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*Am J Physiol Cell Physiol* 293:951-959, 2007. First published May 16, 2007;  
doi:10.1152/ajpcell.00429.2006

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## Different kinases regulate activation of voltage-dependent calcium channels by depolarization in GH3 cells

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Submitted 10 August 2006; accepted in final form 11 May 2007

**Vela J, Pérez-Millán MI, Becu-Villalobos D, Díaz-Torga G.** Different kinases regulate activation of voltage-dependent calcium channels by depolarization in GH3 cells. *Am J Physiol Cell Physiol* 293: C951–C959, 2007. First published May 16, 2007; doi:10.1152/ajpcell.00429.2006.—The L-type  $\text{Ca}^{2+}$  channel is the primary voltage-dependent  $\text{Ca}^{2+}$ -influx pathway in many excitable and secretory cells, and direct phosphorylation by different kinases is one of the mechanisms involved in the regulation of its activity. The aim of this study was to evaluate the participation of Ser/Thr kinases and tyrosine kinases (TKs) in depolarization-induced  $\text{Ca}^{2+}$  influx in the endocrine somatomammotrope cell line GH3. Intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) was measured using a spectrofluorometric method with fura 2-AM, and 12.5 mM KCl ( $\text{K}^+$ ) was used as a depolarization stimulus.  $\text{K}^+$  induced an abrupt spike (peak) in  $[\text{Ca}^{2+}]_i$  that was abolished in the presence of nifedipine, showing that  $\text{K}^+$  enhances  $[\text{Ca}^{2+}]_i$ , preferably activating L-type  $\text{Ca}^{2+}$  channels. H89, a selective PKA inhibitor, significantly reduced depolarization-induced  $\text{Ca}^{2+}$  mobilization in a concentration-related manner when it was applied before or after  $\text{K}^+$ , and okadaic acid, an inhibitor of Ser/Thr phosphatases, which has been shown to regulate PKA-stimulated L-type  $\text{Ca}^{2+}$  channels, increased  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  entry. When PKC was activated by PMA, the  $\text{K}^+$ -evoked peak in  $[\text{Ca}^{2+}]_i$ , as well as the plateau phase, was significantly reduced, and chelerythrine (a PKC inhibitor) potentiated the  $\text{K}^+$ -induced increase in  $[\text{Ca}^{2+}]_i$ , indicating an inhibitory role of PKC in voltage-dependent  $\text{Ca}^{2+}$  channel (VDCC) activity. Genistein, a TK inhibitor, reduced the  $\text{K}^+$ -evoked increase in  $[\text{Ca}^{2+}]_i$ , but, unexpectedly, the tyrosine phosphatase inhibitor orthovanadate reduced not only basal  $\text{Ca}^{2+}$  levels but, also,  $\text{Ca}^{2+}$  influx during the plateau phase. Both results suggest that different TKs may act differentially on VDCC activation. Activation of receptor TKs with epidermal growth factor (EGF) or vascular endothelial growth factor potentiated  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  influx, and AG-1478 (an EGF receptor inhibitor) decreased it. However, inhibition of the non-receptor TK pp60 c-Src enhanced  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  influx. The present study strongly demonstrates that a complex equilibrium among different kinases and phosphatases regulates VDCC activity in the pituitary cell line GH3: PKA and receptor TKs, such as vascular endothelial growth factor receptor and EGF receptor, enhance depolarization-induced  $\text{Ca}^{2+}$  influx, whereas PKC and c-Src have an inhibitory effect. These kinases modulate membrane depolarization and may therefore participate in the regulation of a plethora of intracellular processes, such as hormone secretion, gene expression, protein synthesis, and cell proliferation, in pituitary cells.

phosphatases; protein kinase A; protein kinase C; epidermal growth factor

ENDOCRINE CELLS HAVE THE INTRINSIC capacity for extensive spontaneous activity that is independent of stimulation by external factors (8). In pituitary cells, this activity is charac-

terized by membrane potential oscillations, action potentials, and  $\text{Ca}^{2+}$  oscillations (41). This spontaneous signaling plays an important role in basal hormone release. Moreover,  $\text{Ca}^{2+}$  signaling is involved in virtually all cellular processes: it controls cell survival, proliferation, and death by regulating a plethora of intracellular enzymes in the cytoplasm, nucleus, and organelles. For this reason,  $\text{Ca}^{2+}$  mobilization is delicately controlled, and intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) is maintained at low levels, only to be increased in response to specific  $\text{Ca}^{2+}$ -mobilizing stimuli.

Changes in  $[\text{Ca}^{2+}]_i$  may be due to release from the intracellular  $\text{Ca}^{2+}$  stores or influx from the extracellular medium through the opening of plasma membrane  $\text{Ca}^{2+}$  channels.

Pituitary cells are equipped with voltage-dependent  $\text{Ca}^{2+}$  channels (VDCC), which mediate  $\text{Ca}^{2+}$  influx in response to membrane depolarization (20, 41, 47). VDCC are multimeric proteins composed of  $\alpha_1$ -,  $\beta$ -,  $\alpha_2\delta$ -, and  $\gamma$ -subunits (6). The pore-forming  $\alpha_1$ -subunit accounts for the voltage dependence of the channel. These channels are classified on the basis of the gene encoding the pore subunit and their electrophysiological and pharmacological properties (44). The L-type  $\text{Ca}^{2+}$  channel is the primary voltage-dependent  $\text{Ca}^{2+}$ -influx pathway in many excitable and other secretory cells. Its pore-forming  $\alpha_1$ -subunit contains  $\text{NH}_2$ - and  $\text{COOH}$ -terminal cytosolic domains, which are potential targets for protein phosphorylation (44). Therefore, the activity of L-type  $\text{Ca}^{2+}$  channels can be regulated by different types of kinases, such as PKA and PKC. Both are Ser/Thr kinases that have been reported to mediate activation and/or inhibition of L-type  $\text{Ca}^{2+}$  channel function, depending on the tissue, by phosphorylation of the  $\alpha_1$ -subunit and its auxiliary subunits (27, 29).

An emerging body of evidence suggests that VDCC are also regulated by phosphorylation on tyrosine residues (43, 50). Several growth factors activate receptor tyrosine kinases (RTKs) and trigger complex intracellular signal transduction pathways, finally leading to cell proliferation in different cell types (35).  $\text{Ca}^{2+}$  entry from extracellular medium is known to be a widespread signal that plays a key role in these events. However, the nature of the  $\text{Ca}^{2+}$  channels involved and a possible regulation through direct channel phosphorylation by RTKs remain controversial and differ among tissues. On the other hand, a direct  $\text{Ca}^{2+}$  channel regulation by the c-Src family of cytosolic tyrosine kinases (TKs) has been suggested, but contradictory results have been reported (38, 52).

Less is known about the role of VDCC regulation by TKs in pituitary GH3 cells, a clonal cell line established in 1965 by

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A. H. Tashjian, Jr., from a pituitary tumor carried in a 7-mo-old female Wistar-Furth rat (46) that secretes growth hormone and prolactin.

It has been reported that TK activation increases L-type  $\text{Ca}^{2+}$  channel function, whereas tyrosine phosphatases play an inhibitory role in this cell line (5). However, the nature of the TKs remains unknown.

Because  $\geq 10$  different types of  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunits (6) have been cloned and characterized and their expression differs among tissues, L-type  $\text{Ca}^{2+}$  channel regulation is tissue specific. With regard to GH3 cells, different pore-forming  $\alpha_1$ -subunits of L-type  $\text{Ca}^{2+}$  channels were expressed:  $\alpha_{1.1}$ ,  $\alpha_{1.2}$ , and  $\alpha_{1.3}$  (20).

The aim of our study was to evaluate the participation of several kinases in the depolarization of  $\text{Ca}^{2+}$  channels in GH3 cells.  $[\text{Ca}^{2+}]_i$  was measured second-by-second via a spectrofluorometric method with fura 2-AM as a fluorescent indicator. KCl (12.5 mM) was used as the depolarization stimulus to activate and open VDCC and, thereby, enhance  $[\text{Ca}^{2+}]_i$  in pituitary cells (22).

## MATERIALS AND METHODS

**Cells.** GH3 cells have been adapted to grow in culture with use of Ham's F-12K Nutrient Mixture (Kaighn's modification, GIBCO, Buenos Aires, Argentina) supplemented with 15% horse serum, 2.5% fetal bovine serum, and gentamicin (20  $\mu\text{g}/\text{ml}$ ; GIBCO).

Cells were incubated in a humid atmosphere of 5%  $\text{CO}_2$ -95%  $\text{O}_2$  at 37°C. The incubation medium was changed every 2–3 days. Cells were harvested once per week by treatment with phosphate-buffered saline containing trypsin (2.5 mg/ml; GIBCO), and reseeding was carried out at 20% of the original density.

**Drugs.** Chelerythrine, genistein, and okadaic acid (sodium salt) were obtained from Alomone Labs (Jerusalem, Israel) and tyrphostin AG-1478 and a specific Src kinase inhibitor (PP1) from Biomol Research Laboratories (EI-275, Plymouth Meeting, PA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

**$[\text{Ca}^{2+}]_i$  measurements.** Fura 2-AM (fura 2-tetra-acetoxymethyl ester; Molecular Probes, Eugene, OR) was used as a fluorescence indicator. Cells were harvested and washed in medium and then

redispersed and incubated in a buffered saline solution (BSS; in mM: 140 NaCl, 3.9 KCl, 0.7  $\text{KH}_2\text{PO}_4$ , 0.5  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1  $\text{CaCl}_2$ , 0.5  $\text{MgCl}_2$ , and 20 HEPES, pH 7.5) in the presence of 2 mM fura 2-AM, 10 mM glucose, and 0.1% BSA. Cells were incubated for 30 min at 37°C in an atmosphere of 5%  $\text{CO}_2$ , during which fura 2 is trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS without fura 2-AM and brought to a density of  $2 \times 10^6$  cells/ml BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) equipped with an accessory to measure  $\text{Ca}^{2+}$  with continuous stirring (model CA-261), a thermostat adjusted to 37°C, and an injection chamber.  $[\text{Ca}^{2+}]_i$  was registered every second by exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and the ratio of fluorescence at 340 nm to fluorescence at 380 nm ( $F_{340}/F_{380}$ ) were followed. Drugs were injected (5  $\mu\text{l}$ ) into the chamber as a 100-fold concentrated solution without interruption of recording. The preparation was calibrated by determination of maximal fluorescence induced by 0.1% Triton X-100 and minimal fluorescence in the presence of 6 mM EGTA (pH adjusted to  $>8.3$ ).  $[\text{Ca}^{2+}]_i$  was calculated according to Grynkiewicz et al. (23). Values were corrected for dye leakage as described elsewhere (21, 23) and for autofluorescence using unlabeled cells. Dye leakage and autofluorescence were minimal.

The response of GH3 cells to 12.5 mM KCl in buffer with or without  $\text{Ca}^{2+}$  is shown in Fig. 1. In the latter case, cells were incubated with fura 2-AM, washed, and resuspended in BSS without  $\text{CaCl}_2$ , and 1 min later, 60  $\mu\text{M}$  EGTA was added, as previously described (22, 45).

**Statistical analyses.** Unless otherwise specified, results are expressed as percent increase of  $[\text{Ca}^{2+}]_i$  relative to basal values. Basal values were considered the average of values recorded during the 30 s before the addition of 12.5 mM KCl, which shows the effect of a drug on  $\text{K}^+$  depolarization, or during the 30 s before application of a drug, which shows the effect of a drug relative to basal  $[\text{Ca}^{2+}]_i$  (see insets in Figs. 1, 3, and 4).

The immediate peak  $[\text{Ca}^{2+}]_i$  response was considered the maximal value achieved 8–20 s after  $\text{K}^+$  stimulation, and the sustained plateau phase was defined as the average of values recorded after  $\text{K}^+$  stimulation. When a drug was applied after  $\text{K}^+$  stimulation, the effect of the drug on the plateau phase was defined as the average of values recorded 0.5–3 min after administration of the drug.

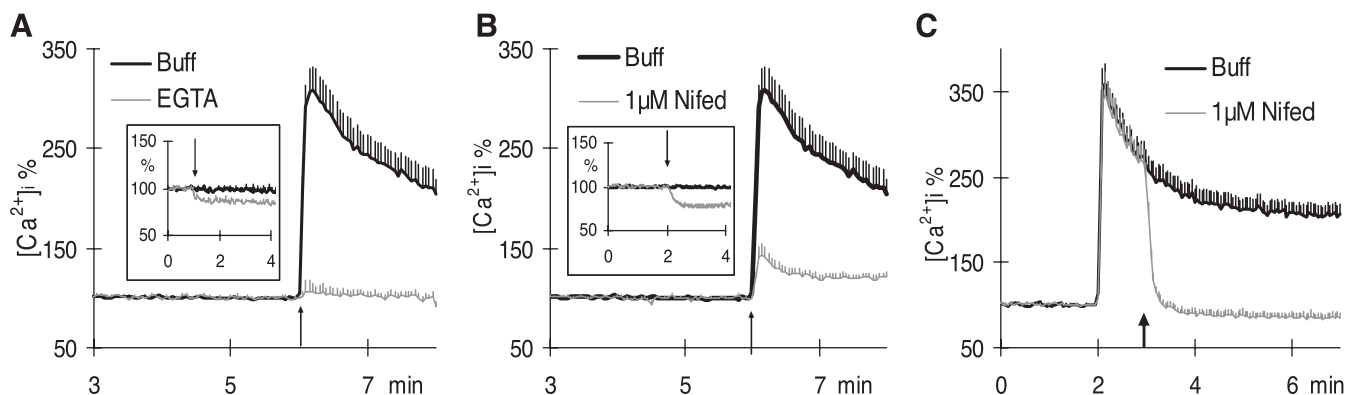


Fig. 1. A: effect of 12.5 mM KCl on intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in buffer with (Buff) or without  $\text{Ca}^{2+}$  (EGTA) in GH3 cells. In the case of buffer without  $\text{Ca}^{2+}$ , cells were incubated with fura 2-AM, washed, and resuspended in buffered saline solution without  $\text{CaCl}_2$ , and 60  $\mu\text{M}$  EGTA was added at minute 1. In both groups, 12.5 mM KCl was applied at minute 6 (arrow;  $n = 6$ ,  $P = 0.00013$ ). Inset: effect of EGTA on basal  $[\text{Ca}^{2+}]_i$  ( $n = 6$ ,  $P = 0.0015$ ). B: effect of nifedipine (Nifed) on  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  influx in GH3 cells. Nifedipine or buffer was applied at minute 2, and 12.5 mM KCl was added at minute 6 (arrow;  $n = 10$ ,  $P = 0.00046$ ). Inset: effect of nifedipine on basal  $[\text{Ca}^{2+}]_i$  ( $n = 10$ ,  $P = 0.00041$ ). C: effect of nifedipine on  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  influx. KCl (12.5 mM) was applied at minute 2, and nifedipine or buffer was applied 1 min later (arrow;  $n = 7$ ,  $P = 0.00018$ ). For this and subsequent figures, traces represent average for each time point, and vertical lines represent SE. Results are expressed as percent increase of  $[\text{Ca}^{2+}]_i$  relative to basal levels (i.e., average of  $[\text{Ca}^{2+}]_i$  at 20 s before  $\text{K}^+$  stimulus).

The peak  $[Ca^{2+}]_i$  response, plateau phase, and basal  $[Ca^{2+}]_i$  were compared by paired *t*-test (see Figs. 1, 3, 4, 5, 7, and 9). The effects of AG-1478, vanadate, and H89 on peak values or plateau levels were analyzed by one-way ANOVA followed by Tukey's honestly significant difference test.  $P < 0.05$  was considered significant.

## RESULTS

High extracellular  $K^+$  concentration depolarizes the cells and stimulates  $Ca^{2+}$  influx. In GH3 cells and under our experimental conditions, 12.5 mM KCl induced an abrupt spike in  $[Ca^{2+}]_i$ , which consisted of an 8- to 11-s delay, an increase of 200% over basal levels, and a gradual decay in  $[Ca^{2+}]_i$  (plateau phase; Fig. 1A, buffer). The average basal value was  $156.6 \pm 11.5$  nM, and the average peak value was  $469.1 \pm 37.1$  nM.

Changes in  $[Ca^{2+}]_i$  may be due to  $Ca^{2+}$  release from the intracellular  $Ca^{2+}$  stores or influx from the extracellular medium through the opening of plasma membrane  $Ca^{2+}$ -permeable channels. To show the importance of extracellular  $Ca^{2+}$  influx in the  $K^+$  response, we demonstrated that the GH3 cells' response to depolarization was lost in  $Ca^{2+}$ -free medium ( $P = 0.00013$ ; Fig. 1A, EGTA). The absence of the peak and plateau phases in the absence of extracellular  $Ca^{2+}$  indicates that the  $[Ca^{2+}]_i$  response to  $K^+$  represents  $Ca^{2+}$  entry. Furthermore, baseline  $[Ca^{2+}]_i$  decreased in  $Ca^{2+}$ -free medium ( $P = 0.0015$ ; Fig. 1A, inset).

To evaluate the contribution of L-type channels to the  $Ca^{2+}$  influx in GH3 cells, we tested the effect of nifedipine, a dihydropyridine that inhibits L-type  $Ca^{2+}$  channels, on  $Ca^{2+}$  influx evoked by 12.5 mM KCl. Nifedipine significantly decreased baseline  $[Ca^{2+}]_i$  by  $24.3 \pm 2.4\%$  ( $P = 0.00041$ ; Fig. 1B, inset, at 2 min), confirming an active participation of L-type  $Ca^{2+}$  channels in the maintenance of basal  $[Ca^{2+}]_i$ . On the other hand, nifedipine markedly (but not totally) decreased  $K^+$ -induced  $Ca^{2+}$  mobilization: maximal peak values were  $308.3 \pm 22.9\%$  with buffer and  $140.8 \pm 9.9\%$  with nifedipine ( $P = 2.4 \times 10^{-5}$ ), and plateau (average at 6.5–8 min) values were  $235 \pm 20.3\%$  with buffer and  $123 \pm 4.2\%$  with nifedipine ( $P = 0.00046$ ; Fig. 1B). In a different set of experiments in which nifedipine was applied 1 min after  $K^+$ -induced depolarization, the return of  $[Ca^{2+}]_i$  to basal values was striking and

the plateau response was totally abolished (Fig. 1C), showing that the contribution of the L-type  $Ca^{2+}$  channel to  $Ca^{2+}$  influx is essential and more important during the plateau phase than in the peak phase. The average plateau values at 3.05–5 min were  $227.2 \pm 16.1$  with buffer and  $97.2 \pm 5.9\%$  with nifedipine ( $n = 7$ ,  $P = 0.00018$ ).

It has been demonstrated that L-type  $Ca^{2+}$  channels can be regulated by several types of kinases in different tissues (7, 12, 28, 36). Previous studies confirmed that PKA and PKC phosphorylate serine and threonine residues on the  $\alpha$ - and  $\beta$ -subunits of the L-type  $Ca^{2+}$  channel (34, 36, 53). Therefore, to evaluate the contribution of PKA to depolarization-induced  $Ca^{2+}$  entry in GH3 cells, we tested the effect of *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide hydrochloride (H89), a selective PKA inhibitor, on  $Ca^{2+}$  mobilization evoked by 12.5 mM KCl.

Application of 0.1, 1, 10, 50, and 100  $\mu$ M H89 1 min before addition of  $K^+$  significantly reduced depolarization-induced  $Ca^{2+}$  mobilization. The effect was a concentration-related event, and the higher doses (50–100  $\mu$ M) abolished  $Ca^{2+}$  entry totally, suggesting that PKA exerted a strong and positive control on VDCC opening (Fig. 2, A and C).

Moreover, when H89 was applied 1 min after addition of  $K^+$ , the plateau phase was immediately reduced in a concentration-related manner (Fig. 2, B and D).

These results show not only a positive effect of PKA on depolarization-induced  $Ca^{2+}$  mobilization but, also, suggest a marked effect on the regulation of channel inactivation because of the important PKA-induced modulation of L-type  $Ca^{2+}$  channel function in GH3 cells.

PKC activation by phorbol esters has been well reported (4). To ascertain whether phorbol ester-sensitive PKC is essential not only in opening of VDCC by depolarization but, also, in regulation of the open state during the plateau phase, we studied the effect of PMA applied before and after  $K^+$  on  $K^+$ -induced  $Ca^{2+}$  mobilization in GH3 cells. Application of 100 nM PMA 2 min before addition of  $K^+$  partially, but significantly, reduced the peak and plateau depolarization-evoked increase in  $[Ca^{2+}]_i$ : relative peak values were  $294.9 \pm 21.4\%$  with PMA and  $358.3 \pm 17.9\%$  with buffer ( $P =$

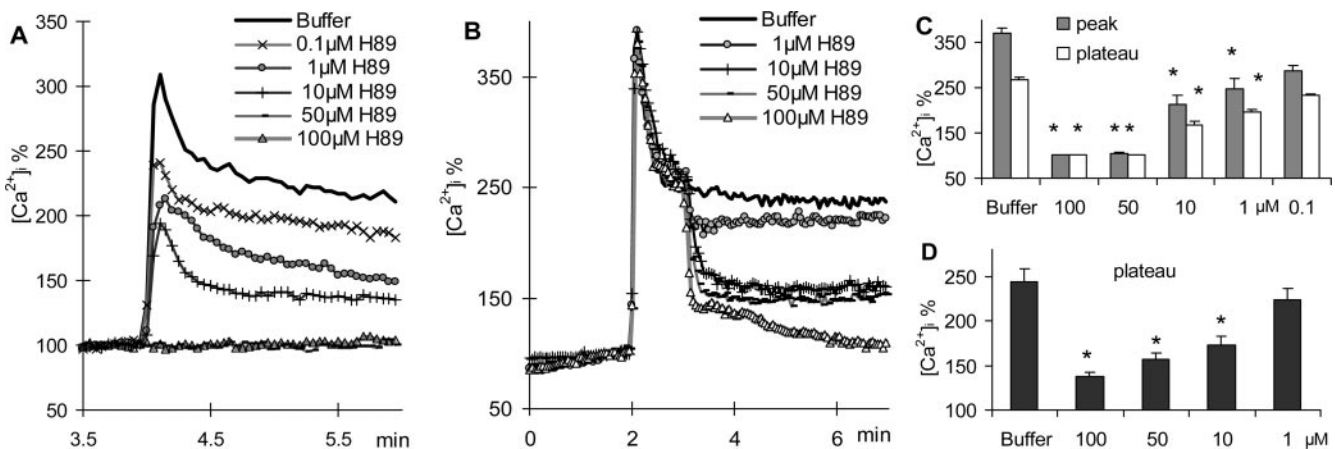


Fig. 2. A: effect of H89 on  $K^+$ -induced  $Ca^{2+}$  influx in GH3 cells. H89 or buffer was applied at minute 2, and 12.5 mM KCl was added at minute 4 ( $n = 6$ ). B: effect of H89 applied 1 min after depolarization induced by 12.5 mM  $K^+$  ( $n = 6$ ). C: average maximal peak values in buffer- or H89-treated cells and average plateau values during minutes 4.5–6 (from A). \* $P < 0.001$  vs. respective buffer. D: average plateau values during minutes 3.05–5.05 (from B). \* $P < 0.001$  vs. buffer.



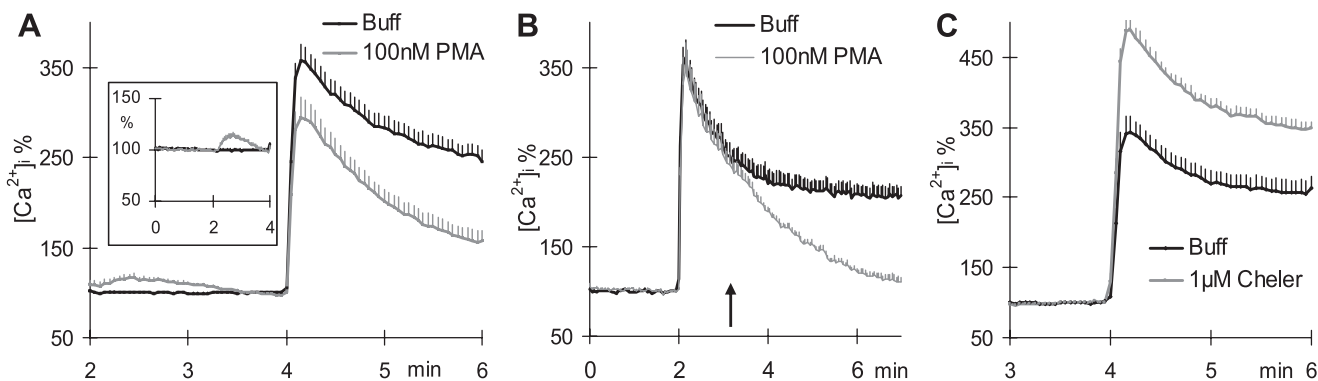


Fig. 3. A: effect of PMA on K<sup>+</sup>-induced Ca<sup>2+</sup> influx in GH3 cells. PMA or buffer was applied at minute 2, and 12.5 mM KCl was added at minute 4 ( $n = 10$ ,  $P = 3.97 \times 10^{-5}$ , for average of plateau values during minutes 4.5–6). Inset: effect of 100 nM PMA on basal  $[Ca^{2+}]_i$  ( $P = 0.006$ ). B: effect of PMA applied 1 min after depolarization induced by 12.5 mM K<sup>+</sup> (arrow;  $n = 8$ ,  $P = 0.00021$ ). C: effect of chelerythrine (Chelerythrine) on K<sup>+</sup>-induced Ca<sup>2+</sup> influx in GH3 cells. Chelerythrine or buffer was applied at minute 2, and 12.5 mM KCl was added at minute 4 ( $n = 6$ ,  $P = 0.0005$ ).

0.0020), and average plateau values at 4.5–6 min were  $275.5 \pm 13.4\%$  with buffer and  $192.4 \pm 12.6\%$  with PMA ( $P = 3.97 \times 10^{-5}$ ; Fig. 3A). Furthermore, when PMA was applied 1 min after the K<sup>+</sup> stimulus, the total Ca<sup>2+</sup> influx during the subsequent minutes of the plateau phase was significantly reduced: average influx at 4–6 min was  $214 \pm 11.8\%$  with K<sup>+</sup> and buffer and  $152 \pm 2.8\%$  with K<sup>+</sup> and PMA ( $P = 0.0021$ ; Fig. 3B). These observations were confirmed using the selective PKC inhibitor chelerythrine (Fig. 3): K<sup>+</sup>-induced Ca<sup>2+</sup> influx was markedly increased when PKC action was inhibited with the application of 1  $\mu$ M chelerythrine 2 min before the K<sup>+</sup> stimulus. In chelerythrine-treated cells,  $[Ca^{2+}]_i$  reached a peak of  $488.9 \pm 17.5\%$  vs.  $342.9 \pm 22.2\%$  in buffer-treated cells ( $P = 0.0019$ ). The average of the plateau values was increased by chelerythrine to  $375.6 \pm 10.2\%$  vs.  $271.4 \pm 16.9\%$  in buffer-treated cells ( $P = 0.00050$ ). These results suggest an inhibitory influence of PKC on VDCC function not only on the activation induced by depolarization but, also, on the maintenance of the open state. Even though chelerythrine did not affect baseline  $[Ca^{2+}]_i$ , PMA evoked a transient increase in basal  $[Ca^{2+}]_i$  during the first 2 min ( $P = 0.0060$ ; Fig. 3A, inset).

To investigate the involvement of TKs on VDCC activity in GH3 cells, we first studied the effect of genistein (a TK inhibitor that acts by binding to the ATP site of the TK) on depolarization-induced  $[Ca^{2+}]_i$  mobilization. Figure 4 shows that application of 25  $\mu$ M genistein 2 min before the K<sup>+</sup> stimulus significantly reduced the depolarization-induced Ca<sup>2+</sup> influx. The average plateau  $[Ca^{2+}]_i$  values were  $233 \pm 11.4\%$  with genistein and  $307 \pm 17.9\%$  with buffer ( $P = 0.0076$ ). Moreover, baseline  $[Ca^{2+}]_i$  was reduced by 18% immediately after genistein ( $P = 0.00073$ ; Fig. 4, inset). This result implies that TKs are involved in the maintenance of baseline  $[Ca^{2+}]_i$  as well as in the depolarization-induced Ca<sup>2+</sup> entry in GH3 cells.

Inasmuch as several growth factors have been reported to induce Ca<sup>2+</sup> influx in many cell types, activating their specific receptors with intrinsic TK activity (RTKs), we studied the involvement of growth factor receptors in modulating the activation of membrane Ca<sup>2+</sup> channels in GH3 cells. Addition of 10 ng/ml epidermal growth factor (EGF; Fig. 5A) or 30 ng/ml type A vascular endothelial growth factor (VEGF; Fig. 6B) to the cell suspension enhanced K<sup>+</sup>-induced Ca<sup>2+</sup> mobilization. The average plateau values were  $350 \pm 12.4\%$  with

EGF vs.  $284 \pm 6.5\%$  with buffer ( $P = 0.0021$ ) and  $325 \pm 9.6\%$  with VEGF vs.  $275 \pm 9.6\%$  with buffer ( $P = 0.0037$ ). On the other hand, 10 ng/ml insulin-like growth factor-I (IGF-I; Fig. 5C) did not modify the depolarization-induced increase in  $[Ca^{2+}]_i$ . None of these growth factors had an effect on basal Ca<sup>2+</sup> levels (not shown).

AG-1478, a selective EGF receptor (EGFR) TK inhibitor, decreased the depolarization-induced Ca<sup>2+</sup> entry (Fig. 6, A and C). The peak  $[Ca^{2+}]_i$  mobilization decreased to  $254 \pm 9.4\%$  in cells treated with 1  $\mu$ M AG-1478 vs.  $345 \pm 4.5\%$  in buffer-treated cells ( $P = 0.015$ ), and the average relative plateau value was reduced to  $165.3 \pm 6.8\%$  in cells treated with 1  $\mu$ M AG-1478 and  $181.0 \pm 6.9\%$  in cells treated with 500 nM AG-1478 vs.  $212.8 \pm 6.8\%$  in buffer-treated cells. Moreover, when AG-1478 was applied 1 min after K<sup>+</sup> stimulus, total Ca<sup>2+</sup> influx during the subsequent 4 min of the plateau phase was significantly reduced at 200, 500, and 1,000 nM AG-1478 (Fig. 6, B and D;  $P < 0.002$ ).

Non-RTKs, such as pp60 c-Src (c-Src), have also been involved in the control of Ca<sup>2+</sup> signal transduction pathways, including interaction with Ca<sup>2+</sup> channels in different tissues (38, 52). To study whether the depolarization-induced activa-

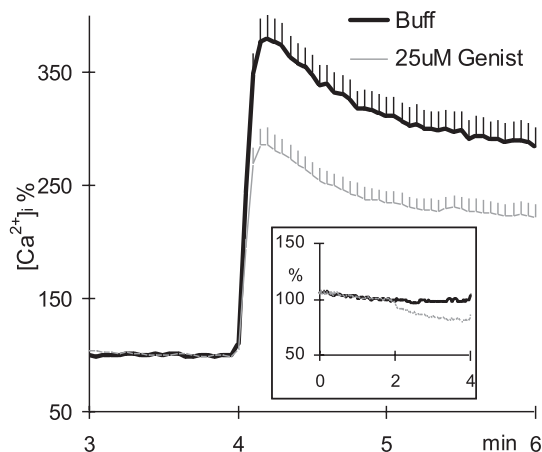


Fig. 4. Effect of genistein (Genist) on K<sup>+</sup>-induced Ca<sup>2+</sup> influx in GH3 cells. Genistein or buffer was applied at minute 2, and 12.5 mM KCl was added at minute 4 ( $n = 6$ ,  $P = 0.0076$ ). Inset: effect of 25  $\mu$ M genistein on basal  $[Ca^{2+}]_i$  ( $n = 6$ ,  $P = 0.00073$ ).

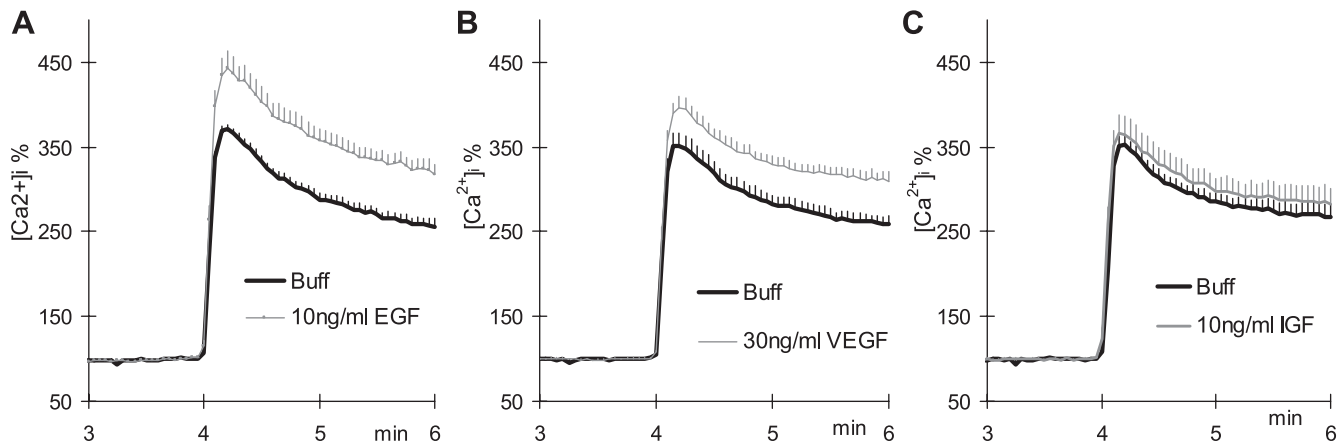


Fig. 5. Effect of epidermal growth factor (EGF, A), vascular endothelial growth factor (VEGF, B), and insulin-like growth factor-I (IGF-I, C) on  $K^+$ -induced  $Ca^{2+}$  influx in GH3 cells. Growth factors or buffer was applied at minute 2, and 12.5 mM  $KCl$  was added at minute 4 ( $n = 8$ ,  $P = 0.0021$ , and  $n = 8$ ,  $P = 0.0037$ , for average of plateau values of EGF and VEGF, respectively, vs. buffer).

tion of VDCC involves c-Src kinase activity, we examined the effect of a selective c-Src kinase inhibitor, type 1 protein phosphatase (PP1), on depolarization-induced  $Ca^{2+}$  mobilization. Unexpectedly, PP1-induced inhibition of c-Src activity enhanced depolarization-induced  $Ca^{2+}$  mobilization. The maximal peak value was  $408.9 \pm 17.3\%$  in PP1-pretreated cells vs.  $334.5 \pm 22.3\%$  in buffer-treated cells ( $P = 0.0023$ ), and the total plateau values during the following 1.5 min were  $339.5 \pm 11.6\%$  in PP1-pretreated cells and  $278.8 \pm 16.9\%$  in cells treated with buffer ( $P = 0.0103$ , Fig. 7).

To ascertain whether phosphatases affect VDCC activity in GH3 cells, we first studied the effect of orthovanadate, a tyrosine phosphatase inhibitor, on depolarization-induced  $[Ca^{2+}]_i$  mobilization. Figure 8, A and C, shows that different concentrations of orthovanadate decreased basal  $Ca^{2+}$  levels significantly ( $P = 2.14 \times 10^{-6}$ , for the effect of treatment). However, when  $K^+$  was applied, the absolute increase (difference between peak and basal values) in depolarization was not altered by vanadate, even though absolute peaks were lower. On the other hand, when vanadate was applied 1 min after the  $K^+$  stimulus, the plateau phase was immediately reduced in a concentration-related manner (Fig. 8, B and D). The average of

plateau values between 3.05 and 5.05 min were  $245 \pm 11.8\%$  with buffer and  $158.2 \pm 6.7\%$  with 1 mM vanadate ( $P = 0.00022$ ).

Finally, we studied the effect of okadaic acid, an inhibitor of Ser/Thr phosphatases, on  $Ca^{2+}$  channel function. Application of 1 or 50 nM okadaic acid 2 min after the  $K^+$  stimulus did not modify basal  $Ca^{2+}$  levels or  $K^+$ -induced  $Ca^{2+}$  mobilization at 4 min (data not shown). Nevertheless, 20 min of pretreatment with 50 nM OK enhanced depolarization-induced  $Ca^{2+}$  influx. Averages of the plateau values at 25–26 min were  $302.69 \pm 6.5\%$  with 50 nM okadaic acid and  $235.5 \pm 6.7\%$  with buffer ( $P = 0.0077$ ; Fig. 9).

## DISCUSSION

VDCC control depolarization-induced  $Ca^{2+}$  entry, triggering essential cellular processes. In the present study, the effect of several kinases and phosphatases on depolarization-induced VDCC activity in pituitary GH3 cells was investigated.

Spontaneous oscillations in  $[Ca^{2+}]_i$  have been shown in the majority (~70%) of GH3 cells. The pattern of these oscillations varies considerably from cell to cell (42, 47), and these

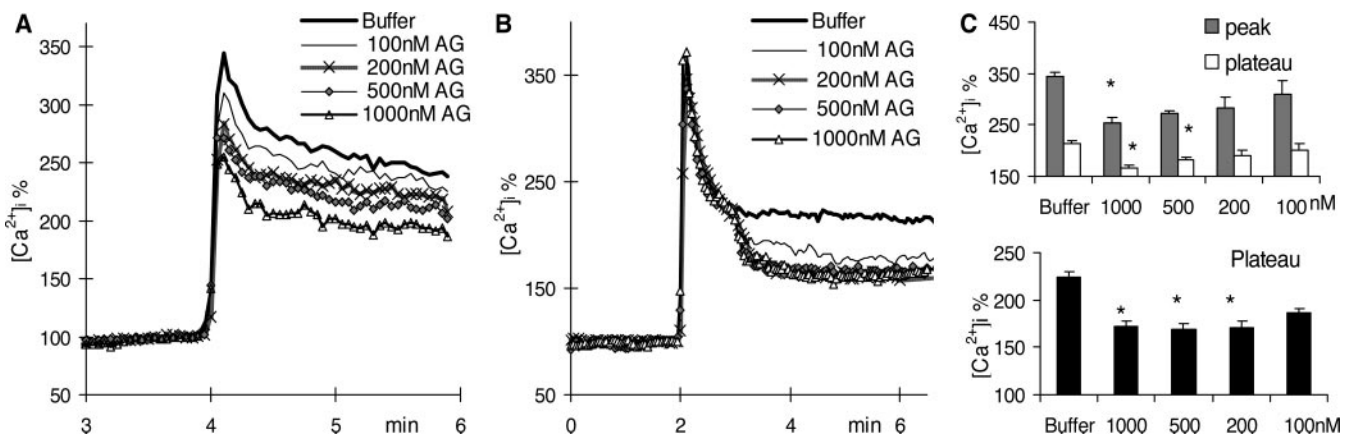


Fig. 6. A: effect of AG-1478 (AG) on  $K^+$ -induced  $Ca^{2+}$  influx in GH3 cells. AG-1478 or buffer was applied at minute 2, and 12.5 mM  $KCl$  was added at minute 4 ( $n = 5$ ). B: effect of AG-1478 applied 1 min after depolarization induced by 12.5 mM  $K^+$  ( $n = 5$ ). C: average peak values in buffer- or AG-1478-treated cells and average plateau values during minutes 4.5–6 (from A). \* $P < 0.02$  vs. respective buffer. D: average plateau values during minutes 3.05–5.05 (from B). \* $P < 0.02$  vs. buffer.

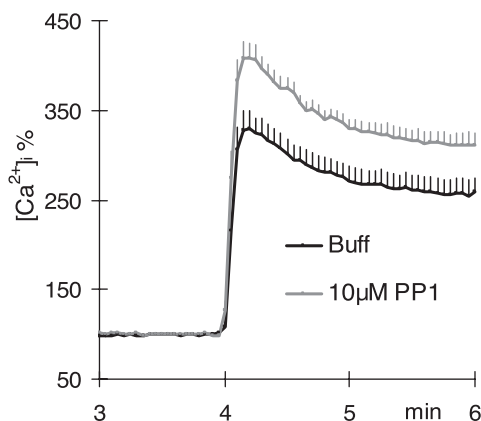


Fig. 7. Effect of a specific Src kinase inhibitor (PP1) on  $K^+$ -induced  $Ca^{2+}$  influx in GH3 cells. PP1 or buffer was applied at minute 2, and 12.5 mM  $KCl$  was added at minute 4 ( $n = 7$ ,  $P = 0.0103$ ).

$Ca^{2+}$  oscillations are completely abolished during perfusion with medium containing no added  $Ca^{2+}$ , confirming that  $Ca^{2+}$  influx is necessary for the maintenance of basal  $[Ca^{2+}]_i$  and  $Ca^{2+}$  oscillations.

In GH3 cells exposed to depolarization induced by 12.5 mM  $K^+$ , there was an immediate spike increase in  $[Ca^{2+}]_i$  followed by a sustained plateau. Changes in  $[Ca^{2+}]_i$  may be due to  $Ca^{2+}$  release from the intracellular  $Ca^{2+}$  stores or  $Ca^{2+}$  influx from the extracellular medium through the opening of plasma membrane  $Ca^{2+}$ -permeable channels. We evaluated the effect of  $Ca^{2+}$ -free medium on the response of GH3 cells to depolarization: the lack of the peak and plateau phases in the absence of extracellular  $Ca^{2+}$  indicates that the  $[Ca^{2+}]_i$  response to  $K^+$  represents  $Ca^{2+}$  entry. The significant reduction of the maximum peak value in nifedipine-pretreated cells shows the importance of the contribution of L-type  $Ca^{2+}$  channels to depolarization-induced  $Ca^{2+}$  and, furthermore, suggests that nifedipine-insensitive channels may also participate in  $K^+$ -induced  $Ca^{2+}$  entry. These results are consistent with the finding that the GH3 cell line expresses different types of VDCC and, primarily, the L-type VDCC, as shown by Glassmeier et al. (20). Using the whole cell configuration of the patch-clamp technique and  $Ba^{2+}$  as the charge carrier, these authors de-

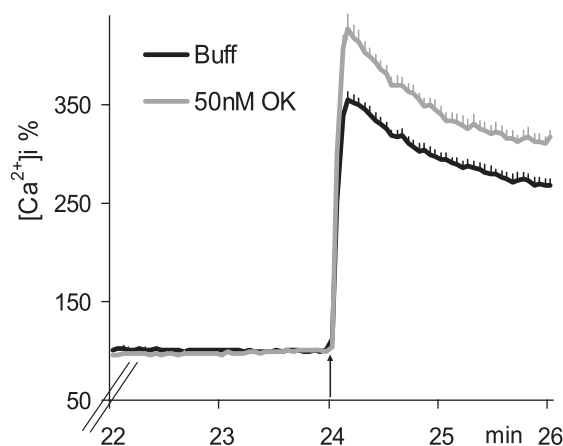


Fig. 9. Effect of 20 min of incubation with 50 nM okadaic acid (OK) on  $K^+$ -induced  $Ca^{2+}$  influx. Okadaic acid was applied at minute 4, and  $K^+$  was added at minute 24 ( $n = 7$ ,  $P = 0.0077$ ). Values represent percent increase relative to basal value recorded during minutes 22–23.

scribed in detail the voltage-activated  $Ca^{2+}$  channel current components in GH3 cells. In addition to the L-type  $Ca^{2+}$  channel, three other high-voltage-activated  $Ca^{2+}$  channel current components were distinguished pharmacologically:  $\omega$ -agatoxin IVA-sensitive current (22%, P-type),  $\omega$ -conotoxin MVIIC-sensitive current (18%, Q-type), and toxin-resistant current (24%). Glassmeier et al. demonstrated that 49% of the total  $Ba^{2+}$  current amplitude in basal conditions is mediated by L-type  $Ca^{2+}$  channels, but they showed that nifedipine totally blocked action potential firing in GH3 cells. In our present study, application of nifedipine after  $K^+$ -induced depolarization completely inhibited the plateau response. This may imply that the contribution of the L-type  $Ca^{2+}$  channel is the most important factor in the maintenance of depolarization-induced  $Ca^{2+}$  influx but that other VDCC, as well as the L-type  $Ca^{2+}$  channels, participate in the immediate peak  $[Ca^{2+}]_i$  response.

Numerous studies have attempted to unravel the mechanisms of VDCC modulation. Phosphorylation is one of the most common mechanisms that modifies ion channel activity. Moreover, L-type  $Ca^{2+}$  channel function is highly regulated by

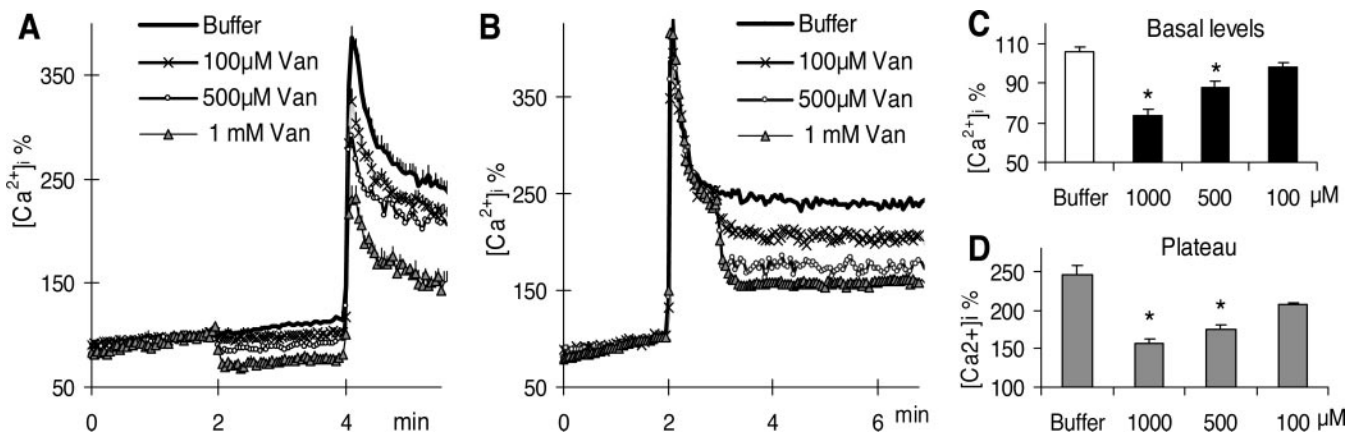


Fig. 8. A: effect of orthovanadate (Van) applied at minute 2 on basal  $[Ca^{2+}]_i$  and  $K^+$ -induced  $Ca^{2+}$  influx at minute 4 in GH3 cells. Basal values were calculated as average of  $[Ca^{2+}]_i$  values obtained during minutes 1–2. B: effect of orthovanadate applied 1 min after depolarization induced by 12.5 mM  $K^+$  ( $n = 6$ ,  $P = 0.00022$ , for effect of drug vs. buffer). C: average basal  $[Ca^{2+}]_i$  obtained 1 min after application of orthovanadate ( $n = 6$ , from A). D: average plateau values during minutes 3.05–5.05 ( $n = 5$ , from B). \* $P < 0.01$  vs. buffer.



hormones and neurotransmitters, mainly through the activation of kinases and phosphatases.

Phosphorylations by PKC and/or PKA are important regulatory pathways; they show widely variable effects on L-type  $\text{Ca}^{2+}$  channel activity, depending, among others factors, on the tissue. Moreover, cross talk between PKA and PKC pathways in the modulation of channel function has been demonstrated (28, 53). These mechanisms have been studied in detail in different tissues, especially cardiomyocytes, but there is little information about kinase regulation of depolarization-induced  $\text{Ca}^{2+}$  influx in GH3 cells. Haymes et al. (24) demonstrated that PKC activation with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate and phorbol 12,13-dibutyrate decreased depolarization-induced  $\text{Ca}^{2+}$  influx in GH3 cells. Using PMA and a PKC antagonist, we have demonstrated that PKC has an inhibitory influence on VDCC function and, furthermore, that PKC participates in the depolarization-induced activation, as well as maintenance, of the open state of the channels. These results were confirmed using the selective PKC inhibitor chelerythrine, which produced a clear increase in depolarization-induced  $\text{Ca}^{2+}$  influx.

MacEwan and Mitchell (33) demonstrated that  $\text{Ca}^{2+}$  influx through L-type  $\text{Ca}^{2+}$  channels in rat anterior pituitary cells can be modulated by PKC in opposite ways, depending on the experimental model. Activators of PKC, similar to phorbol esters, enhanced  $\text{K}^{+}$ -induced  $^{45}\text{Ca}^{2+}$  influx in dispersed anterior pituitary cells. On the contrary, in the GH3 tumor cell line, the same authors showed, in accordance with our results, that phorbol esters inhibited  $\text{K}^{+}$ -induced  $^{45}\text{Ca}^{2+}$  influx. Nevertheless, in this last situation, the cells were subjected to longer (10-min) incubations with phorbol esters, and we have shown that the effect is immediate. We previously demonstrated an overall alteration in  $\text{Ca}^{2+}$  metabolism in tumoral pituitary cells (13). Furthermore, different PKC isoforms are present in each tissue, and each isoform displays differential sensitivity to phorbol esters (32). The  $\alpha$ -,  $\beta$ -,  $\delta$ -,  $\epsilon$ -,  $\zeta$ -, and  $\theta$ -isoforms of PKC are present in rat anterior and posterior pituitary tissue, as well as in the GH3 cell line, but in different proportions (32). In particular, it has been demonstrated that the enhancement of prolactin secretion in tumoral pituitary cell lines is related to overexpression of PKC- $\epsilon$  (1). It may be possible that different PKC isoforms and different regulatory mechanisms (activation/inhibition) on VDCC are involved in the opposite effects observed in normal and tumoral pituitary cells.

On the contrary, a PKA antagonist, H89, had an inhibitory effect on  $\text{K}^{+}$ -evoked  $\text{Ca}^{2+}$  influx, suggesting a positive contribution of PKA to depolarization-induced  $\text{Ca}^{2+}$  entry through VDCC in GH3 cells. Moreover, the high doses of H89 totally abolished  $\text{Ca}^{2+}$  entry. When applied after  $\text{K}^{+}$  stimulation, H89 reduced  $\text{K}^{+}$ -evoked  $\text{Ca}^{2+}$  influx and significantly reduced the total  $\text{Ca}^{2+}$  entry during the plateau phase, and we previously showed that this influx primarily depended on the L-type  $\text{Ca}^{2+}$  channel.

It has been shown that both Ser/Thr kinases, PKC and PKA, phosphorylate the  $\alpha$ -subunit of L-type  $\text{Ca}^{2+}$  channels, even though this is not their only target. Puri et al. (36) demonstrated that all subunits of these channels are excellent substrates for PKA and PKC. Moreover, Gerhardstein et al. (19) showed in intact cardiac myocytes that the  $\beta$ -subunit is a substrate for PKA. The  $\beta$ -subunit contains more than one potential consensus site for PKA-mediated phosphorylation that is not a sub-

strate for PKC. These sites are conserved in most  $\beta$ -subunit isoforms and, thus, represent potential sites for regulation of channel activity.

More recently, it has been reported that PKA and PKC signaling pathways converge on the cardiac L-type  $\text{Ca}^{2+}$  channel complex at Ser<sup>1928</sup> to increase channel activity, and a preassociation of the channel with PKC isoforms has been suggested (53). In the same way, direct phosphorylation of dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels from skeletal muscle by PKA or PKC resulted in an activation of the channels that was observed as an increase in the rate and extent of  $\text{Ca}^{2+}$  influx (7, 12, 27). Nevertheless, our results clearly demonstrate opposite effects of PKC and PKA on depolarization-induced VDCC activation in GH3 cells.

The Ser/Thr phosphatases PP1 and PP2A, but not PP2B or PP2C, have been demonstrated to regulate PKA-stimulated L-type  $\text{Ca}^{2+}$  channels (11). It is well known that okadaic acid inhibits the Ser/Thr phosphatases PP2A ( $\text{IC}_{50} \sim 0.1$  nM), PP1 ( $\text{IC}_{50} \sim 10$  nM), and, also, although much less potently, PP2B (calcineurin,  $\text{IC}_{50} \sim 5$   $\mu\text{M}$ ) (10, 40). Under our experimental conditions, 50 nM okadaic acid increased  $\text{K}^{+}$ -evoked  $\text{Ca}^{2+}$  influx in GH3 cells. At the concentration used, PP2A and PP1 were inhibited, and those conditions favored  $\text{Ca}^{2+}$  entry, probably related to enhancement of PKA action. Nevertheless, the effect of okadaic acid was only observed in 20 min of preincubation assays.

Even though an active and direct participation of TKs in L-type  $\text{Ca}^{2+}$  channel function in different tissues was demonstrated (29, 38, 49, 52), less is known about VDCC regulation through tyrosine phosphorylation in GH3 cells. Cataldi et al. (5) showed that TK inhibition reduced L-type  $\text{Ca}^{2+}$  channel activity evoked by 55 mM  $\text{K}^{+}$  in GH3 cells. This led us to investigate possible TKs involved in VDCC activation. Genistein, a TK inhibitor, decreased total  $\text{Ca}^{2+}$  influx evoked by 12.5 mM  $\text{K}^{+}$  by  $\sim 33\%$ , indicating an involvement of TK in depolarization-induced VDCC activation. However, we observed that sodium orthovanadate, a protein tyrosine phosphatase inhibitor, not only decreased basal  $\text{Ca}^{2+}$  levels in GH3 cells but, also, significantly decreased  $\text{Ca}^{2+}$  influx in the plateau phase when it was applied after  $\text{K}^{+}$  depolarization, suggesting that different kinases might have differential effects on VDCC.

Several growth factor receptors have intrinsic TK activity, and it has been reported that they can regulate ion channel function (9, 18, 39). Tyrosine phosphorylation has been set forth as the mechanism involved in this regulation, because it was demonstrated that the effect of growth factors on channel activity can be blocked by TK inhibitors (9, 39). In GH3 cells, EGF and VEGF increased depolarization-evoked  $\text{Ca}^{2+}$  influx. Furthermore, the tyrphostin AG-1478, which specifically inhibits the EGFR kinase activity by competing for ATP binding, inhibited depolarization-evoked  $\text{Ca}^{2+}$  influx. Moreover, when AG-1478 was applied after the  $\text{K}^{+}$  stimulus, it significantly reduced the total  $\text{Ca}^{2+}$  influx during the plateau phase, suggesting a stimulatory effect of EGFR on L-type  $\text{Ca}^{2+}$  channel activity in GH3 cells, even in the absence of EGF. It is well known that EGFR can be activated by six ligands (EGF, transforming growth factor- $\alpha$ , amphiregulin, heparin-binding EGF-like growth factor,  $\beta$ -cellulin, and epiregulin) (14) and that, at least, transforming growth factor- $\alpha$  is expressed in GH3 cells (15). Moreover, EGFR can be transactivated by other



signaling pathways (14), as we demonstrated in pituitary cells (45). This might explain the mechanism of the inhibitory effect of AG-1478 on VDCC activity in GH3 cells.

In contrast with our results, it has been demonstrated that GH3 cells incubated for several days with 10 nM EGF responded to depolarization with a 30–65% smaller increase in  $^{45}\text{Ca}^{2+}$  uptake than untreated cells, and similar results were obtained by Fu et al. (16) in GH4C1 pituitary tumor cells. However, both studies were done in long-duration incubation conditions. Therefore, a more complex mechanism in the effect of EGF, such as protein synthesis and desensitization, may occur. In our experimental conditions, we describe an immediate and direct effect of EGF on channel activity.

It has been reported that VEGF and its receptor Flk-1 are expressed in GH3 cells (31, 48), and, furthermore, it has been shown that VEGF induces  $\text{Ca}^{2+}$  influx in epithelial cells. Here we show, for the first time, that VEGF receptor (VEGFR) activation stimulates depolarization-induced  $\text{Ca}^{2+}$  influx in GH3 cells, suggesting a positive effect of VEGFR, an RTK, on VDCC activity.

IGF-I has been involved in inhibitory and stimulatory effects on  $\text{Ca}^{2+}$  influx in different tissues (3, 9, 12, 39). Our failure to find an effect of IGF-I on  $\text{K}^{+}$ -induced  $\text{Ca}^{2+}$  mobilization in GH3 cells suggests that the RTK effect is growth factor and tissue specific.

On the other hand, an effect of non-RTKs on channel regulation has been suggested. C-Src is a non-RTK, and it has been shown that c-Src family kinases could mediate tyrosine phosphorylation of the  $\alpha_{1,2}$ -subunit of L-type VDCC in the hippocampus, potentiating the channel currents (25). When we explored the c-Src contribution to VDCC activity in GH3 cells, we found that inhibition of c-Src with PP1 evoked an increase in depolarization-induced  $\text{Ca}^{2+}$  influx, suggesting an inhibitory effect of this kinase. Therefore, the effect of vanadate, a protein tyrosine phosphatase, which decreased VDCC activity, might be explained by the activation of endogenous c-Src kinase activity.

In vascular smooth muscle cells, genistein (a nonselective TK inhibitor), bistrphostin (an RTK inhibitor), and PP1 inhibited L-type  $\text{Ca}^{2+}$  channel activity in a concentration-dependent manner, indicating a stimulatory effect of these kinases (52). On the other hand, an opposite action of endogenous c-Src was found in rabbit ear artery cells, where c-Src increased VDCC currents (51).

The inhibitory effect of this kinase on VDCC in GH3 cells described in the present study is in contrast to the positive action mediated by RTKs coupled to EGF and VEGF. In accordance with our results, two independent and competing TK signaling mechanisms that control of L-type  $\text{Ca}^{2+}$  current in feline atrial myocytes have been described (49): a positive action (channel activation) through membrane-bound TKs and an inhibitory effect by cytosolic TKs. Similar results were found in cardiac and smooth muscle L-type  $\text{Ca}^{2+}$  channel regulation (29), and, more recently, the inhibitory effect of c-Src was described in human atrial myocytes (38).

Precisely how c-Src modulates the L-type  $\text{Ca}^{2+}$  channel is uncertain, but functional and biochemical evidence closely links c-Src to the pore-forming  $\alpha_1$ -subunit of the L-type  $\text{Ca}^{2+}$  channel. c-Src has been found in immunoprecipitates of the  $\alpha_{1,2}$ -subunit of the L-type  $\text{Ca}^{2+}$  channel isolated from colonic smooth muscle (26).

Regulation of ion channel function is complex, differs among tissues, and involves many complex mechanisms, including Ser/Thr and tyrosine phosphorylations, as well as interactions between kinases. Differences among tissues in kinase activity on L-type  $\text{Ca}^{2+}$  channels may be due in part to the  $\alpha$ -subunit subtype expressed in each tissue and the experimental conditions that allow differences in basal kinase and phosphatase activities. At least 10 different types of  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunits (6), four types of  $\beta$ -subunits (2), three types of  $\alpha_2\delta$ -subunits (17), and five types of  $\gamma$ -subunits (30) have been cloned and characterized. However, pharmacological and electrophysiological studies have identified more subtypes of VDCC in excitable cells than the types of  $\alpha_1$ -subunits currently known (37).

In GH3 cells, expression of different pore-forming  $\alpha_1$ -subunits of L-type  $\text{Ca}^{2+}$  channels has been found:  $\alpha_{1,1}$ ,  $\alpha_{1,2}$ , and  $\alpha_{1,3}$  (20). Therefore, if we consider that more than one  $\alpha$ -subunit (and/or their splicing variants) coexist in the same tissue in combination with the variety of the other subunits, it is comprehensible that all may contribute to the pharmacological and electrophysiological diversity of  $\text{Ca}^{2+}$  channels in GH3 cells and to the differences between normal and tumoral cell pituitary responses.

The present study strongly demonstrates that different kinases regulate VDCC activity in the pituitary cell line GH3: PKA and RTKs, such as the VEGFR and EGFR, enhance depolarization-induced  $\text{Ca}^{2+}$  influx, whereas PKC and c-Src have an inhibitory effect. These kinases modulate membrane depolarization and, thereby, may participate in the regulation of a plethora of intracellular processes, such as hormone secretion, gene expression, protein synthesis, and cell proliferation, in pituitary cells.

## GRANTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Técnica (Buenos Aires, Argentina). J. Vela is a research fellow at the Instituto de Biología y Medicina Experimental CONICET, Argentina. M. I. Pérez-Millán is a research fellow at Agencia Nacional de Investigaciones Científicas y Técnicas, Argentina. D. Becu-Villalobos and G. Díaz-Torga are established investigators at CONICET.

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