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FREE INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$) IN OPIOID SENSITIVE CELLS OF THE PORCINE ANTERIOR PITUITARY

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A new experimental model was utilized to study calcium involvement in the mechanism of opioid influence on cultured porcine pituitary cells. The *in vitro* model involved interactive argon laser cytometry of pituitary cells pre-loaded by three dyes (fluo-3AM, fura-red and naloxone-conjugated to fluorescein). We compared: 1) the kinetics of free intracellular calcium ($[Ca^{2+}]_i$) in anterior pituitary cells of pregnant pigs (day 25—30) treated *in vitro* with naloxone (NAL) or gonadotrophin-releasing hormone (GnRH) and 2) the distribution of the opioid-sensitive cells by image analysis of doubly loaded cells. In experiment 1, the changes in $[Ca^{2+}]_i$ of pituitary cells pre-loaded with fluo—3 AM (488_{ex} /520_{em} nm) in response to NAL (10^{-6} M) or to GnRH (10^{-8} M) were compared to a control cell group. Repetitive line scans across cells were performed and the fluorescence emission from individually selected cells was measured in a time-dependent manner (in 0.5 seconds intervals during periods of 50 seconds). Analysis of data indicated significant increases of $[Ca^{2+}]_i$ in NAL- ($P<0.001$) and GnRH-treated cells ($P<0.05$) in comparison to the control group. In experiment 2, the fluorescence intensity of doubly-loaded pituitary cells (fura-red, 488_{ex} /605_{em} nm, as principal calcium indicator and NAL-conjugated with fluorescein, 488_{ex} /520_{em} nm, to distinguish opioid-sensitive cells) were measured using dual detector image analysis. We found that only ~8% of the entire population of anterior pituitary cells exhibited sensitivity to the opioid antagonist treatment.

This paper demonstrates calcium involvement in the opioid action on anterior pituitary cells from pregnant pigs and provides a useful model for studies at the individual pituitary cell level and in time-dependent manner.

Key words: *calcium, fluo-3 AM, fura-red, naloxone, naloxone-fluorescein, opioids, pig, pituitary*

INTRODUCTION

Several studies on anterior pituitary cells have provided increasing evidence that pituitary activity is under the general control of releasing and inhibiting hypothalamic hormones as well as multiple factors that modulate the pituitary response by intercellular communication within the gland. The relationship

that exists between gonadotrophs and other types of pituitary cells creates an ideal situation in which paracrine interaction can occur. Furthermore, the responses of pituitary cells to hypothalamic releasing hormones share some similarities with neurotransmission. Occupation of receptors on heterogeneous pituitary cell populations initiates cellular signaling processes that modulate their different secretory activity. Dynamic alterations at the level of cytoplasmic free calcium ions are probably crucial for coordination of several signaling pathways and their secretory responses. In rat gonadotrophs, during stimulation of the GnRH receptor, G-proteins activate phospholipase C, which catalyzes the hydrolysis of phoshatidylinositol 4,5-biphosphate into the intracellular messengers Ins-1,4,5-P_3, and 1,2-diacylglycerol which mobilize calcium from intracellular stores (1). GnRH stimulates a biphasic rise in calcium concentration in these cells (2, 3), which is associated with hormone biosynthesis (4) and secretion (5). Intracellular calcium receptors in the rat gonadotrophs include calmodulin binding proteins, such as calcineurin, caldesmon and spectrin (6, 7). While cellular mechanisms of GnRH action on gonadotrophin secretion has been widely investigated, modulatory influences of different factors on these processes at the pituitary cell level are poorly understood. Limited studies have been performed on porcine pituitary cells. Barb *et al.* (8), performing *in vitro* studies of entire porcine pituitary cell populations noted the modulatory action of EOP on gonadotrophin secretion. On the basis of studies mentioned above, new questions arise as to whether opioids have a modulatory action on calcium ion concentrations in porcine pituitary cells. These actions would be crucial in signal transduction following GnRH stimulation.

In several earlier studies, intracellular calcium ion dynamics have been measured using different fluorescent calcium indicator dyes. Commonly, acetoxymethyl ester derivatives of fluo-3 (fluo-3 AM) has been used for different types of cells (9—17). Cells pre-loaded with different fluorescent dyes and subjected to interactive argon laser cytometry allow for monitoring intracellular calcium kinetic events in individual cells.

The objective of this study was to demonstrate free intracellular calcium involvement in the opioid signal transduction in cultured porcine anterior pituitary cells treated with NAL or GnRH at the single cell level and in a time-dependent manner. This paper also reports preliminary results of a dual-labeling of pituitary cells of pregnant pigs with fura-red and fluorescein conjugated with NAL that provides useful methodology to define the distribution of opioid-sensitive cell within the cultured anterior pituitary cell population.

MATERIALS AND METHODS

Materials

The calcium indicators, acetoxymethyl esters of fluo-3 AM and fura-red AM, naloxone (NAL) conjugated to fluorescein, dimethyl sulfoxide (DMSO), pluoronic F-127 and ionophore A23187 were purchased from Molecular Probes Inc. (Eugene, OR). Media were purchased from Gibco (Grand Island, NY). Unconjugated NAL and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Pituitary cell cultures

Anterior pituitaries, collected immediately after slaughter from pregnant gilts (Day 25—30) were separated from posterior pituitaries and placed in a sterile solution: 25 mM Hepes (4-2-hydroxyethyl-1-piperazine-ethane sulfuric acid), supplemented with 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 360 μM. $CaCl_2$, 10 mM glucose and 50 IU/ml penicillin-G, 50 μg/ml streptomycin, 0.1% amphotericin and 0.1% BSA. Pituitaries were washed, minced into small pieces (1—2 mm) and dispersed with 0.3% trypsin dissolved in Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+}(HBSS-Ca&Mg-free), in a dilution of 15 ml/g of wet tissue. Trypsinization was performed three-four times, 15 minutes each, at 37°C, and the cell suspension was filtered through a sterile nylon filter (60 μm mesh; Tetko, New York, NY) to remove the remaining clumps and then cells were centrifuged at 375 × g/10 min. Pellets of cells were resuspended and cells were washed three times. Then, cells were counted using a haemocytometer and their viability was determined by 0.4% trypan blue dye exclusion. Finally, cells were resuspended in a plating medium of Dulbecco's modified Eagle's medium (DMEM), pH = 7.5, supplemented with 10 mM Hepes, 10 mM $NaHCO_3$, 1.0 mM sodium pyruvate, 2.0 mM glutamine, 50 IU/ml penicillin-G, 50 μg/ml streptomycin, 0.62 μg/ml amphotericin, 1% MEM (non-essential amino acids). Incubation media were enriched with 10% (v/v) Platelet Derived Horse Serum and 2.5% (v/v) fetal bovine serum (both from HyClone, Logan, UT). To concentrate cells in a smaller area, cloning cylinders (id: 5 mm, Bellco Glass, Vineland, NJ) were placed in the center of 35 mm Petri dish with 2 ml of incubation medium, and the number of cells was 25—50 × 10^3 cells/cylinder. The concentration of cells in the center of Petri dishes made them more available for correct focusing during the scanning procedures. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air. In experiment 1, the cylinders were removed after 24 h and cultures were continued until 48—72 h. In experiment 2, medium was changed after 48 h and culture was extended for additional 48 h.

Preparation of pituitary cells for cytometry and loading cells with calcium indicators:

Loading of cells with fluo-3 AM in Experiment 1

After two-three days of culture, Petri dishes containing attached pituitary cells were washed twice with DMEM serum-free. Then, cells were loaded with medium that contained 10 μM fluo-3 AM, hereafter designated as the dye-loading solution. Preliminary studies were performed to define: a) the required concentration of calcium indicator dye, b) time of incubation with dye, and c) time of dye stability in cells. On the basis of the preliminary studies, final dye-loading solutions were prepared by mixing 5 μl aliquots of a stock solution of fluo-3 AM (10^{-3} M.) with a 2.5 μl aliquot of the surfactant pluoronic F-127 (25% w/v in DMSO), and then diluted to a final volume of 1 ml with DMEM, just before addition to the dish of cultured cells. The surfactant was used to

ensure dispersion of the DMSO-dye solution in the medium. The final concentration of surfactant in media did not exceed 0.125%. To avoid the formation of dye clumps adhering to the cell membranes, the dye/surfactant solutions were mixed vigorously before adding to dishes containing attached cells. Loading cells with dye was accomplished by placing the Petri dishes, protected from light, on the platform of a shaking water bath at 37°C. After shaking for 30 min, cells were washed five times with fresh pre-warmed medium, which did not contain dye. Fluo-3 AM-loaded cells were scanned according to the protocols described below. Maximum fluorescence intensity of dye-loaded cells was measured with 10 μM ionophore A23187 dissolved in 2 mM Ca^{2+} HBSS (pH-indicator-free) and incubated for 1h. Minimum fluorescence intensity was measured with addition of 1M $MnCl_2$ to dishes with dye-loaded cells.

Double-loading of cells with fura-red and NAL-fluorescein in Experiment 2

Because both fluorescent dyes, fluo-3 AM and naloxone-fluorescein conjugate, have similar excitation/emission wavelenghts (see below), loading of cells with fluo-3 AM was replaced with fura-red.

Fura-Red. Petri dishes with adhered pituitary cells were washed twice with DMEM serum-free. Immediately prior to the experiment, incubation medium was changed to a loading-dye solution containing 20 μM fura-red acetoxymethyl ester/dish with the addition of 2.5 μl of the surfactant pluoronic F-127 (0.125%, w/v; dissolved in DMSO). Dishes of pituitary cells, protected from light, were then incubated for 60 minutes at 37°C on the platform of a shaking water bath. After first loading, cells were washed five times with fresh medium to remove excess dye and used for calibration of detector 2 and control tests or subjected to loading with second fluorescent indicator for the double-scanning procedure of cell distribution.

NAL-fluorescein. Pituitary cells, non-loaded with fura-red (for detector 1 calibration and control tests) or pre-loaded with fura-red (for experiments), were incubated with 10 μl NAL-fluorescein conjugate per dish, for 2—3 minutes at room temperature, washed five times and immediately subjected to scanning procedures.

Pituitary cells doubly-loaded with fura-red and NAL-fluorescein conjugate were immediately placed in the pathway of laser beam, focused and simultaneously scanned to measure emissions of both fluorescences, fura-red and fluorescein, by adequate detectors (det. 2 and det. 1, respectively), as described below.

Treatments of pituitary cells

Porcine pituitary cells, pre-loaded with fluo-3 AM in experiment 1, were treated with NAL or GnRH. Both treatment substances were diluted in pre-warmed Hank's Balanced Salt Solution (HBSS-pH-indicator free) which contained 2 mM Ca^{2+}. Control cells were treated with medium alone. All treatments were carried out in locally prepared Petri dish-chambers fitted with inlet and outlet lines, allowing for the replacement of the media with treatments between sequential multiple scans. Only one cell from each culture dish was selected for measurement. During (0—5 sec) and after treatment (5—50 sec) cells were repeatedly scanned (line scans across the cell). Primary studies were performed to establish a dose response of pituitary cells to treatment factors and appropriate time for each measurement (*Fig. 1*). Porcine pituitary cells ($n = 10$ per each group) were treated with NAL (10^{-5}, 10^{-6} and 10^{-7} M/dish) or GnRH (10^{-6}, 10^{-7} and 10^{-8} M/dish) and compared to the control group cells treated with medium alone. Finally, for laser cytometry, the responses of the most effective doses for NAL (10^{-6} M, $n_{exp} = 8$) and GnRH (10^{-7} M,

$n_{exp} = 8$) were used to measure calcium fluxes and compared to the control group (CONT, $n_{exp} = 15$). Fluorescence intensity data were recorded during multiple measurement periods according to procedure described below.

Kinetics of calcium responses

Using an interactive argon laser cytometry (Meridian Instruments, Okemos, MI) and ACAS 570 Software Version 3.0, changes of the free cytoplasmic calcium fluxes were determined in cultured porcine pituitary cells treated with stimulating factors.

Laser setup and calibration

On the day of each experiment the laser beam was calibrated at the wavelength used for excitation of each dye: for experiment 1 with fluo-3 AM ($488_{ex}/520_{em}$ nm, detector 1), or for experiment 2 with fura-red ($488_{ex}/605_{em}$, detector 2) and NAL-fluorescein ($488_{ex}/520_{em}$ nm, detector 1). Dye excitation was achieved by scanning focused cells with the beam of the argon laser. Calibration protocols were completed before each experiment to assure consistency of dye exitation with fluorescence emissions. The laser beam was considered to be calibrated (and thus useable) if the difference between calibration and test data curves did not exceed 5%. Parameters for multiple scans were set after optimization of laser power and photomultiplier tube voltage detector (PMT) sensitivity. After calibration, a lower laser power level was selected (10—100 mWatt) to reduce bleaching of dyes and a 10% neutral density filter was used in the excitation pathway of the beam. Dye-loaded cells were placed in the path of the laser beam, focused, and all scanning procedures performed under ambient conditions at a controlled room temperature at 22—24°C.

Laser setup for serial line scans — experiment 1

Multiple image line scans across a single pituitary cell were performed by using the previously optimized parameters: 35% PMT setting, 1.00 μm step size, 100 scans, 100 points, 0.5 sec delay, 10.00 mm/sec stage speed, 20% scanning strength and 100 mW laser power. These parameters were selected empirically from the results of the primary study with fluo-3AM and were controlled by an optimization protocol. Laser power and detector sensitivity settings were optimized until the last measurement during entire series of scans performed on intact cells did not show any evidence of dye photobleaching. All parameters remained constant for each experiment.

Laser setup for image scans analyses — experiment 2

Image scans were performed with the following parameters: 35—40% PMT, 1.00 μ step size, 20 scans, X/Y points 200—360, 45—100 sec scan delay, 10.00 mm/sec stage speed, 20—25% scanning strength. These parameters were adequately optimized, similar to line scans. Dual detector image scans were performed on the basis of selected colonies of cells pre-loaded with fura-red (to determine the total number of analyzed cells and their areas) and cells that were exposed to NAL-fluorescein conjugate (to determine opioid-sensitive sites on cells).

Statistical procedures

Serial line scans analyses of experiment 1 data

The procedure of repetitive line scans was employed for measurement of the calcium fluxes in pituitary cell pre-loaded with fluo-3 AM in response to NAL and GnRH and compared to the group of control cells. Measurements of serial line scans were performed on the basis of a selected cell (a single cell from each Petri dish) scanned across in 0.5 sec intervals during 50 sec periods. Using the ACAS 570 software kinetics program, monitored changes of $[Ca^{2+}]_i$ in pituitary cells were recorded over scanning time. From the kinetic data recorded during scanning procedure and subtracting the background fluorescence, the normalized fluorescence rates were calculated, and then were used for constructing plots of normalized fluorescence over time. Data were analyzed using general linear models procedures (SAS, 1988).

Dual-image scans analyses of experiment 2 data

Recorded kinetic value data of scanned cells, marked as polygons on the entire image had the background fluorescence subtracted. The kinetic data were quantitated by image analysis of fluorescence intensities of both measurements, separately for each dye by appropriate detector, and calculated as the ratio of normalized fluorescence. Results were presented as a percentage of fluorescence for NAL-sensitive sites of cells (detector 1) versus total fluorescence of entire group of cells pre-loaded with fura-red (detector 2). All data were analyzed using general linear models procedures (SAS, 1988).

RESULTS

Experiment 1

These experiments demonstrate a useful technique for investigation of calcium fluxes, in time-dependent manner, in cultured pituitary cells pre-loaded with fluo 3-AM. Results clearly indicated that influx of extracellular calcium had occurred in porcine pituitary cells exposed to either GnRH or NAL. *Figure 1A* presents an example of data from the primary experiment, depicting free intracellular calcium fluxes (measured as normalized fluorescence) within individual anterior pituitary cells, sequentially treated with NAL (10^{-7}, 10^{-6}, 10^{-5} M, respectively). The most effective dose of NAL, 10^{-6} M that caused approximately a 50% increase in fluorescence intensity by 10—12 seconds post-treatment, was chosen for the entire experiment (*Fig. 1B*). Treatment with NAL or GnRH elevated normalized fluorescence in comparison to the CONT group (*Fig. 2*). Similar patterns of increased fluorescence were observed in both experimental groups of pituitary cells ($P<0.05$). Markedly higher normalized fluorescence during entire measured time was observed in the group of NAL-treated cells ($P<0.001$) than in the group treated with GnRH ($P<0.05$), when compared to the control group. However, significant differences were observed during 15—37.5 seconds ($P<0.001$) and 42.5—50 seconds ($P<0.01$) GnRH post-treatment.

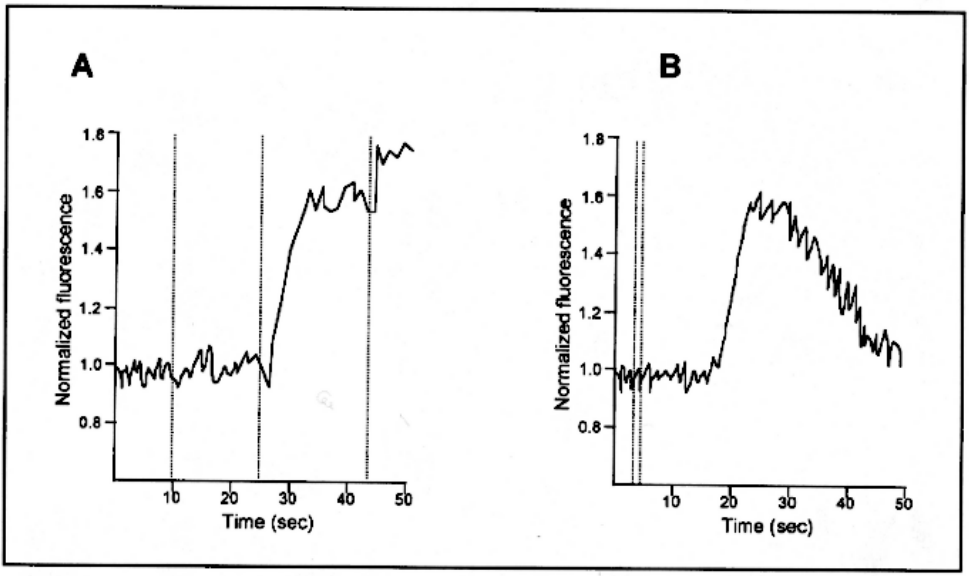

Fig. 1. Cultured porcine anterior pituitary cells pre-loaded with fluo-3 AM and treated with NAL: **A**). An example of normalized fluorescence measurements of pituitary cell in response to different doses of NAL (vertical lanes marked treatments with: 10^{-7}, 10^{-6}, 10^{-5} M, respectively). **B**). Changes of normalized fluorescence measurements of the individual pituitary cell treated with NAL (vertical lanes indicate time of treatment with 10^{-6} M).

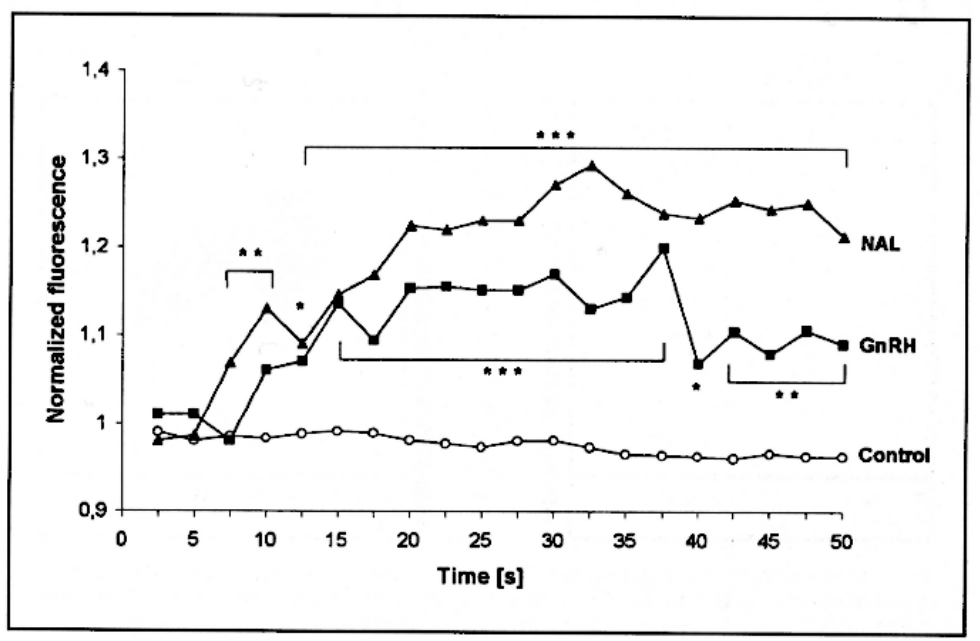

Fig. 2. Experiment 1. Normalized fluorescence of pituitary cells treated with NAL ($n_{exp} = 8$) or GnRH ($n_{exp} = 8$) in comparison to the control group cells ($n_{exp} = 15$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

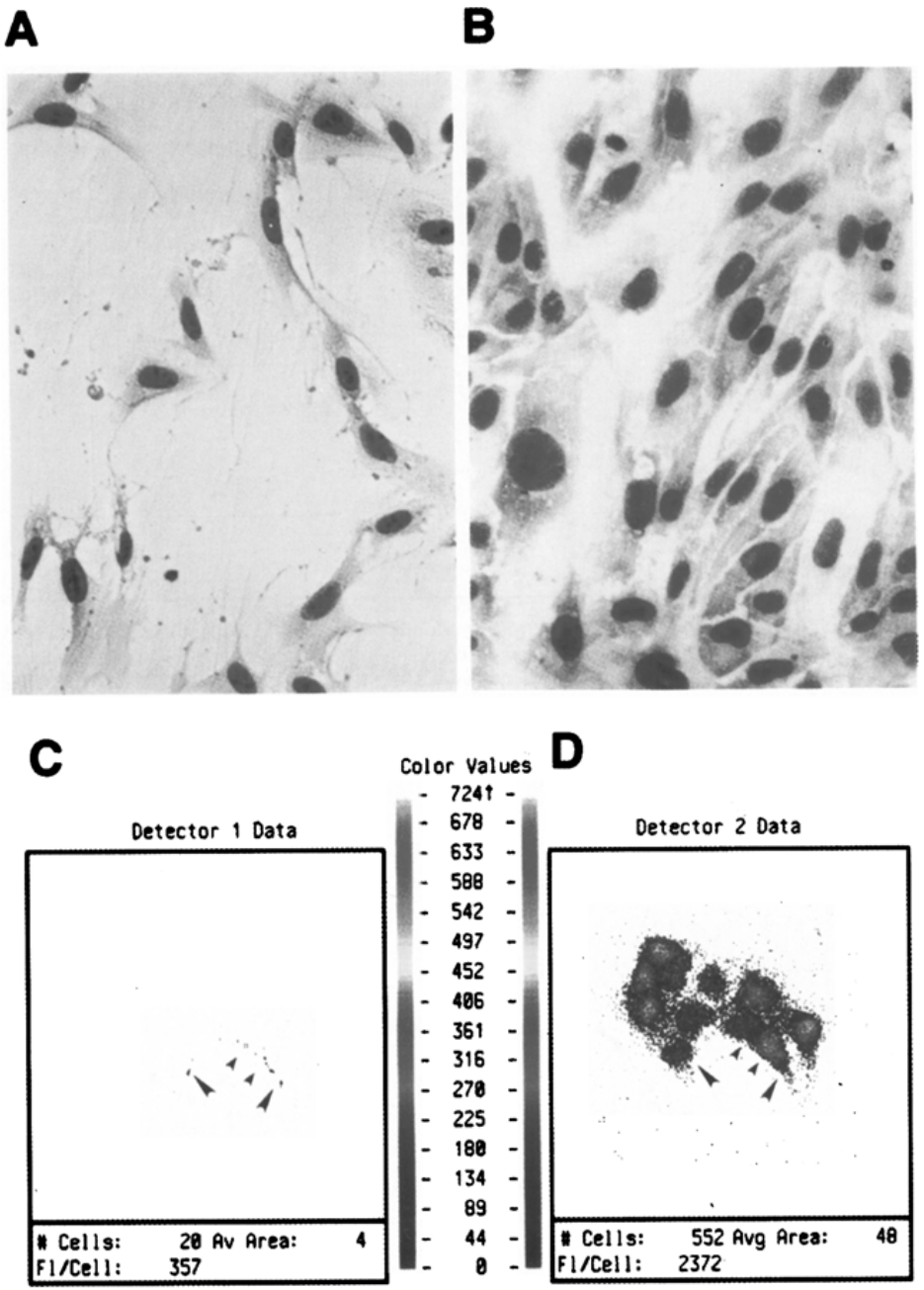

Fig. 3. Cultured porcine pituitary cells (40×) used in experiment 1 and 2 (individual cells in **A** and monolayer in **B**, respectively), stained with Quick-Stain dye. *Figures* **C** and **D** present examples of images during measurements of opioid-sensitive cells distribution in 2 experiment. Dual fluorescence emitted from pituitary cells loaded with NAL-fluorescein (det. 1) compared to fluorescence emitted from the same cells pre-loaded with fura-red (det. 2). Arrowheads indicate the opioid-sensitive sites on cells within the colony.

Experiment 2

Figure 3 presents anterior pituitary cells on day 3 (A) and day 5 (B) of culture. The cells sequentially doubly-loaded with dyes emitting adequate fluorescences are presented in *fig. 3C* and *3D*. Detector 2 data demonstrates pituitary cells pre-loaded with general calcium indicator, fura-red, indicating free calcium concentration within each cell of the colony (*Fig. 3D*). Detector 1 data indicates binding sites for opioids (by NAL-fluorescein) on only some cells of the colony (*Fig. 3C*). Pituitary cells separately pre-loaded with NAL-fluorescein or with fura-red in control experiments (*Table 1*) did not show any fluorescence as measured by alternative detectors. Image analysis indicated that only 30% of studied objects area was occupied by NAL-fluorescein conjugates, which corresponded to approximately 8.1% of the porcine anterior pituitary cell population.

Table 1. The ratio of fluorescence detected for opioid-sensitive sites on cells (NAL-fluorescein) compared to entire population of cultured porcine pituitary cells (pre-loaded with fura-red), measured by detector 1 and by detector 2, respectively.

Groups	N	Detector 1 (530 nm)	Detector 2 (660 nm)
Control fluorescence intensity of:			
1. Cells loaded with fura-red	5	0.0%	100.0%
2. Cells loaded with NAL-fluorescein	5	100.0%	0.0%
Treatment with dual loading	11		
3. Ratio of fluorescence per areas		30.0%	100.0%
4. Distribution of opioid-antagonist sensitive cells		8.1%	0.0%

DISCUSSION

The responses of porcine anterior pituitary cells in our studies (pre-loaded with calcium indicator, fluo-3 AM and measured by argon laser cytometry) to NAL or GnRH treatments have demonstrated an involvement of free intracellular calcium fluxes in these stimulations. The very short-term responses (total time approximately 50 sec) of pituitary cells to both treatments were detectable and thus measurable during experiments performed in a time-dependent manner only (experiment 1). Image analysis (experiment 2) performed on doubly-loaded pituitary cells indicated opioid binding sites (NAL-fluorescein conjugate) on the surface membranes of cells already pre-loaded with fura-red. This dual-labeling experiment allowed us to

distinguish the percentage of opioid-sensitive cells among the heterogeneous cell population of porcine pituitary gland.

Although the involvement of endogenous opioid peptides (EOP) in the regulation of gonadotrophin secretion has been well documented at the level of pituitary or hypothalamus in the pigs in standard *in vitro* studies (8, 18) and in several *in vivo* studies (19—21), direct evidence of calcium involvement in the EOP pathway has not been shown in this species.

One proposed hypothesis is that EOP can act by inhibiting calcium ion influx into neuron terminals, thereby reducing neurotransmitter release (22). Studies *in vitro* have shown that terminals releasing GnRH within the stalk median eminence (SME) of gilts are sensitive to signals coming from EOP system, independently of ovarian steroids. However, these hormones can modulate EOP effect on GnRH release (18). Generally, the complex mechanism of EOP action has been evaluated with different antagonist and agonist treatments. Naloxone, a popular multifunctional opioid antagonist, at low concentrations has a very high affinity for the μ-receptors, but at greater concentrations can also block the κ- and δ-receptor (23). Moreover, NAL specifically bound to human T lymphocytes can be partially displaced by various opiate agonists including morphine — 56%, β-endorphin — 61%, and Met- and Leu-enkephalin — 40% (24). In cultured SME of the pig, GnRH release was sensitive to treatments with NAL and potassium (18). In pituitary cells of rats, GnRH induced an oscillatory release of $[Ca^+]_i$ *via* a G protein-coupled phosphoinositide pathway, and periodically hyperpolarized the gonadotrophs by opening apamin-sensitive Ca^{2+}-activated K^+ channels (25). Thus, the mechanism of EOP action is very complex and depends on several factors regarding the site of action, stage of reproduction, etc.

Studies pertaining to the effects of EOP on endocrine events during pregnancy period are very limited. Only few experiments have been undertaken to define the involvement of EOP in gonadotrophin secretion in the pigs. The administration *in vivo* of WIN-3 (long-acting antagonist of opioids receptors) to early pregnant gilts (Day 12) resulted in LH release in 3 of 4 treated animals (26). Moreover, NAL administered to gilts on Day 40 of pregnancy significantly increased LH secretion. However, on Day 70 of pregnancy, naloxone influence was less effective (27). Before parturition (Day 107—108) naloxone increased LH secretion again (28). The action of EOP system during these periods may depend in part on β-endorphin, probably of pituitary origin (29). It seems that the differentiated opioidoergic influence on LH secretion occurs with progress of pregnancy in the pig. These suggestions were confirmed, at least in part, by our results of pituitary cells responding to naloxone treatment. However, studies concerning the effects of EOP on endocrine events during pregnancy in the pig will need to be elucidated.

In the pig, studies on $[Ca^{2+}]_i$ involvement in anterior pituitary cell functions have not been evaluated. However, studies performed in the rat (2) indicated that pituitary gonadotrophs exhibit episodes of spontaneous fluctuations in cytoplasmic calcium ions due to entry through voltage-sensitive calcium channels. The prominent oscillations of $[Ca^{2+}]_i$ are generated by periodic release of calcium from intracellular pools. Periodical release of calcium from intracellular stores may promote voltage-gated entry of extracellular calcium, which in turn can help to replenish the intracellular stores and induce hormone secretion (25). Different natural oscillations of $[Ca^{2+}]_i$ suggest some additional role of these ions in the cell economy. GnRH may elicit three types of calcium responses: increasing basal $[Ca^{2+}]_i$ at low doses, modulating frequency of oscillation at intermediate doses, and inhibitory to spontaneous fluctuations of $[Ca^{2+}]_i$ at high doses (2). Calcium ions and protein kinase C serve as interacting factors during the cascade of cellular events, in which calcium ions turn cell responses on or off, but protein kinase C amplifies the positive and negative effects of calcium. Moreover, phosphorylation and dephosphorylation of proteins in rat pituitary cells were suggested to be crucial for the modulation of calcium oscillation and patterns of calcium oscillations can produce a code triggering a specific cellular activity, e.g. mitosis, secretion (30). Additionally, some paracrine factors, for instance neuropeptide Y, can enhance pituitary response to GnRH activation probably by increasing extracellular calcium entry (31). Ovarian steroids can also modulate pituitary secretion through an effect on signaling pathway involving calcium ions (32). All aforementioned factors can be incorporated in multiple EOP actions in the hypothalamus and also at the level of heterogeneous pituitary cell populations in the pig.

Our paper provides a useful model for further investigation of EOP influence on gonadotrophin secretion, at the level of individual porcine pituitary cells, and in a time dependent manner. This model can be useful in other studies of neuromediators or neurotransmiters. However, experiments *in vitro* performed at the single cell level require optimization of several factors and conditions that can influence results of $[Ca]_i$, including a choice of appropriate calcium ions indicator non toxic to the cells, time of loading with dye, and time of exposure to a laser beam, etc. Although in other previous studies, monitoring of changes in $[Ca]_i$ by laser cytometry with dye fluo-3 AM in cultured porcine cells: aortic endothelial (12) and granulosa cells (17) indicated a similar profile of fluorescence intensity within the cytoplasm in the perinuclear region as in our recent study. Therefore each type of cells required completely different conditions for the experiments. A time-dependent loss of cellular dye content limits the precision of experiments with longer duration (9). Our period of 50 seconds (in experiment 1) did not expose porcine pituitary cells to toxic calcium levels before activation of mechanisms, which extruded

calcium. Moreover, Saavedra-Molina *et al.* (33) reported release of the calcium indicator over four hours in rat liver. In our studies, we omitted multiple exposure of pituitary cells to stimulants thus avoiding the aforementioned leakage of fluo-3 AM. The extracellular calcium concentration (2 mM) used in our studies did not cause any change of fluorescence intensity in the control group. That condition was instrumental in preventing leakage of fluo-3 AM from cells, as previously observed by Merritt *et al.* (34). The K_d of fluo-3 AM for calcium binding (864 nM at 37°C and 400 nM at 22°C) is temperature-dependent (34), therefore the ratio of the complex (calcium : indicator) was maintained 1 : 1 at either 22 or 37°C (35). Higher K_d and longer excitation wavelengths of fluo-3 AM can have significant advantages over fura-2 (36). Fluo-3 AM measurements by argon laser cytometry seems to be a useful model for study of porcine pituitary cells at the single cell level, as an additional procedure to previously utilized methods such as classical dynamic video imaging of fura-2-loaded cells, microfluorometric measurements of indo-1 fluorescence and classical $^{45}Ca^{2+}$ measurements.

In conclusion, we have presented data of a useful model pertaining to pituitary cells (pre-loaded with fluo-3 AM). It clearly indicates free intracellular calcium involvement in the response to NAL or GnRH stimulation in a time-dependent manner. The dual-labeling of cells (NAL-fluorescein and fura-red) allows one to distinguish the percentage of opioid-sensitive cells within entire heterogeneous population of porcine anterior pituitary cells. Our results suggest an important role of calcium in the opioid-modulated regulation of pituitary activity in pregnant pigs, although a better explanation of the role of calcium in EOP action within the heterogeneous porcine pituitary gland will need much more investigations.

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