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Pregnenolone sulfate increases intracellular Ca^{2+} levels in a pituitary cell line

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Abstract

We have investigated the rapid steroid effects on intracellular calcium ($[Ca^{2+}]_i$ levels in a clonal pituitary cell line (GH3). Among the steroids tested only pregnenolone sulfate induced a rapid and transient $[Ca^{2+}]_i$ increase within 1 min. The specificity of pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase with respect to steroid structure was pronounced. Other steroids (5–40 μM) including pregnenolone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, progesterone, estradiol-17β, testosterone, 5α-dihydrotestosterone, 5α-dihydroprogesterone, and 3α,5α-tetrahydroprogesterone were found to be ineffective. The $[Ca^{2+}]_i$ increase with pregnenolone sulfate (30 μM) was completely abolished in a Ca^{2+}-free medium or in the presence of La^{3+} (0.1 mM) and Co^{2+} (5 mM). The organic Ca^{2+} channel blockers methoxyverapamil (100 μM) and nicardipine (5 μM) both showed similar inhibitions (> 73%). The interaction between pregnenolone sulfate and voltage-gated Ca^{2+} channels (VGCC) was shown by coapplication of pregnenolone sulfate (10 μM) with Bay K 8644 (0.1 mM) or KCl (15 mM). Coapplication of pregnenolone sulfate with KCl increased the $[Ca^{2+}]_i$ in an additive manner. However, with the specific agonist Bay K 8644(±), the pregnenolone sulfate effect was potentiated in a majority of the cells, suggesting cooperative interaction between the two. The results demonstrate that pregnenolone sulfate induces a rapid Ca^{2+} influx in GH3 cells. The marked nicardipine block also suggests that most of the Ca^{2+} influx is mediated through L-type VGCC.

Keywords: Pregnenolone sulfate; Steroid; Ca^{2+}; GH3 cell; Pituitary

1. Introduction

The functional repertoire of steroid hormones includes a broad range of rapid, nongenomic effects along with their well-established genomic activities (McEwen, 1991). The nongenomic effects are rapid in onset (s-min) and short in duration (min-h). This mode of action includes a variety of effects such as the rapid induction of general anesthesia (Phillips, 1975; Bukusoglu et al., 1993) and the modulation of ion-channel activities (Paul and Purdy, 1992; Majewska, 1992). One of the most potent endogenous steroids for the above actions is the reduced progesterone metabolite, 3α,5α-tetrahydroprogesterone. At physiological levels, 3α,5α-tetrahydroprogesterone potentiates the γ-amino-*n*-butyric acid (GABA)-activated chloride currents in neurons and at pharmacological levels it induces rapid loss of righting response in mouse (Mok et al., 1991; Paul and Purdy, 1992; Majewska, 1992). Steroids also modulate neurotransmitter and hormone release in the brain (Drouva et al., 1984, 1985; Dluzen and Ramirez, 1987). In ovariectomized estrogen-primed rat striatal fragments, progesterone shows a bimodal influence on dopamine release (Dluzen and Ramirez, 1987). Continuous infusion of progesterone inhibits whereas intermittent infusion increases the amphetamine-stimulated dopamine release. Moreover, it was recently shown that certain neuroactive steroids are synthesized in the brain (Baulieu and Robel, 1990). In glial cells, pregnenolone is produced and later transformed into progesterone and its reduced metabolites. Pregnenolone and dehydroepiandrosterone are also present in the brain as sulfate esters. Thus, the presence of these neuroactive steroids suggests a new, localized role.

In the hypothalamus-pituitary axis, sex steroids modulate the hormone release in a Ca^{2+}-dependent manner. Progesterone at 0.1 μM enhances luteinizing hormone-releasing hormone (LH-RH) release from estradiol-primed mediobasal hypothalamic slices (Drouva et al., 1985; Ramirez et al., 1990). The Ca^{2+} channel blocker methoxyverapamil (D600) and Na^+ channel blocker

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tetrodotoxin effectively inhibit the progesterone-induced LH-RH release (Drouva et al., 1985; Ramirez et al., 1990). Estradiol-17β at 10 nM does not change the basal levels but effectively increases the KCl (56 mM)-induced LH-RH release (Drouva et al., 1984). Both steroid effects require Ca^{2+} in the medium.

Steroids also modulate the intracellular calcium ($[Ca^{2+}]_i$) and Ca^{2+} currents in certain tissues. In granulosa cells from preovulatory follicles, estradiol-17β increases the $[Ca^{2+}]_i$ levels through phosphoinositide hydrolysis (Morley et al., 1992). In hippocampal and spinal cord neurons, pregnenolone sulfate potentiates *N*-methyl-D-aspartate (NMDA)-induced Ca^{2+} influx and currents, respectively (Wu et al., 1991; Irwin et al., 1992; Bowlby, 1993). In GH3 cells which are a clonal strain of rat pituitary cells, estradiol-17β induces Ca^{2+}-dependent action potentials within 1–2 min (Dufy et al., 1979). Another steroid effect, the induction of sperm acrosome reaction by progesterone also depends on a Ca^{2+} influx (Blackmore et al., 1990).

In the present report, GH3 cells are used to demonstrate rapid steroid effects on the basal $[Ca^{2+}]_i$ levels. It is well established that Ca^{2+} ions mediate the secretory activity of endocrine cells (Albert and Tashjian, 1984; Aizawa and Hinkle, 1985; Gershengorn and Thaw, 1985). In this study, GH3 cells cultured on glass coverslips were treated with steroids and changes in basal $[Ca^{2+}]_i$ levels were monitored by microfluorometry. Among the steroids tested, only pregnenolone sulfate showed a rapid $[Ca^{2+}]_i$ surge. Other steroids including progesterone, 3α,5α-tetrahydroprogesterone, estradiol-17β, pregnenolone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, testosterone, 5α-dihydrotestoserone, 5α-dihydroprogesterone did not increase the basal $[Ca^{2+}]_i$ levels. Organic (D600, nicardipine) and inorganic (La^{3+}, Co^{2+}) Ca^{2+} channel blockers were used to identify the source of pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase.

2. Materials and methods

2.1. Reagents and hormones

Bay K 8644(±), D600, and nicardipine, were purchased from Research Biochemicals International (Natick, MA, USA). Cobalt chloride, dehydroepiandrosterone, dehydroepiandrosterone sulfate, 5α-dihydroprogesterone, dihydrotestosterone, estradiol-17β, lanthanum chloride, pregnenolone, pregnenolone sulfate, progesterone, testosterone, and 3α,5α-tetrahydroprogesterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA). Fura 2/AM and pluronic-F127 were obtained from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle medium was purchased from Life Technologies (Grand Island, NY, USA). GH3 cells were obtained from American Tissue Type Collection (Rockville, MD, USA).

2.2. Cell culture

GH3 cells were grown as monolayers on 12 mm glass coverslips in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5%CO_2-95% air at 37°C as described previously (Wang and Wang, 1994). The cells were replated once a week and the medium was replaced every 2–3 days.

2.3. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was determined by dual excitation microfluorometry using the Ca^{2+}-sensitive fluorescent dye, Fura-2. GH3 cells were incubated with normal buffer solution (140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 2.6 mM dextrose and 10 mM Hepes) containing 0.00125% (w/v) pluronic and 5 μM Fura-2/AM for 30 min at room temperature. The cells were then rinsed with normal buffer solution and incubated for an additional 30 min. Following incubation, the coverslip was transferred into a perfusion chamber (0.4 ml) with a constant flow rate of 4 ml/min. Steroids were applied for 1 min with the same flow rate. Dimethyl sulfoxide (DMSO) was the choice of solvent for the steroids and other drugs because pregnenolone sulfate was freely soluble in DMSO. Ethanol was used for 5α-dihydroprogesterone and Bay K 8644(±). The steroids and Bay K 8644(±) were then mixed with normal buffer solution in a final volume of less than 0.2% DMSO or ethanol. The addition of the vehicles did not change the basal or KCl (15 mM) stimulated $[Ca^{2+}]_i$ increase. Experiments were repeated 5 or more times with the best representative trace being chosen for illustration.

Microfluorometry was performed with an inverted epifluorescence microscope (Nikon DioPhot 200, Japan) connected to a light source capable of providing two rapidly alternating excitation wavelengths (Photon Technology International Deltascan 4000, South Brunswick, NJ, USA). Excitation wavelengths were 340 nM and 380 nM and the emitted light was passed through a 510 nM interference filter to a photomultiplier tube. The data collection was per second and the chopper frequency was 100 Hz. The cells with similar density were used and the field was limited to four to six cells for data collection. The concentration of the $[Ca^{2+}]_i$ was calculated according to the formula (Grynkiewcz et al., 1985):

$$[Ca^{2+}]_i = K_d \times b \times (R - R_{min})/(R_{max} - R).$$

R_{min}, R_{max} (F_{340}/F_{380}) and b (Sf2/Sb2) were determined in separate experiments. The R_{min} valuc (0.4 ± 0.01, mean ± S.E.M., $n = 8$) was measured in a Ca^{2+}-free normal buffer solution with EGTA (1 mM) and ionomycin (10 μM). Then, the R_{max} value (5.2 ± 0.3) was measured in

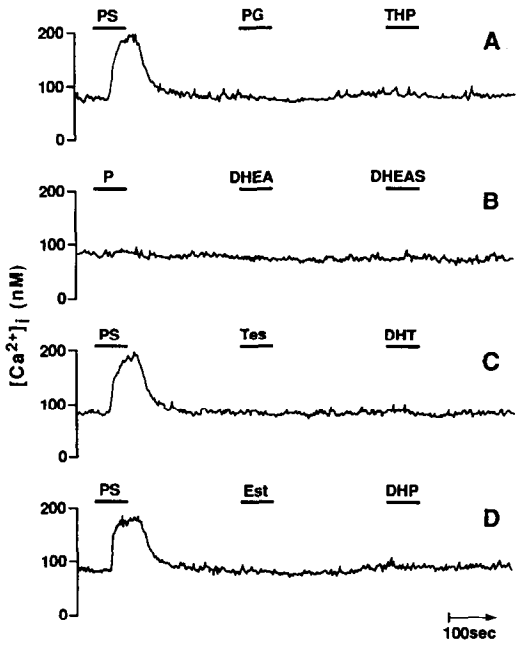

Fig. 1. Rapid steroid effect on $[Ca^{2+}]_i$. Steroids were applied to the same GH3 cells and $[Ca^{2+}]_i$ levels were monitored. The order of steroid application was from (A) to (D). (A) Pregnenolone sulfate (PS) (30 μM), progesterone (PG) (40 μM), 3α,5α-tetrahydroprogesterone (THP) (5 μM). (B) Pregnenolone (P) (40 μM), dehydroepiandrosterone (DHEA) (40 μM), dehydroepiandrosterone sulfate (DHEAS) (40 μM). (C) Pregnenolone sulfate (30 μM), testosterone (Tes) (40 μM), 5α-dihydrotestosterone (DHT) (10 μM). (D) Pregnenolone sulfate (30 μM), estradiol-17β (Est) (5 μM), 5α-dihydrprogesterone (DHP) (5 μM).

normal buffer solution with Ca^{2+} (15 mM) and ionomycin (10 μM). b was found to be 5.5 ± 0.2 (mean ± S.E.M., $n = 8$). K_d is the dissociation constant for Fura-2 and Ca^{2+}. Based on the experimental conditions (buffer composition and room temperature), a K_d of 224 nM was assumed (Grynkiewcz et al., 1985; Williams and Fay, 1990). The background fluoroscence was measured in the presence of digitonin and substracted from all measurements prior to calculations.

3. Results

3.1. Effects of steroids on $[Ca^{2+}]_i$ in GH3 cells

Steroids were applied to the same GH3 cells and the $[Ca^{2+}]_i$ levels were monitored (Fig. 1). Among the ten steroids tested only pregnenolone sulfate showed a rapid and transient increase in $[Ca^{2+}]_i$. Other steroids including progesterone, 3α,5α-tetrahydroprogesterone, pregnenolone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, testosterone, 5α-dihydrotestosterone, estradiol-17β and 5α-dihydroprogesterone did not affect the basal $[Ca^{2+}]_i$ levels. Due to the lack of solubility estradiol-17β, 3α,5α-tetrahydroprogesterone, 5α-dihydroprogesterone and 5α-dihydrotestosterone were applied at a final concentration of 5 and 10 μM, respectively. Pregnenolone sulfate was applied at a final concentration of 30 μM to induce a transient $[Ca^{2+}]_i$ increase. Other steroids were applied at concentration as high as 40 μM. Using separate coverslips, experiments were repeated with different orders of steroid applications and the same results were obtained. The marked specificity with respect to steroid structure demonstrates that the pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge is not a nonspecific effect but rather a specific action.

3.2. Reversibility and dose dependence of pregnenolone sulfate effect

The pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge was found to be transient and reproducible. To establish the reversibility of pregnenolone sulfate effect and the stability of GH3 cells under continuous perfusion, pregnenolone sulfate was repeatedly applied to the same GH3 cells (same coverslip) (data not shown). Two separate concentrations of pregnenolone sulfate (30, 10 μM) were used in order to detect small changes in response due to an inactivation. The pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge was found to be similar in five consecutive applications. In a separate coverslip, the apparent transient response of pregnenolone sulfate effect was further challenged by continuous application of pregnenolone sulfate (30 μM). Pregnenolone sulfate rapidly increased the basal $[Ca^{2+}]_i$ levels by approximately 5 times and then slowly decreased and stabilized at a relatively high $[Ca^{2+}]_i$ level (150 nM).

The dose dependence of pregnenolone sulfate effect was shown in Fig. 2. Increasing concentrations of pregnenolone sulfate were applied to GH3 cells and the percentage of $[Ca^{2+}]_i$ increase according to basal levels were plotted. The $[Ca^{2+}]_i$ increase showed an apparent saturation at the 100–150 μM range, however at 225 and 300 μM concentrations the response was markedly higher. Due to lack of

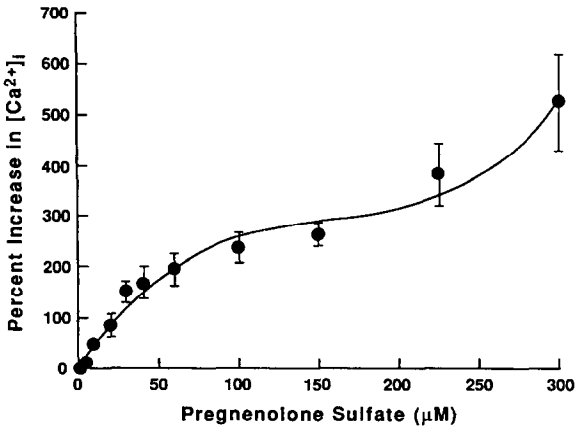

Fig. 2. Dose dependence of pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge. Increasing concentrations of pregnenolone sulfate (5–300 μM) were applied to the GH3 cells and the percentage of $[Ca^{2+}]_i$ increase was plotted. Each point represents the mean ± S.E.M. ($n = 5$–8).

solubility, we were unable to prepare higher concentrations of pregnenolone sulfate than 300 μM. Thus the pregnenolone sulfate response below 150 μM was fitted with a four parameter logistic function (Sigma Plot, Jandel Scientific, Corte Madera, CA, USA). The apparent EC_{50} was found to be 32 μM.

3.3. The contribution of extracellular Ca^{2+} to the pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase

To establish whether an influx of extracellular Ca^{2+} was responsible for the observed pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase, GH3 cells were perfused with Ca^{2+}-free buffer. In the absence of Ca^{2+}, the pregnenolone sulfate (30 μM)-induced $[Ca^{2+}]_i$ surge was completely abolished. After returning to the normal buffer solution, the basal Ca^{2+} level was slightly increased and the cells became responsive to pregnenolone sulfate (data not shown). Pretreating the cells with the inorganic Ca^{2+} channel blocker La^{3+} (100 μM) (Kostyuk et al., 1989; Shibuya and Douglas, 1992) almost completely blocked the pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge (data not shown). In the presence of La^{3+} (100 μM) the basal Ca^{2+} level was slightly reduced and the application of pregnenolone sulfate (30 μM) showed only a small change in $[Ca^{2+}]_i$. In other experiments ($n = 4$), the effect of La^{3+} was complete, in that pregnenolone sulfate did not induce any detectable change at the tracings. Pretreating the cells with another inorganic Ca^{2+} channel blocker, Co^{2+} (5 mM) also completely blocked the pregnenolone sulfate effect (data not shown). In these cells, instead of a Ca^{2+} influx, pregnenolone sulfate (30 μM) induced a Co^{2+} influx which was monitored by the quenching of fluorescence (data not shown). The complete elimination of pregnenolone sulfate effect with Ca^{2+}-free buffer and inorganic Ca^{2+} channel blockers La^{3+} and Co^{2+} demonstrates that the $[Ca^{2+}]_i$ surge is due to an influx from the extracellular medium.

To identify whether pregnenolone sulfate-induced Ca^{2+} influx was mediated by voltage-gated Ca^{2+} channels (VGCC), the GH3 cells were pretreated with D600 (50 μM, Fig. 3A), a nonspecific blocker (Liu and Jackson, 1985; Kostyuk et al., 1989), or nicardipine (5 μM, Fig. 3B), a specific L-type VGCC antagonist (Triggle and Janis, 1987; Kostyuk et al., 1989). The inhibition of KCl (15 mM)-induced $[Ca^{2+}]_i$ increase was used to show the efficiency of D600 and nicardipine. D600 at 50 μM almost completely blocked the KCl (15 mM)-induced Ca^{2+} influx (Fig. 3A). Based on the peak $[Ca^{2+}]_i$ levels, the inhibition was 92 ± 3% (mean ± S.E.M., $n = 5$). The effect of D600 against the pregnenolone sulfate-induced Ca^{2+} influx was significant but partial (Fig. 3A). At 50 μM concentration, D600 inhibited the pregnenolone sulfate effect by 60 ± 9% (mean ± S.E.M., $n = 5$). At the highest dose of 100 μM, D600 antagonized the pregnenolone sulfate effect by 74 ± 2% (mean ± S.E.M., $n = 4$)

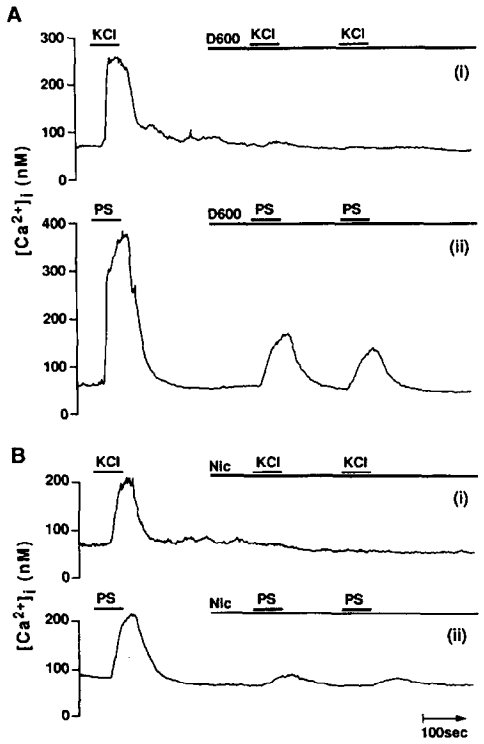

Fig. 3. The effect of D600 and nicardipine on the pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase. (A) (i) KCl (15 mM) and (ii) pregnenolone sulfate (PS) (30 μM) were applied to the GH3 cells in the absence and presence of D600 (50 μM). (B) (i) KCl (15 mM) and (ii) pregnenolone sulfate (30 μM) were applied to the GH3 cells in the absence and presence of nicardipine (5 μM).

(data not shown). D600 at this high dose (100 μM) blocks the hormone release in pituitary cells (Kostyuk et al., 1989). Nicardipine at 5 μM completely blocked the KCl-induced Ca^{2+} influx (Fig. 3B). Nicardipine at 5 μM also effectively inhibited the pregnenolone sulfate-induced Ca^{2+} influx by 73 ± 4% (mean ± S.E.M., $n = 5$). Nicardipine at a high dose of 20 μM blocked 77 ± 5% (mean ± S.E.M., $n = 5$) of the pregnenolone sulfate effect (data not shown). The marked inhibition of organic channel blockers (60–77%) further confirms a pregnenolone sulfate-induced Ca^{2+} influx mechanism and the nicardipine effect is consistent with the fact that most of the $[Ca^{2+}]_i$ surge occurs via L-type VGCC. The possibility that pregnenolone sulfate-induced Ca^{2+} influx is initiated through voltage-gated Na^+ channels is eliminated with the specific blocker tetrodotoxin. Pretreatment of the GH3 cells with tetrodotoxin (2 μM) did not alter the pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge (data not shown).

3.4. Interaction between pregnenolone sulfate and VGCC

Since pregnenolone sulfate induced a Ca^{2+} influx through VGCC, the next question was to ask whether pregnenolone sulfate modulates the Ca^{2+} channel activity. We have used KCl to chemically depolarize the cells or

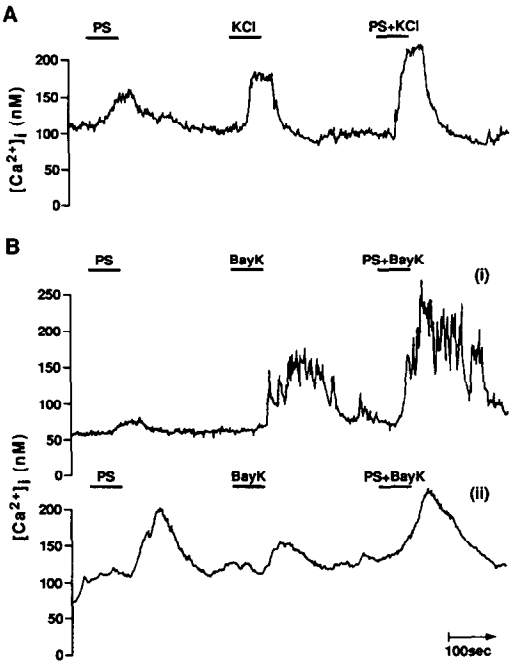

Fig. 4. Coapplication of KCl or Bay K with pregnenolone sulfate. (A) Pregnenolone sulfate (PS) (10 μM) was applied to the GH3 cells with and without KCl (15 mM). (B) Pregnenolone sulfate (10 μM) was applied to the GH3 cells with and without Bay K (0.1 μM) and different responses were shown in (i) and (ii).

Bay K 8644(±), the specific agonist for L-type VGCC (Enyeart et al., 1986; Triggle and Janis, 1987). KCl at 15 mM effectively induced a Ca^{2+} influx (Fig. 3). The nicardipine block (Fig. 3B) showed that the L-type channels were the main Ca^{2+} channels under these conditions. Coapplication of pregnenolone sulfate (10 μM) with KCl (15 mM) showed an increase in $[Ca^{2+}]_i$ as compared to pregnenolone sulfate and KCl alone. The percentage of $[Ca^{2+}]_i$ increase based on peak values by pregnenolone sulfate and KCl were 65 ± 10% and 177 ± 23% (mean ± S.E.M., $n = 8$), respectively. Coapplication of pregnenolone sulfate with KCl increased the $[Ca^{2+}]_i$ by 264 ± 33% (mean ± S.E.M., $n = 8$) suggesting an additive mode of interaction. Coapplication of pregnenolone sulfate with Bay K 8644(±) showed a heterogeneous response in different coverslips (Fig. 4B). Among the eight coverslips tested, pregnenolone sulfate with Bay K 8644(±) showed supra additive mode of interaction in five coverslips. An example is shown in Fig. 4B, (i). In these cells, coapplication of pregnenolone sulfate with Bay K 8644(±) increased the $[Ca^{2+}]_i$ more (111 ± 29%, mean ± S.E.M., $n = 5$) than the sum of the percent increase induced by the two agents. The difference was significant at the 0.01 level (paired, t-test analysis) (Zar, 1984). In other cells, pregnenolone sulfate with Bay K 8644(±) demonstrated an additive mode of interaction (Fig. 4B, ii). In these cells, coapplication of pregnenolone sulfate with Bay K 8644(±) induced approximately the same $[Ca^{2+}]_i$ increase as expected from the sum of pregnenolone sulfate and Bay K 8644(±) responses.

4. Discussion

The results demonstrate for the first time that pregnenolone sulfate induces a dose-dependent $[Ca^{2+}]_i$ increase in GH3 cells. This increase was completely abolished in the absence of Ca^{2+} or in the presence of inorganic Ca^{2+} channel blockers (La^{3+}, Co^{2+}) demonstrating that the pregnenolone sulfate effect was due to a Ca^{2+} influx. GH3 cells possess L- and T-types of VGCC (Armstrong and Matteson, 1985; Suzuki et al., 1990). The nonspecific organic channel blocker, D600 and the specific channel blocker nicardipine significantly attenuated the pregnenolone sulfate-induced Ca^{2+} influx to similar degrees. This suggests that most of the pregnenolone sulfate-induced $[Ca^{2+}]_i$ increase is mediated by L-type VGCC. The pregnenolone sulfate effect was not limited to Ca^{2+} ions. GH3 cells like many other cells are permeable to Co^{2+} ions (Thaw et al., 1984; Shibuya and Douglas, 1992) and as expected pregnenolone sulfate also induced a rapid Co^{2+} influx (data not shown). The interaction between pregnenolone sulfate and VGCC was different with respect to the stimulation used for the Ca^{2+} channels. In the presence of KCl (15 mM), pregnenolone sulfate showed an additive mode of interaction suggesting that under this mild depolarizing condition pregnenolone sulfate effect does not show a significant change. However, when the specific agonist Bay K 8644(±) was used, pregnenolone sulfate potentiated the Bay K 8644(±)-induced Ca^{2+} influx in five out of eight coverslips, suggesting a cooperative type of interaction.

The marked specificity of $[Ca^{2+}]_i$ increase with respect to the steroid structure also suggests a specific target site for pregnenolone sulfate in GH3 membranes. Even at micromolar levels estradiol-17β as well as progesterone, dehydroepiandrosterone sulfate and pregnenolone did not change the basal $[Ca^{2+}]_i$. The lack of effect of estradiol-17β on $[Ca^{2+}]_i$ levels does not correlate with the NMDA-induced Ca^{2+}-dependent spiking activity which was shown by Dufy et al. (Dufy et al., 1979) and may require further research. In a preliminary study, under similar conditions where the estradiol-17β concentration was 10 μM, the basal $[Ca^{2+}]_i$ levels did not change (data not shown). It is possible that the estradiol-17β-induced Ca^{2+}-dependent spiking activity in GH3 cells (Dufy et al., 1979) may generate near membrane Ca^{2+} changes. It has been suggested that during physiological responses, near-membrane Ca^{2+} levels may be markedly different from the whole cytosol levels (Etter et al., 1994). However, in our assay system the overall Fura-signal from four to six cells was measured which might conceal any significant and localized $[Ca^{2+}]_i$ changes within a single cell body.

Specific effects of pregnenolone sulfate were previously

shown in hippocampal and spinal cord neurons. In these studies, pregnenolone sulfate at similar micromolar levels (EC_{50} 5–57 μM) potentiated the NMDA-induced currents and inhibited the GABA- and glycine-induced currents (Majewska and Schwartz, 1987; Wu et al., 1990; Wu et al., 1991; Bowlby, 1993). It is interesting to note that in CA1 neurons, pregnenolone sulfate at 250 μM concentration did not change the basal $[Ca^{2+}]_i$ levels where KCl at 20 mM depolarized and increased the $[Ca^{2+}]_i$ (Irwin et al., 1992). The lack of pregnenolone sulfate effect on $[Ca^{2+}]_i$ levels at 250 μM concentration eliminates any nonspecific pregnenolone sulfate action and suggests specific pregnenolone sulfate effects on target sites. Separately, in a recent study pregnenolone sulfate inhibited the Ca^{2+} currents in CA1 neurons (ffrench-Mullen et al., 1994). The IC_{50} was 11 nM. However, the apparent IC_{100} was reached at approximately 10–100 μM range. As a result, pregnenolone sulfate exhibits a wide range of effects on ligand and voltage-gated ion channels. This level of diversified ion-channel modulation suggests tissue-specific target site(s) for pregnenolone sulfate.

The physiological or pharmacological significance of pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge remains to be elucidated. In humans, the highest anterior pituitary and plasma levels for pregnenolone sulfate are reported to be 0.09 and 0.13 μM, respectively (DePeretti and Mappus, 1983; Lanthier and Patwardhan, 1986). These levels are approximately 100 times lower than the minimum pregnenolone sulfate concentration (10 μM) required for $[Ca^{2+}]_i$ surge, suggesting that the pregnenolone sulfate effect in GH3 cells may not correlate with the normal endogenous pregnenolone sulfate levels. However, under certain physiological or pathological conditions, the pregnenolone sulfate levels may be different. At birth, serum pregnenolone sulfate levels obtained from umbilical cord were found to be in the 2.6–3.9 μM range (Laatikainen and Peltonen, 1975; Chang et al., 1976; Kojima et al., 1981; DePeretti and Mappus, 1983). Micromolar levels of pregnenolone sulfate have also been shown in patients with adrenocortical carcinoma and placental sulfatase deficiency (Hirato and Yanaihara, 1990; Grodal and Curstedt, 1991). In these patients serum pregnenolone sulfate levels were in the range of 1–6 μM which is very close to the pregnenolone sulfate concentrations used in the present study. These high pregnenolone sulfate levels in serum suggest that the pregnenolone sulfate-induced $[Ca^{2+}]_i$ surge may have a physiological relevance at birth or a clinical relevance in certain diseases.

Finally, it has also been pointed out that the effects of pregnenolone sulfate on $GABA_A$, glycine and NMDA receptors may be synergistic (Wu et al., 1991). In neurons pregnenolone sulfate may alter the balance between excitation and inhibition towards excitation. In this hypothesis, low concentrations of pregnenolone sulfate might regulate the excitibility of brain and neuroendocrine functions such as the hypothalamic-pituitary axis. In this kind of interactive regulation, the direct effect of pregnenolone sulfate on $[Ca^{2+}]_i$ levels in anterior pituitary cells may modulate the pituitary hormone release.

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