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Spontaneous Calcium Oscillations Control c-*fos* Transcription via the Serum Response Element in Neuroendocrine Cells*

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Andrés Maturana‡§, Goedele Van Haasteren‡, Isabelle Piuz‡, Cyril Castelbou§, Nicolas Demaurex§¶, and Werner Schlegel‡‖

From the ‡Fondation pour Recherches Médicales, University of Geneva, 1211 Geneva 4, Switzerland and the §Department of Physiology, University of Geneva Medical Center, 1211 Geneva 4, Switzerland

In excitable cells the localization of Ca^{2+} signals plays a central role in the cellular response, especially in the control of gene transcription. To study the effect of localized Ca^{2+} signals on the transcriptional activation of the c-*fos* oncogene, we stably expressed various c-*fos* β-lactamase reporter constructs in pituitary AtT20 cells. A significant, but heterogenous expression of c-*fos* β-lactamase was observed in unstimulated cells, and a further increase was observed using KCl depolarization, epidermal growth factor (EGF), pituitary adenylate cyclase-activating polypeptide (PACAP), and serum. The KCl response was almost abolished by a nuclear Ca^{2+} clamp, indicating that a rise in nuclear Ca^{2+} is required. In contrast, the basal expression was not affected by the nuclear Ca^{2+} clamp, but it was strongly reduced by nifedipine, a specific antagonist of L-type Ca^{2+} channels. Spontaneous Ca^{2+} oscillations, blocked by nifedipine, were observed in the cytosol but did not propagate to the nucleus, suggesting that a rise in cytosolic Ca^{2+} is sufficient for basal c-*fos* expression. Inactivation of the c-*fos* promoter cAMP/Ca^{2+} response element (CRE) had no effect on basal or stimulated expression, whereas inactivation of the serum response element (SRE) had the same marked inhibitory effect as nifedipine. These experiments suggest that in AtT20 cells spontaneous Ca^{2+} oscillations maintain a basal c-*fos* transcription through the serum response element. Further induction of c-*fos* expression by depolarization requires a nuclear Ca^{2+} increase.

How short-lived intracellular signals cause marked alterations in gene expression leading to long-term cellular responses is still a puzzling aspect of cell biology. Immediate early genes (IEGs),[1] such as c-*fos*, function as a relay in this process; the transcription and protein levels of IEGs can be changed rapidly as a direct function of cellular signals. IEGs, which are mostly transcription factors, in turn control the expression of "late" responsive genes, which then modify the cellular functions (1).

The control by intracellular signals of the transcriptional activation of the c-*fos* gene has been widely studied (2, 3, 4). The c-*fos* gene includes in its promoter two major response elements that are targets of phosphorylation cascades, the cAMP/Ca^{2+}response element (CRE) and the serum response element (SRE) (2). These two consensus sequences bind different transcription factors, the activities of which are modulated by Ca^{2+}-dependent (11, 12) as well as Ca^{2+}-independent phosphorylation (13).

The CRE, which is located ~60 nucleotides upstream of the transcription initiation site of the c-*fos* gene, is bound by a leucine zipper transcription factor, CREB (CRE binding protein) (14). Following stimulation, CREB is phosphorylated on a critical residue, the serine 133. This phosphorylation allows CREB to recruit the transcriptional adapter CBP (CREB binding protein) and activates transcription of c-*fos*. Activation of various signaling pathways results in CREB phosphorylation, most notably the cAMP/PKA cascade, Ca^{2+} signaling acting via Ca^{2+}-calmodulin kinases (CaMkinases), as well as the mitogen-activated protein (MAP) kinases.

A second Ca^{2+} responsive element in the c-*fos* promoter is the serum response element, SRE, which is located ~310 nucleotides upstream of the transcriptional initiation site of the c-*fos* gene (15). SRE binds the serum response factor (SRF) and its accessory factor TCF, the ternary complex factor. TCFs are encoded by a family of Ets proteins that includes Elk-1, SAP-1a, and SAP-2. Phosphorylation of SRF on serine residue 103 is crucial for it's transcriptional activity. This residue can be phosphorylated *in vitro* by MAP kinases and CaMkinases. Therefore, as for CREB, the phosphorylation of SRF may implicate various signaling pathways including Ca^{2+} signaling cascades mediated by CaMkinase (16, 17). Physiological activation of transcription via the SRE is thought to occur predominantly following growth factor stimulation of MAP kinase cascades.

Control of gene expression by changes in intracellular calcium (Ca^{2+}) concentration nearly always involves changes in protein phosphorylation. Ca^{2+} signals can cause such changes directly via Ca^{2+}-calmodulin-activated protein kinases, CaMkinases, or calcineurin, a Ca^{2+}-dependent protein phosphatase (6, 7).

A study suggests that finely tuned Ca^{2+} signals triggered in restricted cellular domains may be able to activate processes that are selectively affecting c-*fos* gene transcription (5). An increase of intracellular Ca^{2+} concentration can occur inside or outside of the nucleus. A cytosolic Ca^{2+} increase can activate

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¶ Fellow of the Prof. Max Cloetta Foundation.

‖ To whom correspondence should be addressed: Foundation Pour Recherches Medicales, University of Geneva, 64 av. de la Roseraie, CH-1211 Geneva 4, Switzerland. Tel.: 4122-382-3811; Fax: 4122-347-5979; E-mail: werner.schlegel@medecine.unige.ch.

[1] The abbreviations used are: IEG, immediate early gene; CRE, cAMP/Ca^{2+} response element; SRE, serum response element; PACAP, pituitary adenylate cyclase-activating polypeptide; CREB, CRE binding protein; MAP, mitogen-activated protein; SRF, serum response factor; TCF, ternary complex factor; FRET, fluorescence resonance energy transfer; AM, acetoxymethyl esters; BAPTA-AM, bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; ERK, extracellular signal-regulated kinase.

CaMkinase II and/or calcineurin, which then translocate from their site of activation to the nucleus (8, 7). Transcriptional activation by nuclear Ca^{2+} can occur by a direct binding on DREAM (downstream regulatory element antagonist modulator), a Ca^{2+} binding transcriptional repressor (9), or by the translocation of the Ca^{2+}/calmodulin complex from the cytosol to the nucleus. This translocated complex will then activate a nuclear CaMkinase, such as CaMkinase IV (10).

Based on the stimulus-secretion coupling concept proposed by W. W. Douglas, pioneering studies in the 1960s demonstrated the electrical excitability of endocrine cells of the pituitary, the pancreas, and the adrenal medulla, now termed neuroendocrine cells (18, 19, 20). These early studies showed that voltage-gated Ca^{2+} channels were instrumental in the generation of action potentials in a neuroendocrine cell; depolarizing currents were indeed sodium (Na^+) and Ca^{2+} influxes, whereas repolarization was achieved by potassium (K^+). Later we demonstrated in neuroendocrine cells that a single action potential may cause a well defined intracellular Ca^{2+} signal, that those action potentials occur spontaneously and that they are controlled by somatostatin (21). A wealth of subsequent literature illustrates that the modulation of Ca^{2+} action potentials in neuroendocrine cells is a versatile signaling option utilized by most releasing factors and releasing modulators, but also by growth factors and other extracellular signals (34). Activation or inhibition of neuroendocrine cell activity nearly always involves such modulation, which results in alterations of action potential frequency and/or rhythm. In addition, amplitude, duration, and intracellular propagation of action potential-linked Ca^{2+} signals are modulated as well. In summary, Ca^{2+} action potentials and the resulting intracellular Ca^{2+} transients are fundamental signaling units in neuroendocrine cells.

Transcriptional control by Ca^{2+} has been investigated within the context of Ca^{2+} oscillations generated by intracellular mechanisms originally described in hepatocytes (22). Such Ca^{2+} oscillations are produced following receptor-mediated phospholipase C (PLC) activation and the generation of the second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to the IP_3 receptor at the endoplasmic reticulum (ER) and induces the release of Ca^{2+} from the ER. This Ca^{2+} release from intracellular stores occur in many cell types including pituitary cells (23). Ca^{2+} oscillations may activate gene transcription in a manner dependent on their pattern, as demonstrated for the transcription factor nuclear factor AT (NF-AT), which is activated by a mechanism involving the Ca^{2+}-dependent phosphatase, calcineurin (24, 25).

Action potential-linked Ca^{2+} transients most likely function also for the activation of gene transcription by mechanisms sensitive to frequency and rhythm of oscillatory Ca^{2+} changes. To test this hypothesis, we used a single cell reporter gene approach (26) in order to link the observations on Ca^{2+} transients in individual cells to gene transcription. Furthermore, at the single cell level Ca^{2+} signals can be selectively manipulated by microinjection of high molecular weight Ca^{2+} chelators into specific compartments such as the nucleus.

Here we show that Ca^{2+} transients driven by spontaneous action potentials in the pituitary cell line AtT20 sustain a basal transcriptional activity of the IEG c-*fos* through a mechanism that involves the SRE and does not require changes in nuclear Ca^{2+} concentration.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pBlack-b and the fluorescent substrate CCF-2 AM (CCF-2 acetoxymethyl esters) were purchased from Aurora Biosciences Corp. (San Diego, United States). Fluorescent calcium probes, Fura-2 AM (Fura-2 acetoxymethyl esters) and Fura-2 D70 (Fura-2 Dextran 70 kDa); calcium chelators, Bapta AM (Bapta acetoxymethyl esters), Bapta-D70 (Bapta Dextran 70 kDa), and Texas-Red D70 (TR-D70) were supplied by Molecular Probes (Lucerne, Switzerland). Nifedipine was purchased from Sigma (Buchs, Switzerland).

Plasmid Constructions—We used the bacterial enzyme β-lactamase as a reporter gene. We already dispose of luciferase reporter constructs containing the c-*fos* promoter (27, 28). The luciferase coding sequence from our reporter constructs was then excised by *NocI* and *Xba*I digestion and replaced by the β-lactamase coding sequence.

For our experiments four different constructs were used. The first contains c-*fos* proto-oncogene for the region of the mouse, extending from −379 to +1073. This construct is called c-*fos*-βL and corresponds to the wild type c-*fos* promoter plus part of the gene (first exon and first intron). The second and third constructions contained one each a mutation inactivating the CRE (c-*fos*-ΔCRE-βL) or the SRE (c-*fos*-ΔSRE-βL) response elements. The last construction contained two mutations inactivating both the SRE and CRE response elements (c-*fos*-ΔSRE/CRE-βL). All these constructions are schematically presented in Fig. 5. Mutations were performed by site-directed mutagenesis using the Quick-Change mutagenesis kit from Stratagene (Basel, Switzerland). The strategy of mutagenesis has already been described (5, 27, 28).

Cell Culture—Rat corticotrope AtT20 cells were cultured in a Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Stably transfected AtT20 clones (see below) were selectively maintained with 100 μg/ml G418 (an antibiotic analog to neomycin) added to the culture medium. For all measurements cells were plated on 25 mm coverslips, and serum was removed from the culture medium 24 h before experiments.

Establishment of Stable β-lactamase Expressing AtT20 Pituitary Cell Clones—Five stable AtT20 cell lines were established, four cell lines containing a reporter construct plasmid (c-*fos*-βL, c-*fos*-ΔCRE-βL, c-*fos*-ΔSRE-βL, c-*fos*-ΔSRE/CRE-βL) and one cell line with viral promoter SV40 (SV40-βL). A pcDNA3 vector containing the neomycin resistance gene co-transfected in a mass ratio of 1:10 was used for selection. The DOSPER liposomal transfection reagent (Roche) was used following the manufacturer's recommendations. The cotransfection was performed as follows. AtT20 cells were grown to ~60% confluence in 35 mm diameter Petri dishes. After removal of the culture medium and one wash with HBS (Hepes buffer saline, pH 7.4), cells were incubated with 2 ml of HBS containing the plasmids (0.5 μg of pcDNA3 and 5 μg of reporter plasmid) dissolved previously in 40 μl of DOSPER. After 6 h, cells were washed with HBS, and culture was continued in Dulbecco's modified Eagle's medium-F12 (10% fetal calf serum) containing 400 μg/ml G418.

After 3 weeks of culture in G418 medium, several neomycin-resistant clones were selected and tested for their ability to induce fluorescence changes in CCF-2 in response to stimulation by 20 mM KCl and 3 μM cpt-cAMP (chlorophenylthio-cyclic AMP).

Microinjection—AtT20 cells were injected using an Eppendorf transjector 5246 mounted on a Zeiss Axiovert S100TV microscope. For the measurements of nuclear Ca^{2+}, the injection solution was 50 μM Fura 2-D70, half-strength phosphate-buffered saline, 1 mM $MgCl_2$, pH 7.2. For the nuclear-Ca^{2+} clamp, 2.5 mM Bapta-D70 and 1 mM Ca^{2+} were added (free Ca^{2+} concentration was calculated to be 83 nM). To measure the β-lactamase activity, Fura 2-D70 was replaced by 5% TR-D70 as injection marker. After injection, cells were kept in the incubator at 37 °C for 4 to 5 h before experiments.

Calcium Measurements—Cytosolic calcium concentration variations were measured using the calcium probe Fura-2. Cells were loaded for 30 min at room temperature with the membrane permeant Fura-2 AM in a medium containing NaCl 140 mM, KCl 5 mM, Ca^{2+} 1.2 mM, $MgCl_2$ 1 mM, glucose 10 mM, Hepes 20 mM, pH 7.4.

Changes of nuclear calcium concentration were measured by nuclear injection of the 70-kDa Dextran conjugate Fura-2 (Fura-2-D70; see "Microinjection" under "Experimental Procedures").

Fura-2 fluorescence (excitation 340/380 nm, emission 510 nm) was monitored with an imaging system. Loaded cells plated on coverslips were mounted on an inverted Zeiss Axiovert S100TV microscope coupled to a Princeton Instruments cooled back-illuminated frame-transfer charge-coupled device camera. The light source for illumination came from a Xenon XBO 75-W lamp. Excitation wavelengths were selected with a PTI (Photon Technology International) monochromator. Images were acquired with the Metamorph 4.1 software (Universal Imaging Corp.). Measurements were performed at 37 °C in an open perfusion micro-incubator (Harward Apparatus). The cytosolic and nuclear calcium (Ca^{2+}) concentrations were determined as described (29).

Monitoring β-Lactamase Reporter Activity—AtT20 cells cultured on 25 mm coverslips were loaded with the fluorescent substrate CCF-2AM as described (26). Briefly, cells were incubated for 1 h at room temper-

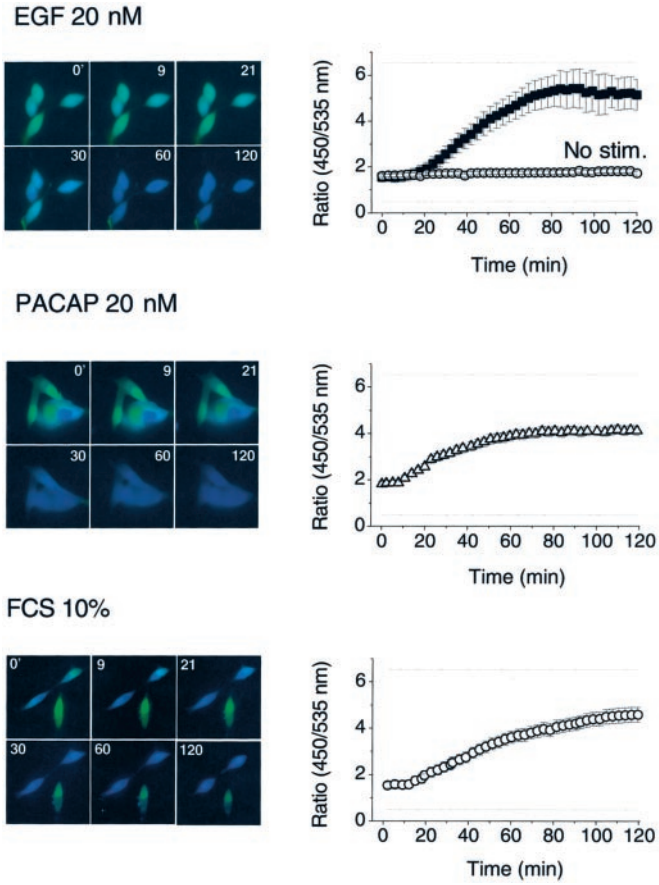


FIG. 1. **Stimulation of c-fos β-lactamase reporter gene expression in AtT20 cells.** AtT20 cells from clones that stably express β-lactamase under the control of the c-*fos* promoter, including part of the c-*fos* gene and the first intron (c-*fos*-βL) as schematically represented in Fig. 5, were loaded with 6 μM CCF-2 for 1 h. Cells were then stimulated with various agonists: 20 nM EGF (n = 30 cells), 20 nM PACAP (n = 67 cells), 10% FCS serum (n = 78 cells). Images of the CCF-2 fluorescence were taken as described under the Experimental Procedures section. A gallery of superimposed images (at times 0, 9, 21, 30, 60, 120 min) representing the change of color caused by FRET suppression in the CCF-2 substrate and a time course of the CCF-2 fluorescent ratio of images taken every 3 min for each agonist are presented. Results from cells in non-stimulated conditions (n = 35, *open circles*, in the upper time-course) are presented. The *broken lines* indicate the minimal and maximal CCF-2 ratios measured in non-transfected AtT20 cells, which do not express any β-lactamase (0.48 ± 0.02, *lower line*), and in AtT20 cells expressing the β-lactamase construct driven by the SV40 promoter (6.5 ± 0.1, *higher line*). Data are mean ± S.E.

ature in culture medium with 1 μM CCF-2AM and 1% pluronic acid. Cells were then washed in the perfusion chamber of the imaging system (as described before for Ca^{2+} measurements). Measurements were made at 37 °C. CCF-2 fluorescence (excitation 405 nm) was imaged alternatively at emission wavelengths 450/530 nm, and data were stored at 3-min intervals. From these raw data in the form of image series at the two wavelengths, the fluorescence emission ratios F(450 nm)/F(530 nm) integrated over the cell area ratio were calculated (Fig. 1). The maximum and minimum CCF-2 ratios were determined by measuring the fluorescence in non-transfected AtT20 cells and in the SV40-βL cell line. The minimum ratio for the CCF-2 fluorescence was 0.48 ± 0.02 (n = 84 non-transfected cells). The maximal CCF-2 ratio was 6.5 ± 0.1 (n = 61 SV40-βL cells). These values are indicated in Fig. 1 (minimum and maximum) and Fig. 5 (minimum only) as dotted lines.

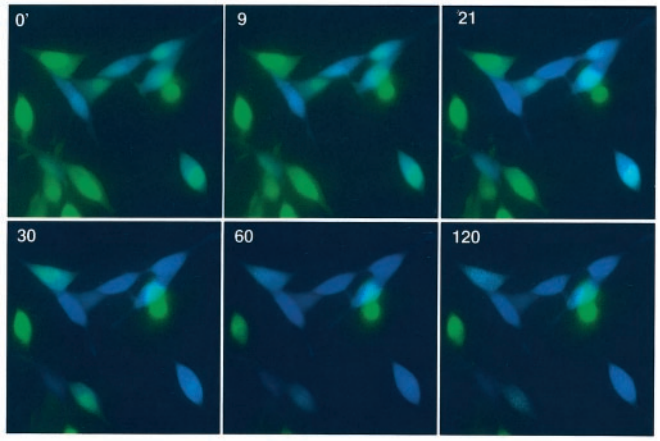
RESULTS

Activation of c-fos Transcription in AtT20 Cells—To visualize the activation of gene transcription at the single cell level, we used β-lactamase as a reporter gene (26). Expression of this bacterial enzyme can easily be detected by monitoring the fluorescence of a cell-permeant substrate, CCF-2, composed of two fluorophores linked by a β-lactame ring. The uncleaved CCF-2 emits green light (530 nm) because of fluorescence resonance energy transfer (FRET) between the two fluorophores. Upon β-lactamase expression, the substrate is cleaved and emits blue light (450 nm) because of the loss of FRET. β-lactamase activity is then measured as the change in green to blue CCF-2 fluorescence. To assess c-*fos* transcription, the β-lactamase coding sequence was placed under control of the promoter and first intron of the c-*fos* gene, yielding the reporter construct c-*fos*-βL that is drawn schematically in Fig. 5. Our earlier studies had shown that the first intron of the c-*fos* gene contains important regulatory elements without which transcription control by Ca^{2+} is incomplete (27, 28).

We generated AtT20 cell clones stably expressing c-*fos*-βL, which were loaded with CCF-2 AM. As seen in Fig. 1 (*left panels*), stimulation of the CCF-2-loaded c-*fos*-βL cells with agonists induced a change in the color of CCF-2 fluorescence from green to blue within 15–20 min. The degree of β-lactamase expression was quantified as the ratio of blue (450 nM) to green (530 nm) fluorescence intensities, an increase in CCF-2 ratio (F450/F530) thus reporting c-*fos*-βL activation. Following cell stimulation, CCF-2 fluorescence images were acquired every 3 min, and CCF-2 ratios were calculated to obtain a time course of c-*fos*-βL transcriptional activation (Fig. 1, *right panels*). In the absence of any stimulation, no change in CCF-2 ratio could be detected (*top right panel*), indicating that the degree of basal c-*fos*-βL transcriptional activation was stable in AtT20 cells over the 2-h measurement period. In contrast, c-*fos*-βL reporter expression was markedly enhanced by stimuli known to activate transcription of the c-*fos* gene in AtT20 cells (Fig. 1, *middle* and *lower panels*), such as EGF (20 nM), PACAP (20 nM), and FCS (10%). All stimuli induced a strong change in the CCF-2 ratio, from a mean basal value of 1.8 ± 0.06 (± S.E.) to maximal values after 2 h of stimulation. Other values were 5.4 ± 0.8 for EGF, 4.13 ± 0.1 for PACAP and 4.6 ± 0.1 for serum. These results validate the use of c-*fos*-βL as a reporter gene, which has the advantage of resolving the kinetics of transcriptional activation of the c-*fos* gene at the single cell level.

To study the effect of Ca^{2+} on c-*fos* transcription, cells were depolarized with 20 mM KCl in order to open voltage-gated Ca^{2+} channels (VOCs) and trigger Ca^{2+} influx. As shown in Fig. 2, KCl depolarization also powerfully activated c-*fos*-βL transcription, causing a near doubling in the CCF-2 ratio after 2 h of stimulation. This increase was almost completely abolished by preloading the cells with the Ca^{2+}-chelator BAPTA-AM (Fig. 2, *bottom panel*), confirming that the KCl response was mediated by an increase in cellular Ca^{2+}. Thus, in AtT20 cells a sustained increase in intracellular Ca^{2+} strongly activates the transcription of the c-*fos* gene.

Spontaneous Ca^{2+} Transients Regulate Basal c-fos Transcription—Although the basal CCF-2 ratio remained stable for up to 2 h in the absence of exogenous stimulus (Fig. 1), there was considerable cell-to-cell heterogeneity among non-stimulated cells (*cf.* the time 0′ images in Figs. 1 and 2). This suggested that c-*fos*-βL might already be activated in a fraction of the resting AtT20 cells. Accordingly, the histogram distribution of the CCF-2 ratios could be separated into three subpopulations centered at ratio values of 0.7, 1.8, and 3.5, and comprising 29%, 59%, and 12% of resting c-*fos*-βL AtT20 cells, respectively (Fig. 3*A*). This suggested that in the absence of stimulation transcription was strongly repressed in only approximately one-third of the cell population, whereas c-*fos*-βL was transcribed at intermediate levels in 59% of cells and at high levels in 12% of cells. Interestingly, the ratio value of the "active" population (3.5) was similar to the average CCF-2 ratio



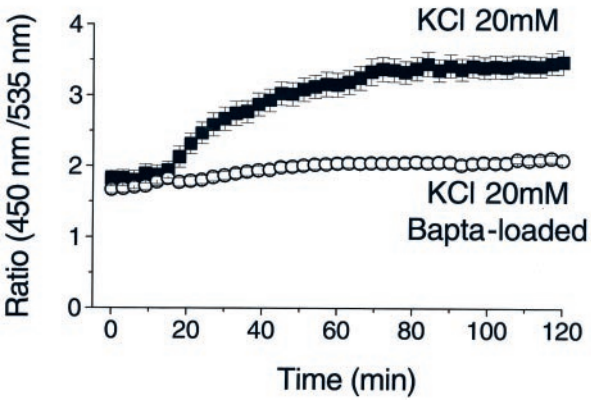


FIG. 2. **Depolarization stimulates c-fos β-lactamase transcription.** Cells were stimulated with 20 mM KCl added in the perfuse solution. A gallery of superimposed images of images taken at 0, 9, 21, 30, 60, 120 min is presented. Time course of the fluorescent ratio of images taken every 3 min shows the mean and S.E. of 126 cells treated with 20 mM KCl (*filled squares*) and 27 cells pre-loaded with 30 μM Bapta-AM (*open circles*).

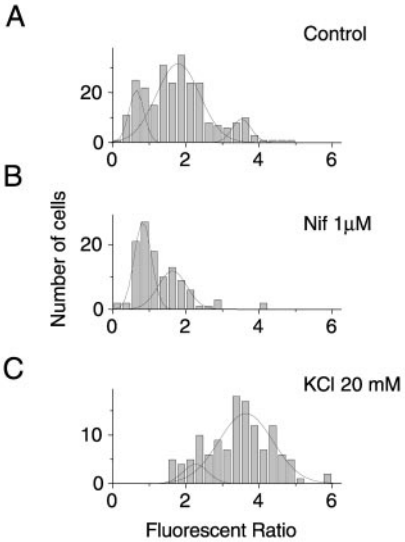


FIG. 3. **c-fos β-lactamase is expressed in most of the non-stimulated AtT20 cells.** Stable c-*fos*-βL AtT20 clones were loaded with CCF2-AM, and fluorescent images (450 nm and 530 nm) were captured with an imaging system as described under Experimental Procedures. To obtain numerical values for the fluorescence of CCF-2 (F450 and F530) for each cell, intensities were integrated over a surface interactively superimposed over the image of each cell. The CCF-2 ratio corresponds to F450 nm/F530 nm. This figure shows the histograms of cellular CCF-2 fluorescent ratio in AtT20 cells: *A*, non-stimulated (n = 258 cells); *B*, in the presence of the Ca^{2+} channel blocker nifedipine (n = 115 cells); *C*, stimulated with 20 mM KCl (n = 126 cells).

measured upon KCl stimulation (Fig. 3*C*), suggesting that c-*fos* transcription was fully activated in 12% of unstimulated cells. To assess whether this heterogeneity could reflect spontaneous Ca^{2+} transients caused by the basal electrical activity of AtT20 cells, we used the L-type Ca^{2+} channel inhibitor, nifedipine. Exposure of cells to 1 μM nifedipine for 4 h, a concentration that completely blocks L-type Ca^{2+} channels, shifted the CCF-2 ratio to lower values, leaving nearly no transcriptionally fully active cells (Fig. 3*B*). Most cells (61%) now displayed a CCF-2 ratio of 0.8, and the proportion of cells with intermediate c-*fos*-βL activity (1.6) was reduced to 34%. An opposite effect was observed with a 2-h KCl stimulation (Fig. 3*C*); the proportion of cells with fully activated c-*fos*-βL increased from 12 to 69% (ratio 3.64), whereas intermediate c-*fos*-βL activity was observed in the remaining 31% of cells (ratio 2.26). Thus, in AtT20 cells, basal c-*fos* transcription is relatively high as a result of electrical activity. Ca^{2+} channel blockers strongly down-regulate this endogenous activity, whereas a long-lasting depolarization powerfully activates c-*fos* transcription.

Spontaneous action potentials in pituitary cells generate characteristic Ca^{2+} transients (21, 30, 31). These Ca^{2+} elevations could be readily measured in c-*fos*-βL AtT20 cells using the ratiometric Ca^{2+} indicator fura-2 (Fig. 4). As observed for basal c-*fos*-βL activity, the Ca^{2+} activity was heterogenous. 87% of cells (55/63) exhibited Ca^{2+} transients with mean amplitude and frequency of 118 ± 87 nM and 0.1 ± 0.04 Hz, respectively (mean ± S.D., n = 1622, Fig. 4*A*), whereas 13% of cells (8/63) lacked electrical activity (not shown). The Ca^{2+} transients ranged from 30 nM to 400 nM, resembling the distribution of basal c-*fos*-βL activity (Fig. 4*C*). As expected, the spontaneous Ca^{2+} activity was completely abolished by the addition of 1 μM nifedipine (n = 19, Fig. 4*C*). Stimulation by 20 mM KCl elicited a transient increase in the cytosolic Ca^{2+}, to 630 ± 200 nM (mean ± S.D., n = 7, Fig. 4*D*), followed by a sustained plateau (200 ± 70 nM). Note the disappearance of the spontaneous Ca^{2+} oscillations during the KCl stimulation.

Basal c-fos Transcription Is Mediated by the SRE Element—The convergent effect of nifedipine on basal Ca^{2+} transients and c-*fos*-βL activity suggested an important role for the spontaneous Ca^{2+} transients in the regulation of gene expression. To assess which element within the c-fos promoter mediated this Ca^{2+} response, we separately mutated two Ca^{2+} response elements within the c-fos-βL promoter (SRE and CRE, Fig. 5*A*). The CRE and SRE elements were inactivated by site-directed mutagenesis (see Experimental Procedures), and AtT20 clones stably expressing the mutated constructs were established. These two clones (c-*fos*-ΔCRE-βL and c-*fos*-ΔSRE-βL) also displayed spontaneous Ca^{2+} transients, with amplitude and frequency similar to those observed in c-*fos*-βL or wild type AtT20 cells (data not shown). As shown in Fig. 5*B* (*hatched bars*), deletion of the CRE element only marginally increased the basal CCF-2 ratio and did not affect the ability of cells to respond to nifedipine, but precluded their activation by KCl (2.69 ± 0.37 *versus* 2.15 ± 0.24, p = 0.07, unpaired t test). In contrast, deletion of the SRE element had a profound effect on endogenous c-*fos*-βL activity, decreasing the basal CCF-2 ratio from 1.80 ± 0.06 to 0.83 ± 0.03 (*filled bars*, $p < 10^{-8}$, unpaired t test). Basal c-*fos*-ΔSRE-βL transcription could not be further reduced by nifedipine (0.80 ± 0.04), but the cells were still able to respond to a depolarizing stimulus. The ratio increased from

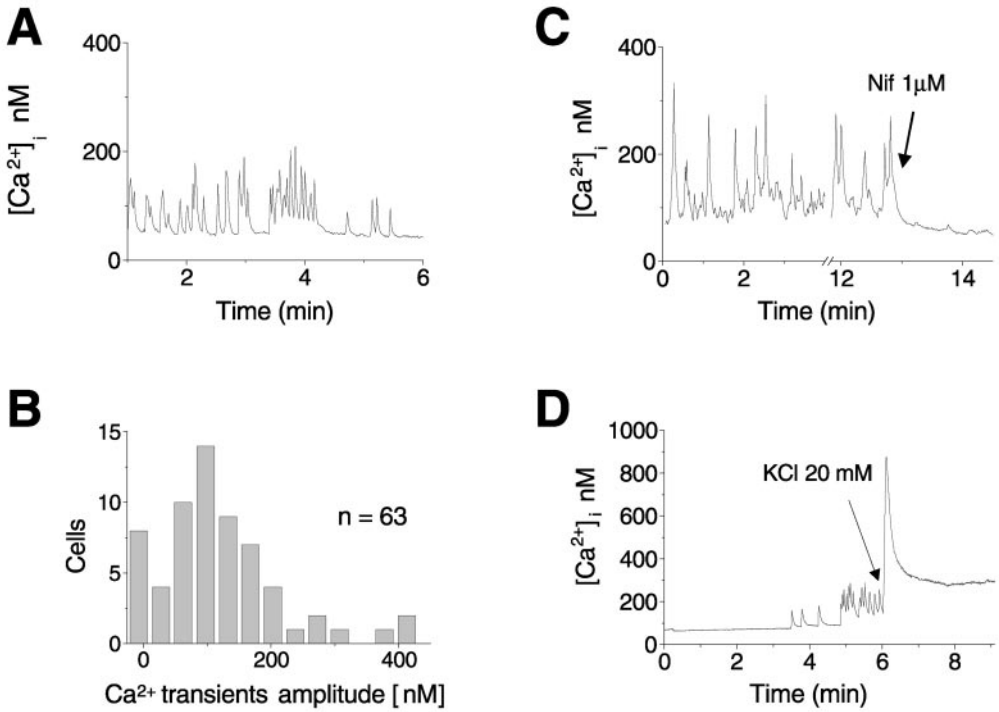


FIG. 4. **Spontaneous Ca^{2+} transients in single AtT20 cells.** Ca^{2+} was measured in 63 single AtT20 cells loaded with the Ca^{2+} probe Fura-2/AM as described under Experimental Procedures. The basal Ca^{2+} concentration was 70 ± 20 nM (mean ± S.D., n = 63 cells). Ca^{2+} transients were measured during a period of 5–15 min. *A*, trace representative of 55 cells with spontaneous Ca^{2+} transients corresponding to 87% of the cells. The mean amplitude of the Ca^{2+} transients was 118 ± 87 nM (mean ± S.D., n = 1622) and the frequency 0.1 ± 0.04 Hz (mean ± S.D., n = 55). *B*, histogram of the mean Ca^{2+} transients' amplitude for the 63 measured cells. *C*, addition of the voltage-gated Ca^{2+} channel blocker nifedipine (1 μM) completely abolished the Ca^{2+} transients (n = 19). *D*, representative Ca^{2+} response induced by KCl stimulation (n = 7 cells).

0.83 ± 0.03 to 2.07 ± 0.1 in the presence of KCl. The inactivation of both SRE and SRE elements (c-*fos*-ΔSRE/CRE-βL, Fig. 5*A*) strongly reduced the expression of β-lactamase (*dark bars*), to values only slightly higher than the minimal values measured in non-transfected cells (*dotted line*). These cells were insensitive to nifedipine and to KCl stimulation. These data suggest that the CRE element is not required for the basal activation of c-*fos*. Instead, the SRE element appears to mediate the basal c-*fos* transcription driven by spontaneous action potentials. In contrast, the SRE element is less essential for the Ca^{2+}-mediated transcriptional activation induced by a large and long-lasting depolarization.

Ca^{2+} Transients Caused by Spontaneous Action Potentials Propagate Poorly to the Nucleus—The differential regulation of the spontaneous and KCl-induced c-*fos* transcription suggested that the two types of Ca^{2+} signals might propagate differently to the nucleus. In particular, because of their relatively low amplitude (118 nM, Fig. 4), we suspected that the spontaneous Ca^{2+} transients might fail to reach the nuclear compartment because of space restriction and buffering effects (32, 33). To test this hypothesis, the Ca^{2+} concentration was measured in the nuclear compartment by nuclear microinjection of Fura-2 Dextran (70 kDa). The large dextran molecule allowed a stable confinement of the Ca^{2+} probe within the nuclear compartment for more than 4 h (Fig. 6*A*). In the absence of stimulation, spontaneous nuclear Ca^{2+} transients were observed only in 5 of 19 microinjected cells (26%), a proportion much lower than the corresponding cytosolic Ca^{2+} transients (87%, Fig. 4). Furthermore, although the two compartments had similar basal Ca^{2+} levels (75 ± 22 *versus* 70 ± 20 nM), the amplitude of the measured Ca^{2+} transients was lower in the nucleus than in the cytosol (80 ± 26 *versus* 118 ± 87 nM). The failure to detect higher spontaneous nuclear activity was not caused by a lack of sensitivity of the microinjected Fura-2-dextran probe, because subsequent KCl stimulation caused a large increase in nuclear Ca^{2+}, regardless of the basal activity of the cells (Fig. 6*A*). Thus, spontaneous Ca^{2+} transients propagate poorly to the nuclear compartment, whereas the KCl-induced Ca^{2+} transients produce large Ca^{2+} signals within the nucleus.

To assess whether Ca^{2+} propagation to the nucleus was required for the KCl response, we then attempted to prevent the nuclear Ca^{2+} signals by co-injecting a dextran-coupled Ca^{2+} chelator (BAPTA-D70) together with the nuclear Fura-2 probe. As shown in Fig. 6*B*, no Ca^{2+} transients were observed under these conditions (n = 9), although the nuclear Ca^{2+} clamp did not affect the cytosolic Ca^{2+} transients (data not shown). More importantly, although the cytosolic KCl response was preserved (data not shown), the nuclear Ca^{2+} signal was strongly reduced (Fig. 6*B*, mean amplitude = 72 ± 23 nM), indicating that the nuclear Ca^{2+} clamp achieved with BAPTA-D70 injection was highly effective.

A Rise in Nuclear Ca^{2+} Is Required for KCl-Induced, but Not for Basal, c-fos Expression—To assess the effects of a nuclear Ca^{2+} clamp on basal- and KCl-induced transcription, c-*fos*-βL activity was measured in cells injected with BAPTA-D70 or with the injection marker Texas-Red-D70 (TR-D70) alone. As shown in Fig. 7*B*, the nuclear Ca^{2+} clamp did not affect basal c-*fos*-βL activity, the CCF-ratio averaging 1.79 ± 0.12 and 2.05 ± 0.17 in BAPTA-D70- and control-injected cells, respectively. Furthermore, basal c-*fos*-βL activity could still be inhibited by nifedipine in BAPTA-D70-injected cells, the CCF-2 ratio decreasing to 1.37 ± 0.1 *versus* 1.41 ± 0.1 in control-injected cells. In sharp contrast however, the nuclear Ca^{2+} clamp completely abolished the KCl-induced c-*fos* activation (Fig. 7*B*). Whereas the ratio values achieved in TR-D70-injected cells were similar to those observed in non-injected cells (3.3 ± 0.15), the KCl stimulation failed to elicit any significant increase in c-*fos*-βL in BAPTA-D70-injected cells. Thus, the nuclear Ca^{2+}

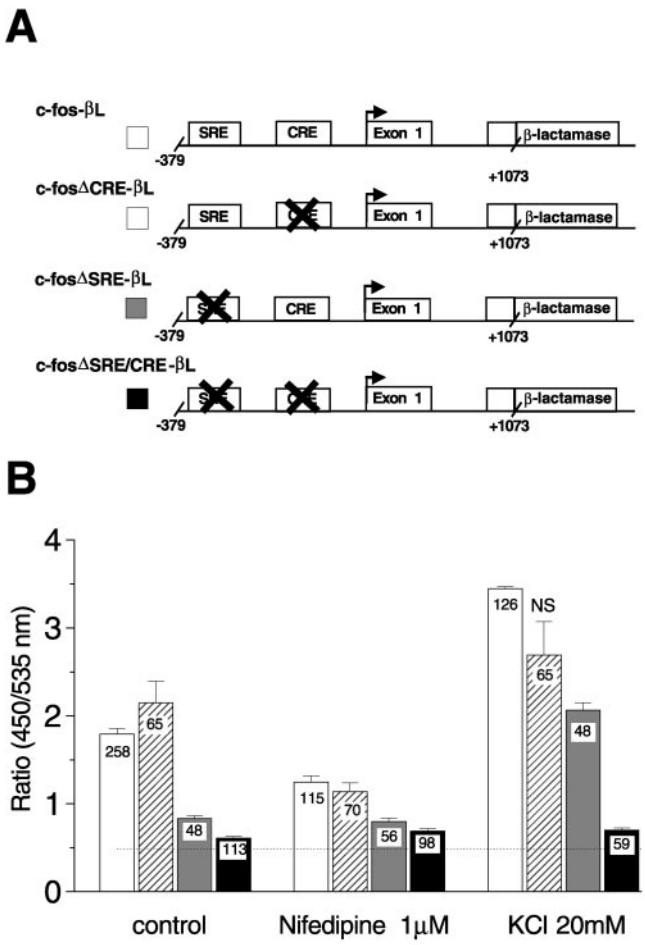


FIG. 5. **The SRE, but not the CRE element, is required for basal c-fos-βL expression.** *A*, the c-*fos* promoter was mutated to inactivate the SRE (c-*fos*-ΔSRE-βL) and the CRE element (c-*fos*-ΔCRE-βL) and both elements (c-*fos*-ΔSRE/CRE-βL) (27). AtT20 clones stably transfected with c-*fos*-ΔSRE-βL or c-*fos*-ΔCRE-βL or c-*fos*-ΔSRE/CRE-βL were established as described under Experimental Procedures. These clones were loaded with CCF-2AM, and the ratio (F450 nm/F530 nm) was measured (see Fig. 3). *B*, shown are the mean CCF-2 ratios (± S.E.) for the c-*fos*-βL (*open bars*), the c-*fos*-ΔCRE-βL (*hatched bars*), the c-*fos*-ΔSRE-βL (*shaded bars*), and the c-*fos*-ΔSRE/CRE-βL clones (*dark bars*). The *dotted line* indicates the miminal CCF-2 ratio measured in non-tranfected Att20 cells. Conditions were non-stimulated (*left*), in the presence of 1 μM nifedipine (*middle*), and 2 h after stimulation with 20 mM KCl (*right*). *NS*, not significant *versus* control c-*fos*-ΔCRE-βL.

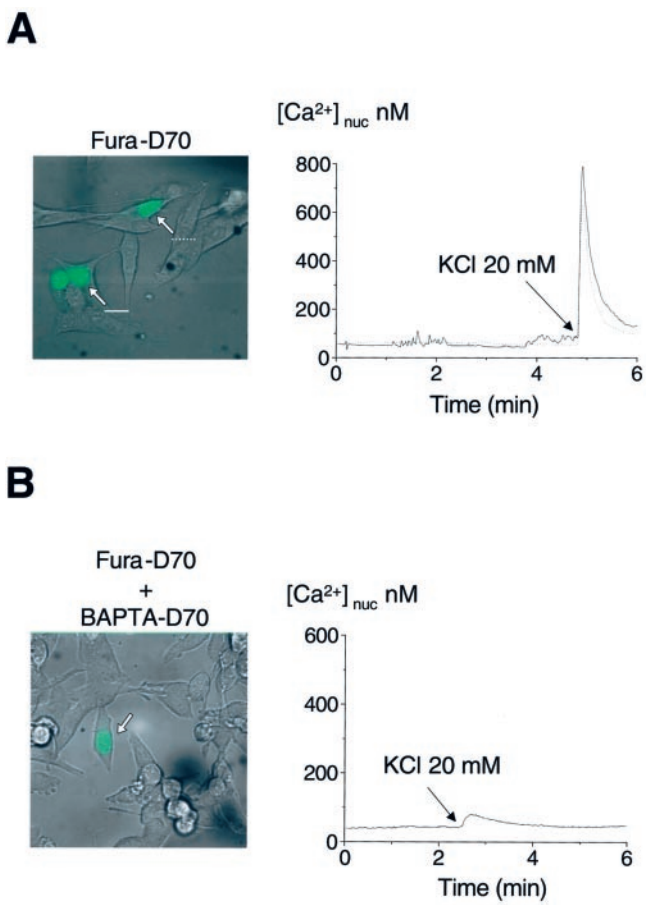


FIG. 6. **Spontaneous Ca^{2+} transients measured in the nucleus of AtT20 cells.** *A*, nuclear Ca^{2+} was measured by intranuclear injection of Fura-2 coupled to dextran D70 (Fura-D70, 50 μM concentration in the pipette). *Left*, the dextran-coupled dye remained confined to the nucleus for more than 2 h. *Right*, changes in nuclear Ca^{2+} concentration in the absence of stimulation and following KCl depolarization in two cells (*arrows*), one displaying spontaneous nuclear oscillations (*unbroken line*, representative of 5 cells) and one lacking nuclear oscillations (*dotted line*, representative of 14 cells). *B*, change in nuclear Ca^{2+} concentration in cells co-injected with Fura-D70 and BAPTA-D70 in order to clamp the nuclear Ca^{2+} concentration to about 80 nM. The trace is representative of 8 tested cells.

clamp had no effect on the basal c-*fos*-βL activity, indicating that endogenous c-*fos* activity, driven by the spontaneous activity of AtT-20 cells, does not require a rise in nuclear Ca^{2+}. In contrast, large and sustained increases in Ca^{2+}, such as those elicited by the KCl-induced depolarization, cause enhanced c-*fos* transcription in a manner stringently dependent on a nuclear Ca^{2+} increase.

A Nuclear Ca^{2+} Rise Is Required for CRE, but Not for SRE, Mediated c-fos Transcription—To determine whether a rise in nuclear Ca^{2+} activates the SRE or the CRE element, we performed nuclear Ca^{2+} clamp experiments with the mutated constructs. As shown in Fig. 8*A*, nuclear microinjection of BAPTA-D70 had no effect on the construct bearing an inactivated CRE element (c-*fos*-ΔCRE-βL). This construct displayed a strong basal expression sensitive to nifedipine and failed to respond to KCl, regardless of the nuclear clamp. In contrast, nuclear Ca^{2+} chelation nearly abolished the KCl-induced activation of the construct bearing an inactivated SRE element (c-*fos*-ΔSRE-βL). This construct had a reduced basal expression insensitive to nifedipine and a strong KCl response that was completely prevented by nuclear microinjection of BAPTA-D70 (Fig. 8*B*). Thus, a rise in nuclear calcium mediates the KCl response, which does not require the SRE element but does require the CRE element. Conversely, a rise in nuclear Ca^{2+} is not necessary for basal c-*fos* expression, which does not require the CRE element but does require the SRE element.

DISCUSSION

Important elements of Ca^{2+} signaling in neuroendocrine cells can be studied only in individual cells. To link such elements to the control of transcription, tools are required to study the latter at the level of the single cell. In the past repeated attempts have been made to study gene transcription at the single cell level, and several very different techniques have been engaged.

Some techniques aim to directly read out the mRNA of interest. Those are based on "*in vivo*" hybridization of fluorescent DNA or RNA probes. Hybridization generates a fluorescence signal by eliminating self-quenching in probes with a "molecular beacon" design (44). Alternatively, hybridization of two probes in close vicinity can be monitored by FRET from the FRET photon donor probe to the FRET photon acceptor probe (45). Direct detection of mRNA in live single cells using those

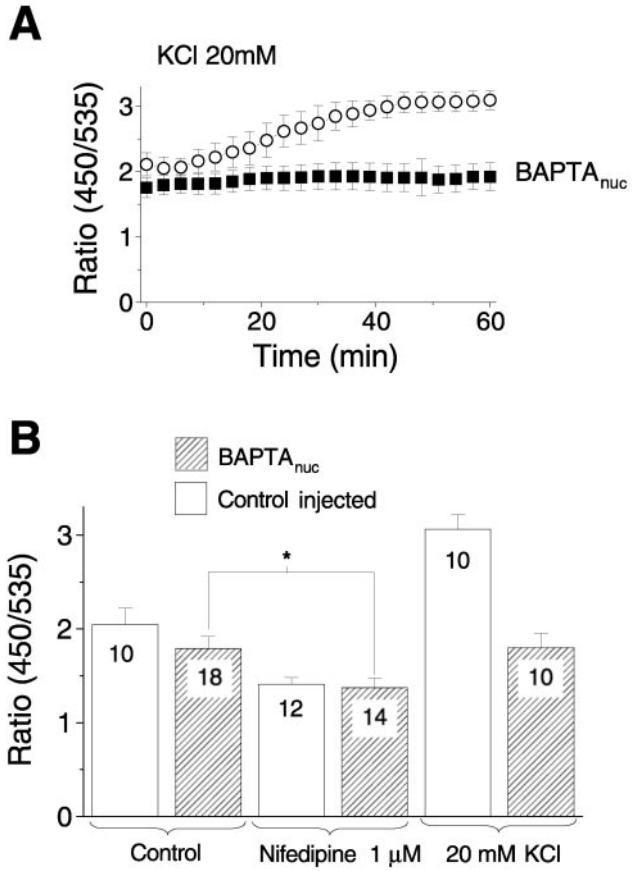


FIG. 7. **A Nuclear Ca^{2+} clamp does not affect c-fos basal expression but prevents activation by depolarization.** Stable c-*fos*-βL AtT20 clones were loaded with CCF2-AM after intranuclear microinjection of BAPTA-D70 and TR-D70 (injection marker). *A*, mean CCF-2 ratios (± S.E.) of BAPTA-D70-injected (*filled squares*, $n = 10$) and control-injected (*open circles*, $n = 10$) c-*fos*-βL AtT20 cells. Cells were stimulated with 20 mM KCl at time 0. *B*, mean CCF-2 ratios (± S.E.) of control-injected (*open bars*) and BAPTA-D70-injected (*hatched bars*) c-*fos*-βL cells measured in the absence of stimulation, in the presence of 1 μm nifedipine, or after stimulation with 20 mM KCl (1 h). For each condition, 10–18 injected AtT20 cells were analyzed. *, $p < 0.0001$ *versus* control-injected cells, unpaired *t* test.

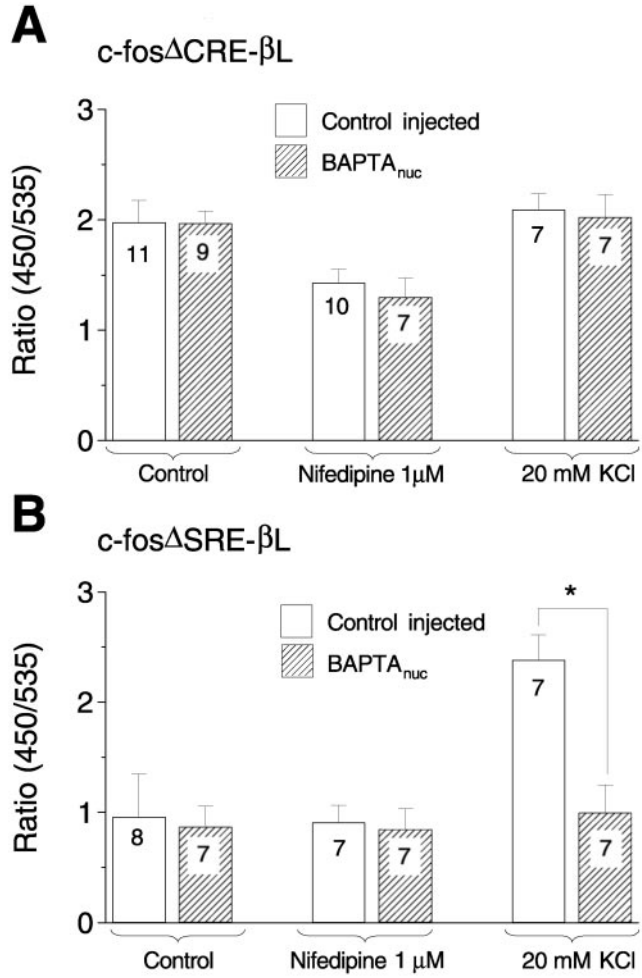


FIG. 8. **A nuclear Ca^{2+} rise is required for CRE, but not for SRE-mediated c-fos transcription.** Stable c-fosΔCRE-βL or c-fosΔSRE-βL AtT20 clones were loaded with CCF-2 after nuclear microinjection of BAPTA-D70 and TR-D70 (injection marker). *A*, mean CCF-2 ratios (± S.E.) of control-injected (*open bar*) and BAPTA-D70-injected (*hatched bars*) c-fosΔCRE-βL AtT20 cells measured in the absence of stimulation, in the presence of nifedipine, and after 1 h of KCl stimulation. *B*, the same experiment was performed with c-fosΔSRE-βL AtT20 clones. *, $p < 0.002$, unpaired *t* test.

techniques has not been reported by many groups, most likely because of technical difficulties. For example, probes need to be injected, and their design is difficult because they have to hybridize selectively at low temperatures; furthermore, only a substantial increase in mRNA will generate a detectable signal.

The few studies addressing gene transcription control mechanisms in single cells are based mostly on the quantification of a "reporter" protein. Three types of reporter genes have been used, namely luciferase and β-lactamase that code for enzymes whose activity can be monitored (25, 26, 42, 43) or GFP whose product can be directly detected by its fluorescence. Finally, in some studies exogenous gene products carrying an epitope tag have been quantified by immunocytochemistry (5).

Among reporter proteins, β-lactamase has some clear advantages, namely the high sensitivity of its detection with the non-toxic fluorescent β-lactamase substrate CCF-2 and the possibility of quantification based on a ratio-metric measurement that eliminates nonspecific effects on total fluorescence. By comparison with microinjected luciferase reporter vectors using the same regulatory elements[2] or very similar elements (42), we estimate that β-lactamase provides for a 10–50-fold increase in sensitivity over luciferase, which turns out to be a reporter too weak to be used to study induction of IEGs (46).

In this single-cell study of transcription control it was indeed possible to exploit individual cell activities and relate them to gene expression (Figs. 3 and 4). Also, signal compartmentalization was addressed using microinjection, a powerful single cell technique. Note that microinjection of the reporter plasmids should permit reporter studies with preparations close to the physiological reality (*e.g.* fully differentiated neurons in brain slices). The fluorescence readout of reporter β-lactamase activity provides only a semi-quantitative result, the relevance of which relies on statistics. This limit, however, is inherent to all reporter gene studies; furthermore, the majority of currently published work on gene expression uses largely semi-quantitative data such as densitometric evaluation of Northern blots.

With the β-lactamase reporter gene, c-*fos* expression was studied in individual cells of the pituitary line AtT20. The releasing factor PACAP as well as serum caused a strong induction of c-*fos* reporter expression. Spontaneous Ca^{2+} transients caused by Ca^{2+} action potentials (21) which occur in a large fraction of the cells, sustained the basal rate of c-*fos*

[2] A. Maturana, G. Van Haasteren, I. Piuz, C. Castelbou, N. Demaurex, and W. Schlegel, unpublished data.

reporter transcription (Fig. 4). Mutation of the c-*fos*-promoter, which inactivates the SRE element, strongly reduced basal transcriptional activity; in contrast, an inactivating mutation on the CRE element had no effect (Fig. 5). Nuclear injection of a slowly diffusing Ca^{2+} chelator, BABTA-D70, strongly attenuated spontaneous as well as depolarization-induced nuclear Ca^{2+} transients measured with coinjected fura-D70. Nuclear BAPTA-D70 had no significant effect on basal c-*fos* reporter transcription, whereas it strongly reduced KCl-stimulated c-*fos*-βL because of sustained depolarization, consistent with the notion that cytoplasmic rather than nuclear Ca^{2+} signals are responsible for basal c-*fos* transcription (Figs. 6 and 7).

IEG transcription factors such as c-*fos* link cellular activity to gene expression. Transcription of IEGs is triggered by short lived intracellular signals that accompany cell activity; IEG transcription factors in turn control a multitude of cell type-specific genes, the transcription of which is adapted to cell activation. We show here a link between Ca^{2+} action potentials and basal c-*fos* transcription. Physiologically, such a link can provide for a precise transcriptional readout of a finely tuned phasic Ca^{2+} signal. Indeed, frequency, rhythm, and amplitude of Ca^{2+} action potentials are extremely well controlled by a multitude of factors that regulate hormone secretion by these same cells (34). It thus appears that secretory activity and immediate gene expression are sensitive to the same Ca^{2+} signals, which is a means for coordinate control.

What is the potential role of the "basal" expression of IEGs such as c-*fos* in non-stimulated neuroendocrine cells? In the pituitary, inhibitors such as somatostatin and dopamine play an important part in the regulation of hormone secretion. Their action is based largely on the suppression of spontaneous action potentials. Indeed, spontaneous action potentials are likely a rare physiological event for cells in an intact pituitary in which tonic inhibition by dopamine and adenosine predominate most of the time. In the AtT20 cell model, spontaneous Ca^{2+} transients that cause the basal c-*fos* expression just illustrate how IEG levels may reflect precisely the "activity status" of each individual cell.

The c-*fos* promoter element SRE appears essential to sustain a basal transcription of the c-*fos* gene because of action potential-linked Ca^{2+} transients in AtT20 cells. The transcription factors SRF and it's cofactor TCF (*i.e.* ELK-1) bind to the SRE element of the c-*fos* promoter. In response to serum or purified growth factors, the transcription activation by SRF is principally controlled by the ras-raf-ERK MAPkinase pathway or by rhoA GTPase-dependent mechanisms (35, 36).

The activation of SRF by Ca^{2+} signals was demonstrated with a c-*fos* reporter gene including a minimal promoter and a single SRE inserted 42 base pairs upstream of the transcription start site (16). Moreover, in a promoter lacking the SRE element, even in presence of the CRE the Ca^{2+}-activated transcription was significantly reduced. Activation of SRF by Ca^{2+} depends on CaMKinases, and not on the ras-raf-ERK pathway known to mediate Ca^{2+} activation of TCF in PC12 cells (37). In AtT20 cells the Ca^{2+}-dependent c-*fos* gene transcription activation via SRE appears to be TCF-independent (17). The expression of constitutively active CaMKinases II and IV in AtT20 cells (38) showed that CaMKinase IV repressed transcriptional activity of a c-*fos*-SRE-CAT reporter activity, whereas CaMKinase II activated a c-*fos*-SRE-CAT reporter transcription.

SRE-dependent transcription of the c-*fos* gene in AtT20 cells appears possible by Ca^{2+} signals of moderate amplitude that are limited to the cytosol, as is evident from our data as well as from an earlier study (5). It is therefore likely that Ca^{2+} transients are linked to c-fos transcription via CaMKinase II. This enzyme is located in the cytosol (8), and it's activity is modulated by the pattern of Ca^{2+} oscillations (39). A further potential mediator is the Ca^{2+}-activated tyrosine kinase PYK-2, a member of the non-receptor tyrosine kinase family Fak, which is highly expressed in brain (40). Ca^{2+}-activated PYK-2 leads to the activation of the Ras-MAP kinase signaling pathways.

Ca^{2+} signals outside the nucleus can lead to enhanced transcription also via CRE (41). This has been elegantly demonstrated by a recent study in neurons (47) where calmodulin associated to L-type voltage-gated Ca^{2+} channels and released upon Ca^{2+} flux through them is capable of causing the phosphorylation of CREB and thereby the activation of CRE-driven reporter gene transcription. Interestingly, this selective activation mechanism based on a very localized rise in Ca^{2+} involves the ERK-MAP kinases cascade.

For the spontaneous Ca^{2+} transients and "basal" c-*fos* transcription studied here, CRE does not seem to be an important target, as it can be inactivated by mutation without significant consequences. However, this finding does not exclude a certain contribution of an ERK-CREB-CRE pathway to the physiological regulation of steady-state c-*fos* mRNA.

In conclusion, this study shows how Ca^{2+} action potentials, which are a reflection of neuroendocrine cell activity even in a non-stimulated "basal" state, control transcription of the immediate early gene c-*fos* via cytoplasmic Ca^{2+} transients signaling to the SRE element in the c-*fos* promoter. In our view this mechanism is involved in the adaptation of gene expression to cell activity and may well apply to many more IEG transcription factors in all cells that show electrical excitability of their plasma membrane.

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