (1 μM) has recently been shown to release Ca^{2+} from internal stores in these cells (Hertelendy et al., 1992); internal Ca^{2+} handling is preserved with the perforated patch recording method; and Ca^{2+} currents are inhibited by intracellular Ca^{2+} in many preparations. Consistent with this idea, we found that oleic acid was much less effective in reducing Ca^{2+} currents measured with the whole cell recording method, in which intracellular Ca^{2+} is well buffered. Even a concentration of oleic acid (0.1 μM) that is five times higher than those added along with the oleic-acid saturated BSA caused only small reductions of the Ca^{2+} currents

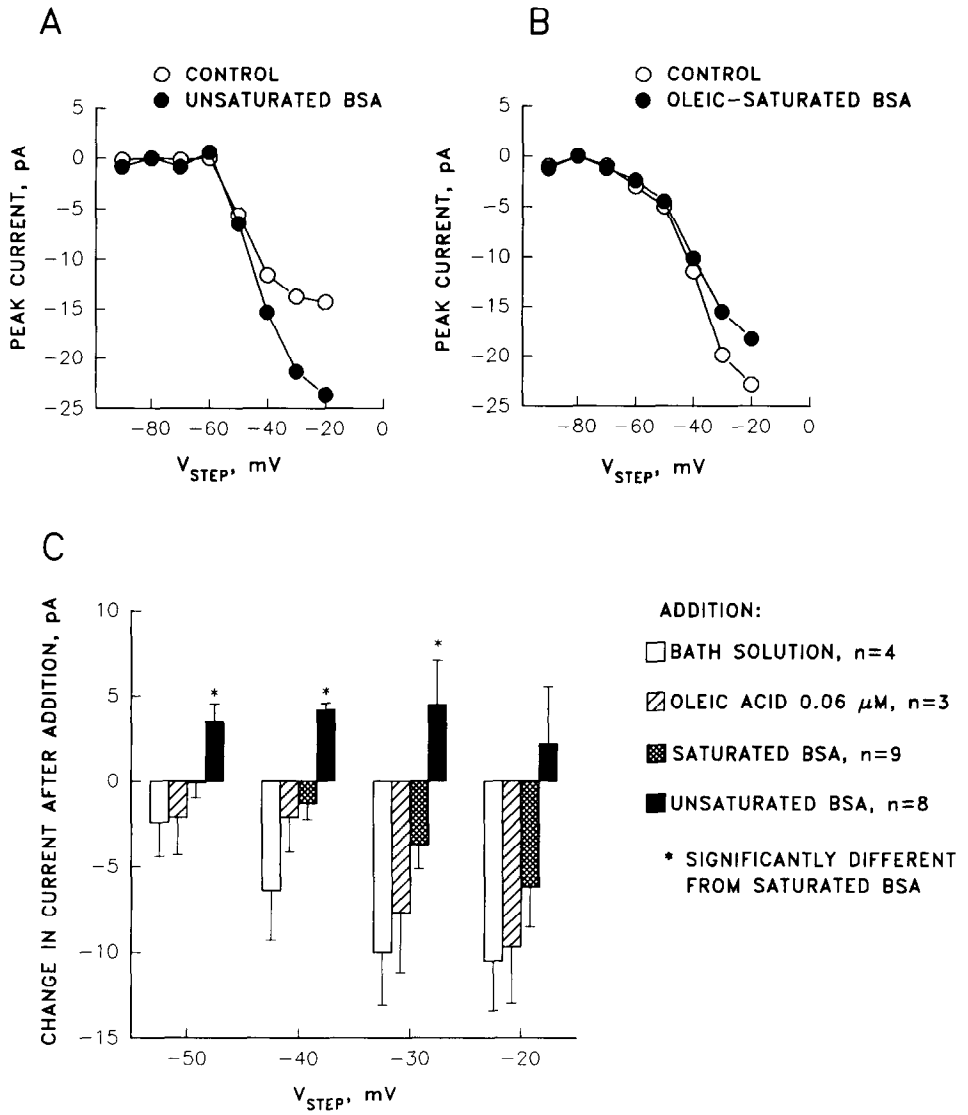

Fig. 4. Comparison of the effects of laboratory-prepared oleic acid-saturated BSA and FAF-BSA, measured with the conventional whole cell recording technique. Panel A: unsaturated FAF-BSA enhances peak inward current in a single cell. Currents were measured only at potentials below -10 mV in order to improve the stability of the whole cell recording. Panel B: in another cell, BSA saturated with oleic acid failed to enhance inward current. Panel C: summary of data from 8 experiments with unsaturated BSA (includes the experiment shown in panel A, with BSA concentrations from 0.003% to 0.015%), 9 experiments with oleic acid-saturated BSA (includes the experiment shown in panel B, with the same range of BSA concentrations), 3 control experiments in which 0.1 μM oleic acid was added to the bath, and 4 control experiments in which the rate of Ca^{2+} current rundown was measured by simply adding aliquots of bath solution, using a protocol identical to that used in the BSA experiments. The difference in magnitude between the control currents and those after addition of BSA, oleic acid, or bath solution is shown for each step potential. The effects of oleic acid-saturated BSA and FAF-BSA were significantly different ($P < 0.02$) at potentials of -50 to -30 mV and approached significance at -20 mV ($P < 0.058$). There were no significant differences between oleic acid-saturated BSA, oleic acid alone, and bath solution at any potential. In this series of experiments, the FAF-BSA was subjected to incubation in buffer under heptane, as described in the methods, as a control for the methods used to prepare the oleic acid-saturated BSA.

($n = 4$) measured using the conventional whole cell recording method. These small reductions were not discernible from the time-dependent rundown of the Ca^{2+} current, (i.e., were indistinguishable from the reductions of current seen with control applications of bath solution). Therefore, we used the conventional whole cell recording method to compare the effects of saturated and unsaturated BSA, as shown in Fig. 4.

Discussion

These data show clearly that BSA enhances voltage-activated Ca^{2+} currents in chicken granulosa cells. The primary effect of BSA was to enhance the amplitude of the inward current at all potentials, without shifting its voltage dependence. The currents also showed increased inactivation during a depolarization.

In the present study, Ca^{2+} currents recorded with the perforated patch method were similar to those recorded with the conventional whole cell technique. With regard to the activation of whole cell Ca^{2+} current, the present results confirm the findings of Schwartz et al. (1988), who, using the conventional whole cell recording technique, demonstrated that chicken granulosa cells express Ca^{2+} currents activated by depolarizing voltage steps. Previously published single channel studies in these cells (Schwartz et al., 1989) showed that, like many cell types (Tsien and Tsien, 1990), the granulosa cells have two different types of Ca^{2+} channels: high threshold, large conductance channels (i.e., L-type) which open only with stronger depolarizations, and which do not inactivate; and low threshold, small conductance channels (i.e., T-type) which can open with more negative depolarizations and which tend to inactivate during a maintained depolarization. Our observation that little inward current was observed with holding potentials above -40 mV indicates that most of the Ca^{2+} channels in these cells are of the low-threshold type (T-channels). This is in agreement with the single channel experiments in which high threshold channels were seen in less than 10% of patches studied and low threshold channels predominated (Schwartz et al., 1989).

One possible explanation of the present findings is that the effects of BSA are due to its actions on some lipophilic component(s) of the plasma membrane. Thus, BSA, due to its ability to bind lipophilic molecules, may have altered or removed some lipophilic molecules in the plasma membrane which in turn affected the Ca^{2+} channels, either through a direct physical interaction or indirectly through some biochemical or regulatory pathway. This contention is supported (at least in part) by the observation that fatty acid free BSA was actually more effective in enhancing the Ca^{2+} current, and that complete saturation of the fatty acid binding sites of this BSA preparation with oleic acid reduced its ability to enhance Ca^{2+} currents, as measured with conventional whole cell recording methods. This could also explain the lack of a clear dose-response relationship (above the threshold concentration needed to get a response), since the BSA in the bath solution represents an essentially infinite sink relative to the plasma membrane volume of the plated cells. The observation that the effects of BSA are not readily reversible further supports the notion that albumin may have been acting by altering the properties of the plasma membrane. However, the present results have certainly not elucidated the exact mechanisms by which BSA enhanced the inward currents. Since some commercial BSA preparations have been shown to be contaminated with other proteins and polypeptides (e.g., see Kane and Headon, 1980) the albumin preparations employed in the present studies were tested for possible LH contamination. These BSA preparations are free from LH contamination as determined by a specific radioimmunoassay. In addition, one major contaminant (globulin) which was tested failed to mimic the BSA effect on Ca^{2+} currents. However, the possibility that other biologically active contaminants of the commercial BSA preparations are responsible for the observed effects cannot be ruled out completely.

The present findings may provide a partial explanation for the observations that BSA enhanced steroidogenesis in rabbit testis (Ewing et al., 1976); and in corpus luteal cells obtained from ovine and bovine ovaries (Caffrey et al., 1979; Condon and Pate, 1981). In particular, our data could explain the reported effects of rat testicular fluid albumin or serum albumin on the enhancement of LH-stimulated steroidogenesis in rat Leydig cells (Melsert et al., 1988, 1989, 1991). Albumin (0.1 to 1%) purified from rat testicular fluid increased dose-dependently LH-promoted pregnenolone production in rat Leydig cells in short-term (4 h) culture (Melsert et al., 1988). Similarly, fatty acid free bovine serum albumin (BSA-FAF; Sigma Chemical Co.) at concentrations of 0.4 and 1% enhanced significantly LH-stimulated pregnenolone synthesis in rat Leydig cells in short-term incubations (Melsert et al., 1991). The effect of BSA on steroidogenesis in Leydig cells was mimicked by albumin from rat (Melsert et al., 1988, 1989) or human (Melsert et al., 1991) serum. Similar to the present findings, Melsert et al. (1991) found that saturation of albumin (BSA-FAF; Sigma Chemical Co.) with oleic acid inhibited the effect of this protein on steroidogenesis in Leydig cells. However, it is noteworthy that the concentrations of BSA-FAF used in the study by Melsert et al. (1991) were significantly greater than those that enhanced Ca^{2+} currents in the present studies.

In the present work, the primary motivation for examining the effects of BSA was that this is a common constituent of incubation media used in several

laboratories. Bovine serum albumin at concentrations as low as 0.003 to 0.03% was able to enhance Ca^{2+} currents in our experiments. For comparison, 0.1–0.2% BSA is commonly added to incubation media. The concentration of albumin in serum is in the range of 6–7% (6–7 g/100 ml); and it is also present in the follicular fluid bathing mammalian granulosa cells (Shalgi et al., 1973; Andersen et al., 1976). Although we have focused on the in vitro use of BSA, it is interesting to note that a number of recent reports have ascribed important functional roles to this abundant serum protein other than its commonly known role as a carrier protein. For example, plasma membrane of adipocytes (Brandes et al., 1982) and liver cells (Weisiger et al., 1981) have been shown to possess specific binding sites for serum albumin. Furthermore, it has been shown that human serum albumin (HSA) is present in the cytoplasm of MCF-7 breast cancer cells (Singh et al., 1992). The breast cancer cell-derived HSA as well as commercially purified HSA preparations increased the activity of 17β-hydroxysteroid dehydrogenase, the enzyme that catalyzes the reductive conversion of estrone to estradiol-17β (Singh et al., 1992). Other serum proteins that have hitherto been presumed to be reservoirs or carriers of substances, such as sex steroid hormone-binding globulins (SHBG) and corticosteroid-binding globulins (CBG) are also now known to interact with plasma membranes of cells through specific receptors (Hyrb et al., 1985, 1986; Naklah et al., 1988; Singer et al., 1988). Moreover, it has been shown that the internalization of CBG occurs via its membrane binding sites in MCF-7 (Naklah et al., 1988) and rat hepatoma FAO (Kuhn, 1988) cells. It is noteworthy that divalent cations Ca^{2+} and Mg^{2+} play significant roles in the interactions of SHBG and CBG with cell surfaces (Hyrb et al., 1986; Singer et al., 1988). These reports support the notion that certain abundant serum "binding" proteins may have additional, specific physiological effects on cells, including the regulation of activities of enzymes.

In summary, voltage-activated Ca^{2+} currents in granulosa cells are rapidly enhanced by commercially purified bovine serum albumin and essentially fatty acid free BSA that are common components of incubation media used extensively in the authors' and other laboratories to study the regulation of chicken ovarian cell function. These results suggest that BSA has the potential to alter many physiological processes in these cells, since Ca^{2+} is a major intracellular regulator of granulosa cell function.

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