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L- and T-Type Voltage-Gated Ca^{2+} Channels in Human Granulosa Cells: Functional Characterization and Cholinergic Regulation

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Using the whole-cell configuration of the patch-clamp technique, we have characterized two types of ionic currents through voltage-dependent Ca^{2+} channels in human granulosa cells. One is long-lasting, activates at approximately −20 mV, reaches the peak at approximately +20 mV, has an inactivation time constant of 132.5 ± 5.6 msec at 20 mV, and is sensitive to dihydropyridines. The other is transient, activates at approximately −40 mV, peaks at approximately −10 mV, has an inactivation time constant of 38.8 ± 1.8 msec at −10 mV, displays a voltage-dependent inactivation, and is sensitive to 100 μM Ni^{2+}, but not to dihydropyridines. Biophysical and pharmacological properties of these currents indicate that they are gated through L- and T-type calcium channels, respectively. The cholinergic receptor agonist carbachol (50 μM) reduces the amplitude of the currents through both L-type (−34.7 ± 6.4%; n = 10) and T-type (−52.6 ± 7.4%; n = 8) channels, suggesting a possible role of these channels in the cholinergic regulation of human ovarian functions. (*J Clin Endocrinol Metab* 90: 2192–2197, 2005)

GRANULOSA CELLS (GC) surround the oocyte and aid in its maturation by secreting hormones and paracrine growth factors mainly in response to gonadotropins (1). Steroidogenesis is clearly Ca^{2+}-dependent, and several studies indicate that besides Ca^{2+} release from cytosolic stores (2–5), Ca^{2+} influx from the extracellular environment through voltage-dependent calcium channels (VDCC) plays an important role in this process. In particular, gonadotropin-induced progesterone production has been shown to be significantly inhibited by calcium channel blockers in human (6, 7), hen (5), swine (8), and rat (9) GC. Calcium entry through VDCC also contributes to apoptosis (10) and may play a role in other Ca^{2+}-dependent processes, such as cytoskeleton rearrangements (11) and modulation of cell proliferation (12) in physiological (*e.g.* ovulation and corpus luteum formation) and pathological (*e.g.* polycystic ovary) conditions.

Despite the importance of these processes related to variation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), little was known about VDCC in human GC until a very recent, independent, patch-clamp and molecular study (7) published just after the completion of the present investigation. Only the existence of the high voltage-activated (HVA) L-type Ca^{2+} channels had previously been proposed on the basis of the observation that in these cells L-type Ca^{2+} channel blockers inhibit both gonadotropin-stimulated steroidogenesis (6) and the androstenedione-induced increase in $[Ca^{2+}]_i$ (13). Furthermore, previous electrophysiological studies performed in animal preparations had indicated that another VDCC may also be expressed in GC: the low voltage-activated (LVA) T-type channel, characterized in porcine (14, 15) and chicken (16, 17) GC. The first purpose of the present work was to characterize the ionic currents through VDCC in human ovarian GC using the whole-cell configuration of the patch-clamp technique. In agreement with the recent electrophysiological and molecular observations in human GC (7), the biophysical and pharmacological properties presented here indicate that these currents are gated not only through L-type, but also through T-type, VDCC.

Several studies have shown that Ca^{2+} entry through VDCC in endocrine cells can be regulated by hormones, autocrine/paracrine factors, and neurotransmitters (18–21) and that this modulatory action plays an important role in the control of secretion (22). In human GC, T-type Ca^{2+} channels are modulated by chorionic gonadotropins, and this regulation influences progesterone production (7). Other factors have recently been shown to be involved in the regulation of ovarian physiology, such as growth factors (1, 23, 24) and neurotransmitters, including catecholamines (25–28), histamine (28, 29), and acetylcholine (12, 28, 30–38). Interestingly, recent data suggest that the cholinergic modulation of ovary functions is not due to a parasympathetic innervation, but, rather, to an intraovarian, nonneuronal, cholinergic system. In fact, the acetylcholine-synthesizing enzyme, choline-acetyl transferase, has been found in GC (12, 35), but not in ovarian nerve fibers or neuron-like cells (35). The second aim of the present work was to verify whether VDCC play a role in the cholinergic modulation of human GC functions. The results presented here support this hypothesis.

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Abbreviations: $[Ca^{2+}]_i$, Intracellular Ca^{2+} concentration; GC, granulosa cell(s); HVA, high voltage activated; IVF, *in vitro* fertilization; VDCC, voltage-dependent calcium channel.

Materials and Methods

Granulosa cell preparation

Human GC were collected from 34 ovulatory women undergoing hormone treatment for *in vitro* fertilization (IVF) at the Reproductive Medicine Unit, Società Italiana Studi di Medicina della Riproduzione (Bologna, Italy). Only a few of them underwent this procedure because of female infertility (one for endocrine pathology and five for tubal obstruction), the most common reasons being male infertility (n = 24, in five cases associated with tubal obstruction). The other indications were idiopathic in two cases and genetic in the remaining two couples (fragile X and hemophilia, respectively). Patients received pituitary desensitization with a GnRH analog and ovarian stimulation with urinary human FSH (Metrodin, Serono, Milan, Italy), followed by administration of human chorionic gonadotropin. Informed consent for the use of GC (which would otherwise be discarded) for research purposes was obtained from each patient. Single cumula were isolated from follicular fluid and kept in IVF medium for 5 h in the presence of the oocytes. Then the oocytes were removed for IVF procedures, and single cumula were transferred in 0.5 ml DMEM supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Inc., Milan, Italy), fungizone (1 ml/100 ml), and penicillin/streptomycin (6 mg/ml) (Invitrogen Life Technologies, Inc., Grand Island, NY) and dissociated by gentle pipetting (modified from Refs. 11 and 39). According to size, each cumulus was plated onto one or two 35-mm petri dishes previously coated with poly-L-lysine (Sigma-Aldrich Corp., Milan, Italy). Cells were grown at 37 C in an incubator gassed with 95% air/5% CO_2 in a humidified atmosphere. Culture medium was replaced every other day. Following this protocol, the majority of the cells did not reach confluence even after several days in culture, enabling the study of single cell ionic currents. Cells were used for electrophysiological experiments between d 1 and 8, with d 1 being the day of collection.

Electrophysiology

Ba^{2+} currents through voltage-gated calcium channels were monitored using the whole-cell configuration of the patch-clamp technique (40). Petri plates (35 mm) with cultured cells were placed on a moving support mounted on a TMS inverted microscope (Nikon, Melville, NY) for whole-cell voltage-clamp analyses and were perfused (see below) using a gravity-driven system. Recording pipettes were made from borosilicate glass capillary tubing (CG150–15, Harvard Apparatus, Natick, MA) and had resistances 3–8 MΩ when filled with the internal solution. Currents were recorded using a voltage-clamp amplifier, filtered at 5 kHz, and acquired using a PC compatible with a Digidata 1200 interface and pCLAMP 7.0 software (Axon Instruments, Union City, CA). Currents were not leak-subtracted on line. Current-voltage (I/V) relations were elicited from a holding potential of −80 mV using 1) a voltage-ramp protocol ranging from −80 to +80 mV in 400, 200, and 100 msec; and 2) 200-msec steps (10 sec between steps) to test potentials over a range of −50 to +60 mV in 10-mV increments. All experiments were performed at room temperature (22–23 C).

Solutions

The extracellular bath solution contained 20 mM $BaCl_2$ (or $CaCl_2$ in the preliminary experiments), 115 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, and 10 mM HEPES (pH 7.2) (modified from Ref. 15). The intracellular solution had the following composition: 130 mM CsCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, and 4 mM ATP, and the pH was adjusted to 7.2 (modified from Ref. 15). To minimize the cell toxicity induced by the high Ba^{2+} concentration during current recording, EGTA (10 mM) was added to this solution in most of the experiments. After each recording, the cells were washed with Tyrode's solution of the following composition: 128 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 27 mM $NaHCO_3$, and 10 mM glucose, pH 7.3.

Drugs

All chemicals were purchased from Sigma-Aldrich Corp. (Milan, Italy). Carbachol was stocked in bidistilled water at a concentration of 10 mM. Nifedipine and (±)-Bay K 8644 were stocked in absolute ethanol at concentrations of 10 and 5 mM, respectively. Stock solutions were stored at 4 C and diluted in the external solution to the final concentration before use. The experiments with dihydropyridines were performed in semidarkness.

Kinetics

The inactivation time constant of the currents (τ_{inact}) was calculated by fitting a first order exponential [$I = I_{max} \exp(-t/\tau_{inact}) + c$] to the experimental points of the current decay. The fitting was calculated using a subroutine of Clampfit 6.0.5 included in the pClamp 7.0 software (Axon Instruments, Union City, CA).

Statistical analysis

Where appropriate, values are expressed as the mean ± SEM.

Results

Inward currents

Physiological concentrations of Ca^{2+} were insufficient to detect Ca^{2+} currents in our experimental conditions. For this reason we used an external recording solution containing 20 mM of the more permeant ion Ba^{2+}. Despite this elevated concentration, recorded cells did not suffer from Ba^{2+} toxicity (we also used 10 mM bivalent ion chelator EGTA in the pipette in most of the experiments), and some recordings lasted more than 1 h. We found barium currents in only 24.8% of the tested cells. Currents recorded using a ramp protocol with a 0.4 mV/msec steepness displayed the same voltage-dependency and position of the peak as those recorded with the step protocol (Fig. 1A). Therefore, both protocols were used for pharmacological and biophysical characterization of the currents. Among the analyzed cells (n = 98), 55.1% (n = 54) had only a long-lasting component, 11.2% (n = 11) had only a transient component, and 33.7% (n = 33) had both of them. No correlation was found between the presence of these components and the physiopathological features of the donors.

The long-lasting component of the inward barium current activated at approximately −20 mV and peaked at 20.4 ± 1.3 mV (n = 24) (Fig. 1A). The analysis of decay times during depolarizing steps led to a τ_{inact} value of 132.5 ± 5.6 msec (n = 6 at 20 mV). This value was obtained by fitting a first order exponential [$I = I_{max} \exp(-t/\tau_{inact}) + c$] to the experimental points of the current decay (an example is given in Fig. 1B). As shown in Fig. 1C, this current was blocked by 5 μM nifedipine and increased (+351.6 ± 72.5%; n = 9) by 10 μM (±)-Bay K 8644. The latter treatment also shifted the voltage-sensitivity of the channel activation: the membrane potential of the peak current changed from 17.9 ± 2.4 to 3.0 ± 3.2 mV (n = 8). The biophysical and pharmacological properties of this current indicate that it is gathered through the HVA L-type Ca^{2+} channels.

When present together, L-type and transient currents could be easily distinguished using the ramp protocol (Fig. 2A). The transient component activated at approximately −40 mV and peaked at −17.9 ± 1.8 mV (n = 18). During 200-msec squared pulse, it rapidly inactivated with a τ_{inact} of 38.8 ± 1.8 msec (n = 5) at −10 mV when the experimental trace was fitted with a single exponential curve (Fig. 2B). Holding the cell membrane to depolarized potentials (*e.g.* −40 mV for 30 sec) led to complete inactivation of the transient component, whereas the long-lasting component was

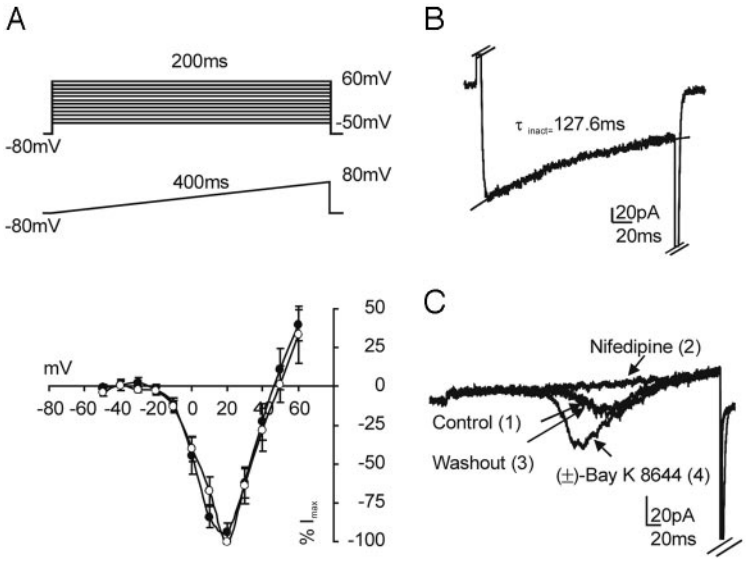

FIG. 1. Ba^{2+} currents through VDCC in human GC expressing only the L-type component. A, Normalized I/V plot of the peak currents recorded after the application of a 200-msec step protocol (●; n = 5), superimposed to the I/V plot obtained by measuring, at the same potentials, the currents elicited using a 400-msec ramp protocol (○; n = 4). *Error bars* represent the SEM. The two protocols of stimulation are shown on *top* (holding = −80 mV). B, A representative trace of a Ba^{2+} current elicited by a 200-msec step protocol to +20 mV (holding = −80 mV). The fitting curve of current inactivation superimposes the experimental trace in its decaying phase (τ = 127.6 msec). For calculation of the constant of inactivation, see *Materials and Methods*. C, Recordings of currents elicited in a GC by a ramp test (same protocol as in A). Perfusion with 5 μM nifedipine completely abolished the current, whereas the application of 10 μM (±)-Bay K 8644 increased the current and shifted the voltage-dependence of the channel activation to more negative potentials.

unaffected. This voltage-dependent inactivation was completely reversible when the holding potential was set back to −80 mV (Fig. 2C). As shown in Fig. 3, the kinetics of the transient and long-lasting components could be studied in isolation using a depolarizing step protocol in the presence of saturating doses of the selective inhibitors nifedipine (5 μM) or $NiCl_2$ (100 μM), respectively.

Because of the small size of whole-cell Ba^{2+} currents recorded in the majority of cells, we were not able to study the selectivity for different permeant divalent cations. However, the biophysical and pharmacological properties of the transient current indicate that it is gated through LVA T-type Ca^{2+} channels.

Cholinergic modulation of inward currents

Application of the cholinergic receptor agonist carbachol (50 μM) to the external solution significantly reduced the amplitude of both Ba^{2+} current components. The effect had a fast onset (reaching the maximum effect within ~30 sec) and was reversed by washout. The peak current amplitudes of the L- and T-type components were decreased by 34.7 ± 6.4% (n = 10) and 52.6 ± 7.4% (n = 8), respectively. Representative examples are shown in Fig. 4. Higher doses of carbachol (*e.g.* 500 μM) dramatically affected the stability of the baseline during recordings and caused variable reduction of the currents. Both effects were reversible and were pos[ps-

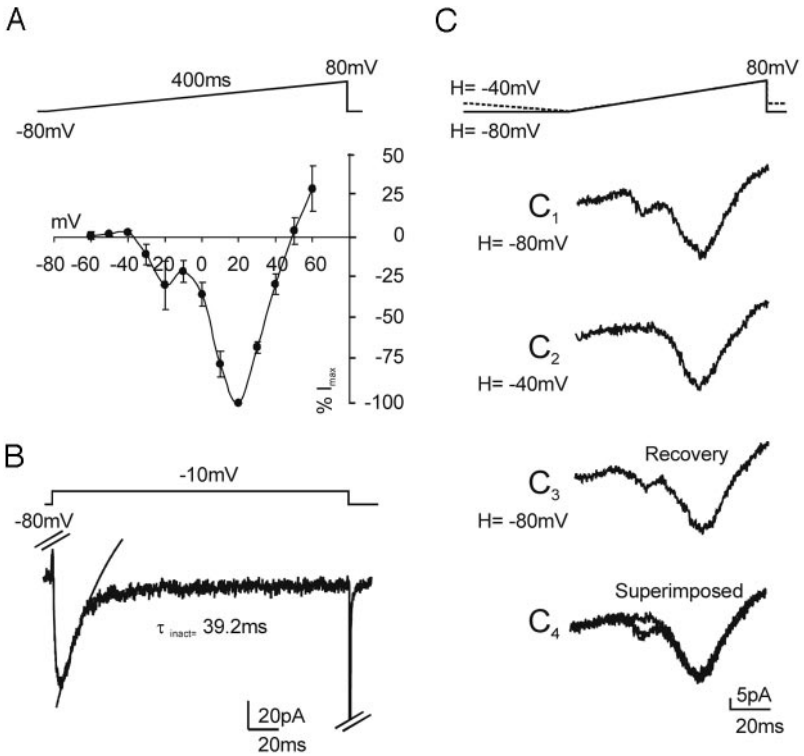

FIG. 2. Ba^{2+} currents through VDCC in human GC expressing both L- and T-type components. A, Normalized I/V plot obtained by measuring the currents elicited by the 400-msec ramp protocol shown on *top* (holding = −80 mV; n = 3). *Error bars* represent the SEM. B, A representative trace of a Ba^{2+} current recorded at −10mV using the step protocol shown on *top* (holding = −80 mV). The fitting curve of current inactivation superimposes the experimental trace in its decaying phase (τ = 39.2 msec). For the calculation of τ, see *Materials and Methods*. C, Sensitivity of T-type channels to membrane depolarization. Two 100-msec ramp protocols (shown on *top*) from −80 to +80 mV were used: one from holding = −80 mV (*continuous line*) and one from holding = −40 mV (*dotted line*). Cells were held at the different potentials for 30 sec before delivering the ramp. Both T- and L-type Ba^{2+} currents were elicited by the −80 mV holding protocol (C_1), whereas only the L-type Ba^{2+} currents were elicited by the −40 mV holding protocol (C_2). The T-type Ba^{2+} current was recovered when the −80 mV protocol was applied after the −40 mV holding protocol (C_3). Traces shown in C_1–C_3 are superimposed in C_4.

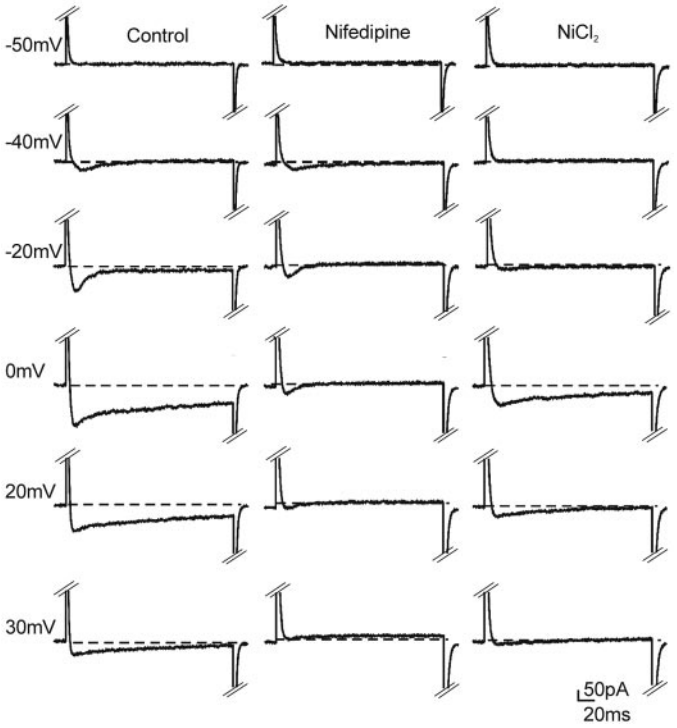

FIG. 3. Pharmacology of voltage-dependent Ba^{2+} currents in human GC expressing both L- and T-type channels. Currents were elicited by 200-msec depolarizing steps (holding = −80 mV) at selected potentials under control condition, or during perfusion with either 5 μM nifedipine or 100 μM $NiCl_2$ (a washing with Ba^{2+} solution was performed after nifedipine application of the drugs to restore control condition).

ibly related to a massive release of calcium from the internal stores that was not rapidly and/or completely buffered by the 10 mM EGTA contained in the recording pipette (data not shown).

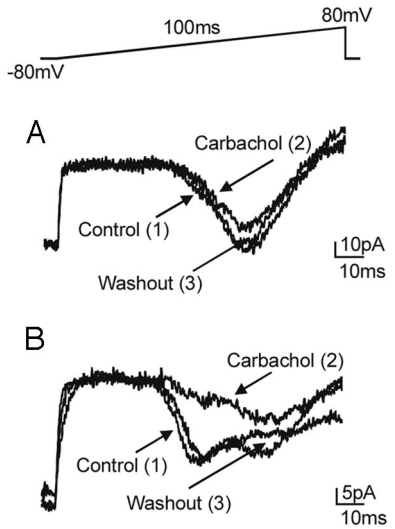

FIG. 4. Effect of the cholinergic agonist carbachol on L- and T-type voltage-dependent Ba^{2+} currents in human GC. Representative examples of the effect of 50 μM carbachol on an L-type Ba^{2+} current (A) and on both L- and T-type Ba^{2+} currents (B). The effect is reversed after washout. The ramp protocol applied is shown on *top* (holding = −80 mV).

Discussion

The present results indicate that human GC express functional L- and T-type VDCC whose conductance can be reduced by the activation of cholinergic receptors. In the cell population analyzed, we observed a certain degree of variability in the composition of the whole current, not related to the physiopathological features of the donors. Although the small proportion of female endocrine pathologies in our donor population precluded strong conclusions, it might be supposed that the variability simply reflects different states of the cell cycle (and, therefore, different functional states). The long-lasting component was predominant over the transient one in our sample. Agoston *et al.* (7) also found a significant variability in the composition of the whole current in human GC, but the transient component was largely predominant. This discrepancy might reflect the different methodological conditions (we only used cumula GC plated on poly-L-lysine-coated dishes, whereas Agoston *et al.* (7) used GC obtained from follicular aspirates, which may also include mural GC, and cultured them on glass coverslips), but other factors, including a different distribution of physiopathological conditions in the two donor populations, cannot be excluded. Differences in the composition of the whole current have also been reported for pig GC. Kusaka *et al.* (14) and Mattioli *et al.* (15) found only the transient component, whereas both transient and long-lasting components were pharmacologically differentiated by Lieberherr *et al.* (41).

The presence of L-type Ca^{2+} channels in GC is supported by previous studies performed with different methodological approaches in human (6, 7, 13), hen (16, 17), pig (8, 41), and rat (9) preparations. In human GC, these channels seem to play a role (even though not essential) in gonadotropin-stimulated progesterone secretion (6) and to be involved in the androstenedione-induced increase in $[Ca^{2+}]_i$, an important modulatory mechanism of human ovarian functions in both physiological and pathological conditions (13).

T-type Ca^{2+} channels have been found in human (7) as well as animal (14–17) GC, and their functional role is still under investigation. A large number of observations made in neuronal, muscular, and endocrine cells (42) indicate that the low threshold of activation of this channel subtype enables it to generate spontaneous depolarizing waves, which may trigger other cellular events, leading to action potential generation and/or hormone secretion. Interestingly, experiments carried out in chicken GC provide direct evidence of spontaneous Ca^{2+}-dependent action potentials preceded by a slow, 4- to 6-mV depolarization (43), possibly resulting from the activation of T-type calcium currents and/or the reduction of delayed outward K^+ currents. However, it should be noted that action potentials could not be elicited in human GC by depolarizing current injections, despite the presence of functional voltage-dependent Na^+ channels (44). A recent study of human GC has shown that T-type calcium currents also play a critical role in gonadotropin-stimulated progesterone production (7). Thus, the modulation of this channel subtype appears of particular importance, because it would affect both spontaneous and stimulus-triggered changes in calcium influx from extracellular fluid.

Our experiments on Ca^{2+} channel modulation were focused on the cholinergic system, recently characterized in the human ovary by Mayerhofer and colleagues (12, 35, 37, 38). The present results clearly indicate that both T- and L-type Ca^{2+} channels are under an inhibitory cholinergic control in human GC: 50 μM carbachol decreased Ba^{2+} currents through these channels by 34.7 and 52.6%, respectively. In partial accordance with our observations, Wan *et al.* (20) reported that 500 μM carbachol caused a 90% reduction of Ca^{2+} currents in hen GC under patch-clamp conditions. On the other hand, the observation that cholinergic receptor stimulation increases $[Ca^{2+}]_i$ in human GC (30) is not in contrast with our results, because it is due to Ca^{2+} release from intracellular stores. Furthermore, if the depolarization caused by this intracellular Ca^{2+} release was sufficient to open VDCC, it would trigger Ca^{2+} influx from the extracellular compartment (resulting in additional $[Ca^{2+}]_i$ increase) even if these channels were simultaneously (partly) inhibited by the same cholinergic receptor stimulation. This hypothesis is supported by data obtained in hen GC. In this preparation, where carbachol strongly decreases Ca^{2+} currents (18), Morley *et al.* (31) described a biphasic increase in $[Ca^{2+}]_i$ after 1 μM to 1 mM carbachol application: a fast increase due to Ca^{2+} release from intracellular stores and a delayed, long-lasting phase of increase due to Ca^{2+} influx from the extracellular milieu. However, a question arises: why does cholinergic receptor stimulation lead to opposite effects on VDCC permeability and Ca^{2+} release from intracellular stores? A possible explanation is that the effect on VDCC could be a *protective* action against calcium overload, which could cooperate with the negative feedback driven by Ca^{2+} on its own release from intracellular stores (45). VDCC inhibition might reduce Ca^{2+} influx elicited by depolarization due to different factors, including intracellular Ca^{2+} mobilization and changes in Na^+ (44) and K^+ (46) channel permeability in response to neurotransmitters, hormones, and other modulatory molecules. Interestingly, a recent study demonstrates that human GC express Ca^{2+}-activated K^+ channels (which play a prominent role in the cessation of Ca^{2+}-induced cellular responses by repolarizing the plasma membrane), and that these channels are activated by carbachol via elevated intracellular Ca^{2+} levels (46). Indeed, the opening of these K^+ channels might represent a third *protective* mechanism against Ca^{2+} overload during cholinergic receptor activation. The functional role of cholinergic modulation on human GC has been investigated by several authors in the recent years and will not be discussed in detail. The main outcome of these studies is that cholinergic receptor stimulation increases cell proliferation (12) and estradiol (36) and progesterone release (28, 36) (but the latter effect was not observed in a previous study) (12). Within this frame, we propose that the cholinergic inhibition of VDCC conductance might be a modulatory mechanism capable of preventing excessive changes in $[Ca^{2+}]_i$ and cell excitability during intracellular Ca^{2+} mobilization after autocrine/paracrine cholinergic stimulation.

Acknowledgments

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