THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 29, Issue of October 15, pp. 13920-13927, 1987
Printed in U.S.A.


# Dopamine Inhibits Cytosolic $Ca^{2+}$ Increases in Rat Lactotroph Cells

## EVIDENCE OF A DUAL MECHANISM OF ACTION*

(Received for publication, March 30, 1987)

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**Single rat lactotroph cells were studied after loading with the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) indicator fura-2 either 1 or 3 days after cell dispersion. Under unstimulated conditions, two groups of lactotrophs were observed, the first (predominant at day 1) with large $[Ca^{2+}]_i$ fluctuations (peaks up to 300 nM) probably due to spontaneous action potentials and the second (predominant at 3 days) with stable $[Ca^{2+}]_i$ (values variable between 65 and 200 nM). The effect of dopamine on the resting $[Ca^{2+}]_i$ was different in the two groups. Even at high dopamine concentrations, no change occurred in the second group; whereas in the first, disappearance of fluctuations and marked decrease of $[Ca^{2+}]_i$ were observed. These effects of dopamine appear to be due to hyperpolarization that was demonstrated by the use of a specific fluorescent indicator, bis(oxonol). Two types of triggered $[Ca^{2+}]_i$ transients were studied in detail: those due to redistribution of $Ca^{2+}$ from the intracellular stores (induced by thyrotropin-releasing hormone) and those due to $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (induced by high $[K^+]$). Dopamine (1 μM) markedly inhibited both these transients by the action of D2 receptors (blocked by l-sulpiride and domperidone). All effects of dopamine were prevented by treatment of the cells with pertussis toxin, indicating the involvement of one (or more) GTP-binding protein(s). Another consequence of D2 receptor activation is the inhibition of adenylate cyclase. Treatments (cholera toxin, forskolin), known to raise cAMP levels, were found to dissociate the effects of dopamine on $[Ca^{2+}]_i$ inasmuch as they markedly relieved the inhibition of the redistributive transients by thyrotropin-releasing hormone but left hyperpolarization and inhibition of $K^+$ transients unaffected. The spectrum of intracellular signals elicited by the activation of D2 receptors is therefore complex and includes at least two mechanisms that involve $[Ca^{2+}]_i$, one related and the other independent of the decrease of cAMP levels.**

Inhibition by dopamine, released by hypothalamic neurons to the hypophyseal portal circulation, is known to be the major control mechanism of PRL[1] secretion at the LC of the anterior pituitary (1). LC are endowed with a D2-type dopamine receptor that previous studies from our as well as other laboratories have demonstrated to be negatively coupled to adenylate cyclase (2–4). Whether this mechanism is responsible for the entire effect of dopamine is, however, doubtful because (i) dopamine remains active in cells with high levels of cAMP (5, 6), and (ii) results have been reported suggesting a role of $Ca^{2+}$ in the dopamine effect (5–9). Such a role, however, has not been demonstrated directly yet because, so far, measurements of $[Ca^{2+}]_i$ have been carried out only on mixtures of dissociated anterior pituitary cells (10, 11) that include LC together with other endocrine cell types, some of which are sensitive to dopamine (1). Other studies were performed on rat tumoral cell lines ($GH_3$ and $GH_4$) that secrete PRL, growth hormone, or both, but do not express dopamine receptors (12–17). In order to investigate the effects of D2 receptor activation on $[Ca^{2+}]_i$ homeostasis, highly purified rat LC preparations were studied by microfluorometry at the single cell level after loading with the fluorescent indicator fura-2 (18). The results obtained demonstrated that dopamine induces a marked inhibition of both the resting $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ transients triggered by two different mechanisms: redistribution from the intracellular $Ca^{2+}$ stores and stimulation of $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels. Evidence is presented indicating that these effects of dopamine are due to at least two intracellular mechanisms that appear to operate in parallel.

## EXPERIMENTAL PROCEDURES

*Preparation of LC*—Anterior pituitaries from 10 female Wistar rats weighing 250–300 g were dissociated enzymatically (19), applied over an albumin (0.3–2.4%) gradient (prepared according to a slight modification of Ref. 19), and sedimented at 1 × *g* for 3.2 h at room temperature. Nine 100-ml fractions were collected. The cells of the LC- and somatotroph cell-enriched fractions (fractions 5 and 7–9, respectively) were attached to glass coverslips coated with polyornithine. For immunofluorescence, cells were fixed with 4% paraformaldehyde in phosphate buffer (1 h, 0 °C) and then permeabilized with Triton X-100 (0.1%) in phosphate-buffered saline, washed with phosphate-buffered saline, reacted with the specific antibodies (either anti-PRL or anti-growth hormone antibodies (20); 1 h, room temperature), washed repeatedly with hypertonic phosphate-buffered saline containing bovine serum albumin, and finally decorated with rhodamine-labeled anti-Ig antibodies. The other coverslips were cultured in Dulbecco's modified Eagle's medium supplemented with 12% fetal

* This work was supported in part by Special Project Oncology Grant 85.02148.44.115.04912 from the Cosiglio Nazionale delle Ricerche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PRL, prolactin; LC, lactotroph cells; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; PTx, pertussis toxin; TRH, thyrotropin-releasing hormone; EGTA, [ethylenebis(oxy-ethylenenitrilo)]tetraacetic acid.

calf serum for either 1 or 3 days and then rinsed with serum-free Dulbecco's modified Eagle's medium and used for the measurement of $[Ca^{2+}]_i$ and membrane potential. In a few experiments, part of the cells were not attached to coverslips but were maintained in spinner culture for 24 h, after which they were used for $[Ca^{2+}]_i$ measurement in cell suspension.

*Measurement of $[Ca^{2+}]_i$*—Cell monolayers were covered with 0.5 ml of Dulbecco's modified Eagle's medium that had been mixed with 0.1% volume of fura-2/acetoxymethyl ester in dimethyl sulfoxide (final dye concentration, 2 $\mu$M). The cell loading incubation was carried out at 37 °C for 30 min. Spectrofluorometric analysis of the monolayers (carried out as described in Ref. 21) revealed that under these conditions, the fluorochrome undergoes extensive cleavage to yield the intracellularly trapped probe, fura-2-free acid (18). The latter appeared uniformly distributed within the cytosol when the cells were appropriately viewed in the fluorescence microscope. Loading of LC suspensions with fura-2/acetoxymethyl ester was carried out similarly to monolayers but in small Eppendorf tubes.

$[Ca^{2+}]_i$ measurements were carried out on either cell suspensions in the cuvette of a conventional fluorometer or single cells in a microscopic fluorometer. The technique used for the cuvette measurements has been described (22). For microscopic measurements, fura-2-loaded cells were positioned (facing downward) in a thermostated (32 °C) chamber (1 ml) sitting on the stage of a fluorescence microscope (Zeiss photomicroscope III, objective Zeiss Plan Neofluar 40X) equipped with a device to alternate quickly two narrow-band filters (340 and 380 nm; Oriel) in the pathway of the exciting beam, as well as with a photomultiplier (Zeiss PMT system) connected to an A-D converter interfaced to a personal computer. The emitted fluorescence, before feeding into the photomultiplier, was filtered through a cutoff filter (490 nm).

The field of analysis (5–20 $\mu$m of diameter) was delimited by a pinhole diaphragm placed in the image plane in front of the photomultiplier. The personal computer controlled filter alterations and calculated 340/380 ratios from light intensities (subtracted from background) averaged over excitation periods (0.5–2 s) at each of the two wavelengths. Incubations were carried out in medium containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM Hepes/NaOH, pH 7.4, 6 mM glucose. Additions of test substances were made directly into the chamber. At appropriate times, the chamber was washed with large volumes of warm medium (5–10 ml/min). Data were stored in sequential files. At the end of each experiment, they were plotted and calibrated in terms of $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz *et al.* (18).

*Measurement of Membrane Potential*—LC monolayers on glass coverslips were placed in the microscope stage as described above for $[Ca^{2+}]_i$ measurements and loaded with bis(oxonol) (23) and mixed in the medium in the incubation chamber at the final concentration of 100 nM. After 5 min at 32 °C, the chamber was washed, and the cells were exposed to various treatments. Fluorescence (excited at 546 nm) was recorded from single cells and expressed in arbitrary units.

*Materials*—Fura-2 and fura-2/acetoxymethyl ester were purchased from Molecular Probes (Junction City, OR). Anti-growth hormone and PRL antibodies were the kind gift of Dr. A. Zanini (Consiglio Nazionale delle Richerche, Milano, Italy), affinity-purified PTx of Dr. R. Rappuoli (Sclavo, Siena, Italy), bis(oxonol) of Dr. R. Y. Tsien (University of California, Berkley, CA), and cholera toxin of Dr. M. Tomasi (I.S.S., Roma, Italy). TRH, dopamine, verapamil, sulpiride, forskolin, and polyornithine were from Sigma; domperidone was from Janssen Pharmaceutica (Beerse, Belgium). Culture media were from Flow Laboratories (Mackenheim, West Germany); all other chemicals were reagent-grade.

## RESULTS

*Somatotroph Cells*—The cells of fractions 7–9, shown by immunofluorescence to be primarily (over 80%, four preparations) somatotrophs, were loaded with fura-2, and their $[Ca^{2+}]_i$ were studied in the microfluorometer both before and after various treatments. Only a short account of these results will be given here (not illustrated in the figures) to serve as a reference to the data on LC. Fluctuations $[Ca^{2+}]_i$ of unstimulated somatotrophs were usually (10 out of 12 cells) similar to those described below for a fraction of the LC population. These fluctuations subsided when the cells were treated with verapamil ($10^{-5}$ M) or incubated with an excess of EGTA. Neither TRH (100 nM) nor dopamine (10 $\mu$M) induced appreciable effects on the $[Ca^{2+}]_i$ in any of the cells investigated. In contrast, these cells responded to the application of somatostatin (0.1 $\mu$M) with the loss of fluctuations and decrease of average $[Ca^{2+}]_i$.

*Unstimulated LC*—Immunofluorescence of dissociated cells separated by the albumin gradient sedimentation (four preparations) revealed that in fraction 5, the vast majority (80–90%) of the cells were LC (Fig. 1), the contaminants being somatotroph cells (not shown). As the latter fail to respond to TRH (Ref. 24 and result above), the ability to respond to this neurohormone was taken as an additional criterion for LC identification. All cells investigated for $[Ca^{2+}]_i$ measurements were therefore treated at least once with TRH (100 nM), and only those that responded with the typical large TRH-induced transient (see below) were considered.

$[Ca^{2+}]_i$ measurements carried out 1 and 3 days after cell dispersion yielded different results. At day 1, the majority of the cells analyzed (22 out of 30) exhibited an unstable resting $[Ca^{2+}]_i$, fluctuating from values of 60–100 nM up to 260–300 nM (mean ± S.D.: 161 ± 51 nM). By increasing the time resolution of the measurements (one 340:380 nm ratio/s), the fluctuations were shown to be due to discrete, rhythmic oscillations of $[Ca^{2+}]_i$, each lasting several seconds (Fig. 2*A*). The fluctuations rapidly disappeared when extracellular $[Ca^{2+}]_i$ was chelated with excess EGTA or voltage-gated $Ca^{2+}$ channels were blocked by verapamil. Following either of these treatments, $[Ca^{2+}]_i$ of LC became stable at values in the 60–90 nM range (Fig. 2, *B* and *D*). These results indicate that the fluctuations are due to $Ca^{2+}$ influx from the medium. In the rest of the LC analyzed at day 1, $[Ca^{2+}]_i$ was stable, *i.e.* with fluctuations around 20–30 nM or less. In the cell population analyzed at day 3, the resting $[Ca^{2+}]_i$ could be classified in the same two groups, but the relative proportion was markedly changed. Thus, almost all (75 out of 80) LC exhibited a stable $[Ca^{2+}]_i$. The measured values were quite variable from cell to cell, ranging from 60 to 260 nM (mean ± S.D.: 143 ± 40 mM). In these cells with stable $[Ca^{2+}]_i$, verapamil had no effect (Fig. 2*E*), and chelation of extracellular $Ca^{2+}$ with EGTA induced only a slow decline of $[Ca^{2+}]_i$ (not shown). Only in very few (5 out of 80) LC analyzed at day 3 were fluctuations observed that resembled those predominating at day 1 with regard to their sensitivity to verapamil and excess EGTA.

Treatment with dopamine (1 $\mu$M) was found to affect differently the resting $[Ca^{2+}]_i$ of the two groups of LC described above. As shown in Fig. 2*C*, in the cells of the first group, catecholamine caused the rapid conversion of the fluctuating

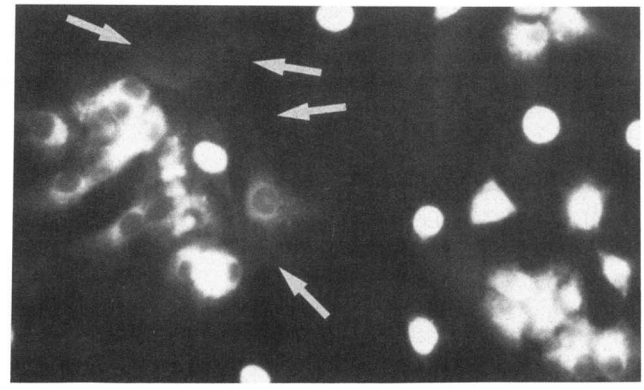

FIG. 1. **Indirect anti-PRL immunofluorescence in the LC-enriched fraction separated by albumin gradient (fraction 5).** The field illustrates the typical appearance of highly fluorescent LC. Fluorescence-negative contaminants (4 out of 36 cells) are indicated by *arrows*. Magnification × 550.

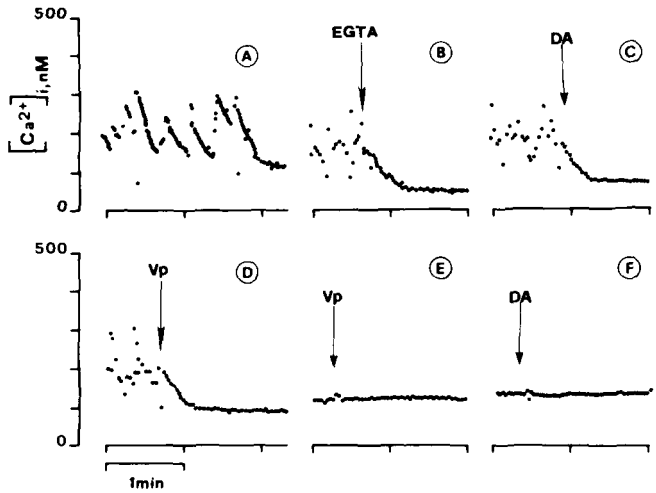


FIG. 2. **Resting $[Ca^{2+}]_i$ in single LC.** Cells were loaded with fura-2 and analyzed by the microspectrofluorometric technique described in detail under "Experimental Procedures." *Symbols* correspond to individual values calculated from the 340:380 nm ratios measured every 1 s (*A*) or 2 s (*B–F*). The traces shown in *A–D* were obtained in cells maintained in culture for 1 day, and those of *E–F* in cells cultured for 3 days. Where indicated, the various agents were added at the following final concentrations: verapamil (*Vp*), 10 μM; EGTA, 3 mM; and dopamine (*DA*), 1 μM.

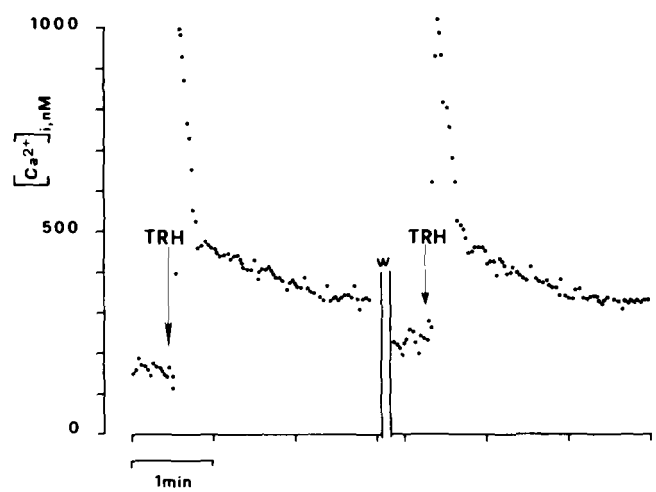


FIG. 3. **TRH-induced $[Ca^{2+}]_i$ transients in single LC.** The fura-2-loaded cell was analyzed as described in the legend to Fig. 2. TRH was used at 100 nM. Between the two consecutive TRH stimulations, the cell was washed with warm medium (5 ml/min) for 5 min (*W*).

to the stable pattern, with the disappearance of the peaks and a decrease in $[Ca^{2+}]_i$ to values approaching those observed after verapamil. It should be mentioned, however, that a small inhibitory effect of dopamine on $[Ca^{2+}]_i$ remained appreciable even after applications of an optimal concentration (10 μM) of verapamil. In contrast, in the cells showing stable $[Ca^{2+}]_i$ from the very beginning, dopamine failed to cause any detectable decrease (Fig. 2*F*).

In a few experiments (not shown), LC that had been maintained in suspension for 1 day were analyzed in a conventional fluorometer cuvette. The results obtained were consistent with those on single cells as suspensions showed comparable resting $[Ca^{2+}]_i$ (mean ± S.D.: 210 ± 40 nM; $n = 5$) that were substantially decreased by treatment with dopamine.

*THR- and $K^+$-induced $[Ca^{2+}]_i$ Transients*—TRH, applied in the $Ca^{2+}$-containing medium, caused, in most (50 out of 62) cells of the LC-enriched fraction 5, typical biphasic $[Ca^{2+}]_i$ transients composed by a rapid increase, followed within a few seconds by a decrease to a lower and sustained plateau that was maintained for many minutes (Fig. 3). In a population of 50 cells, the initial $[Ca^{2+}]_i$ peak induced by 100 nM TRH was quite variable, ranging from 0.65 μM to values approaching dye saturation around 20 μM (mean ± S.D.: 6.7 ± 6 μM). Repeated (up to three) exposures of the same cell to the same concentration of TRH after 5–10 min of washing yielded $[Ca^{2+}]_i$ increases that were almost identical (difference <10%) in size and time course (Fig. 3). Also, the plateau that followed the initial TRH-induced peak was quite variable in the cell population (200–650 nM; mean ± S.D.: 430 ± 150 nM; $n = 40$), but highly reproducible in individual cells. In a group of cells (4 out of a total of 44), the plateau was not seen, but was replaced by $[Ca^{2+}]_i$ fluctuations of various size and frequency that, in some cases, reached values as high as 1 μM. These fluctuations slowly subsided when TRH was washed away, leaving a plateau only slightly above the resting level (not shown).

When TRH (100 nM) was applied in a $Ca^{2+}$-free medium (containing 3 mM EGTA), the initial $[Ca^{2+}]_i$ peak was largely maintained (mean ± S.D.: 5.3 ± 4.8 μM; $n = 32$), but the subsequent plateau (or the fluctuations) was not seen (Fig. 4*A*). A similar result was obtained when the cells were exposed to the $Ca^{2+}$ channel blocker verapamil (10 μM) before exposure to TRH (not shown). On the other hand, when EGTA and verapamil were applied after TRH, they caused the $[Ca^{2+}]_i$ plateau to dissipate rapidly (Fig. 4, *B* and *C*). Taken together, the results with TRH indicate that the $[Ca^{2+}]_i$ transient induced by the neurohormone has two components. The initial peak appears to be due primarily to redistribution from

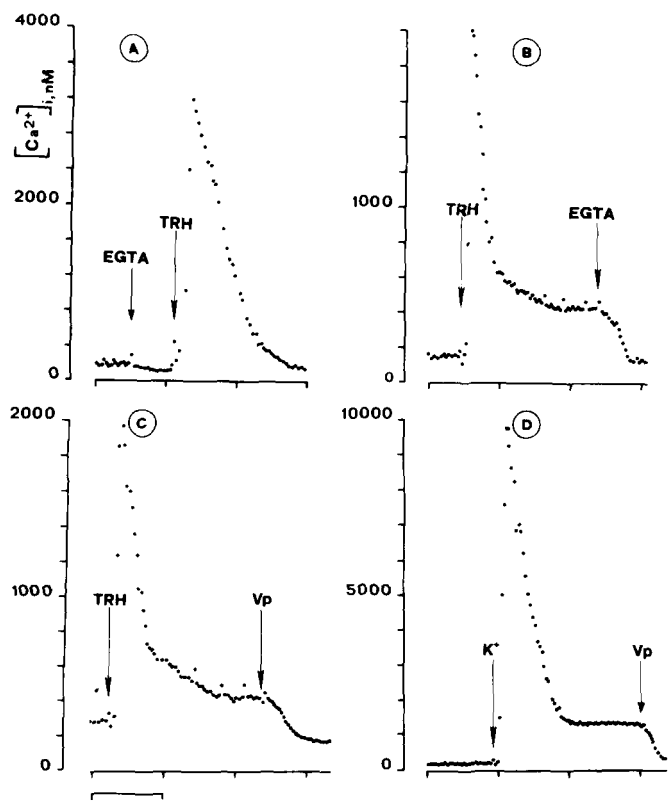


FIG. 4. **Effects of EGTA and verapamil on TRH- and $K^+$-induced $[Ca^{2+}]_i$ increases.** $[Ca^{2+}]_i$ was monitored from single fura-2-loaded LC as described in the legend to Fig. 2. EGTA (3 mM) was added either before (*A*) or after (*B*). TRH. Verapamil (*Vp*) applied at 10 μM after TRH (*C*) or $K^+$ (*D*) stimulation. TRH and $K^+$ concentrations were 100 nM and 55 mM, respectively.

intracellular stores, whereas the subsequent plateau and the fluctuations depend on influx from the extracellular space, most probably through voltage-gated $Ca^{2+}$ channels.

$[Ca^{2+}]_i$ transients could be induced in LC also by depolarization. Switching of extracellular free $[K^+]$ from 5 to 55 mM caused $[Ca^{2+}]_i$ to rise abruptly to values even higher than those induced by TRH (mean ± S.D.: 11.3 ± 11 μM; range: 1 μM to dye saturation; $n = 20$). The initial spike was followed by a plateau (mean ± S.D.: 720 ± 420 nM). These increases were eliminated or quickly interrupted by EGTA or verapamil applied either before or after high $[K^+]$ (Fig. 3*D* and data not shown). Similar to those by TRH, the $[Ca^{2+}]_i$ transients induced by $K^+$ were reproducible in a single cell.

*Dopamine Inhibition of TRH- and $K^+$-induced $[Ca^{2+}]_i$ Transients in LC*—The effects of dopamine on the $[Ca^{2+}]_i$ increases induced by either TRH or high $[K^+]$ are shown in Fig. 5 and Table I. Because of the variability of both resting and stimulated $[Ca^{2+}]_i$ in different cells and, in contrast, the reproducibility in individual cells, the effects of dopamine are expressed in terms of percent inhibition with respect to a previous transient induced in the same cell by the stimulatory agent without dopamine (Table I). Since the results obtained on cells cultured for 1 or 3 days were similar, they will be described together.

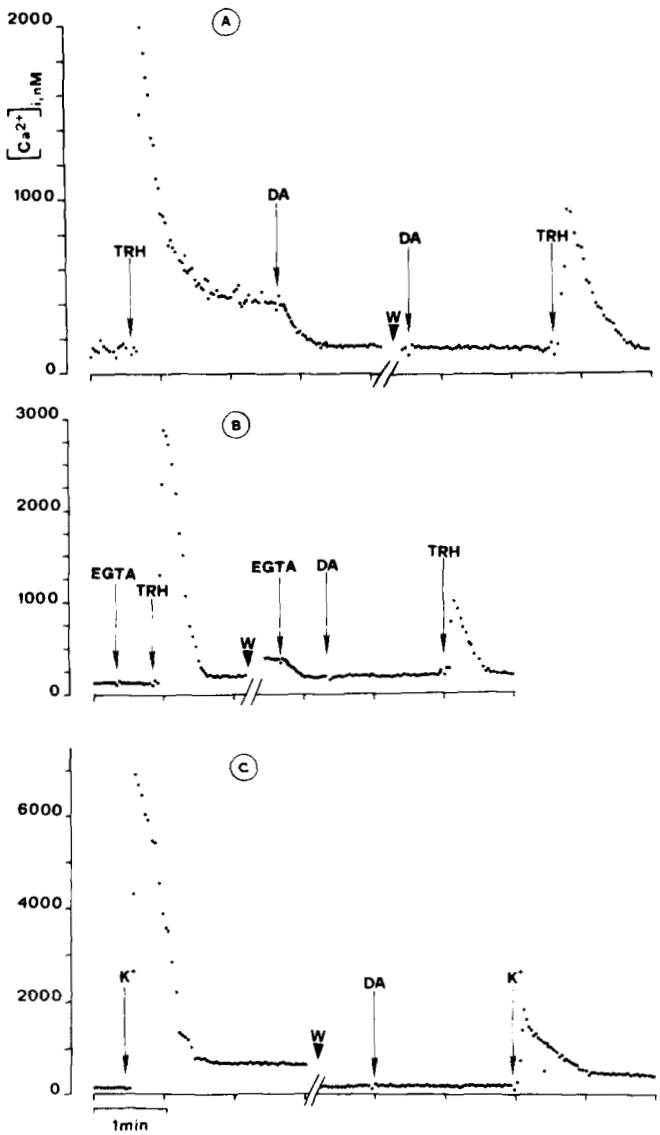


FIG. 5. **Dopamine inhibition of TRH- and $K^+$-induced $[Ca^{2+}]_i$ transients.** Single fura-2-loaded LC were analyzed as described in the legend to Fig. 2. *A*, dopamine (*DA*) either after or before TRH stimulation; *B*, dopamine followed by TRH in the presence of EGTA; *C*, dopamine followed by $K^+$. Dopamine was used at 1 μM; other concentrations were as described in the legend to Fig. 4. Where indicated (*W*), the chamber was washed with warm medium (5 min, 5 ml/min).

Dopamine (1 μM), administered in the $Ca^{2+}$-containing medium before TRH, caused a substantial inhibition of both the $[Ca^{2+}]_i$ peak and the plateau phases induced by the neurohormone (Fig. 5*A*) (mean: −80%, range: 70–90%; and mean: −96%, range: 80–100%, respectively; $n = 10$). The inhibition of the plateau was also observed when dopamine was applied after TRH (Fig. 5*A* and Table I). In order to define in more detail the inhibitory effect of dopamine on stimulated $Ca^{2+}$ homeostasis, catecholamine was applied in the conditions in which $[Ca^{2+}]_i$ transients depend exclusively on either intracellular redistribution (TRH applied in the $Ca^{2+}$-free/EGTA medium) or $Ca^{2+}$ influx (high $[K^+]$). In either case, dopamine proved to maintain its inhibitory effect (Fig. 5, *B* and *C*, and Table I).

*Dopamine-induced Hyperpolarization of LC*—The effects of dopamine on the depolarization-induced $Ca^{2+}$ influx, revealed by the results with both TRH and $K^+$, could be due to various mechanisms. In particular, a direct inhibition of voltage-gated $Ca^{2+}$ channels or an indirect effect mediated by a change of the LC membrane potential can be envisaged. Changes of membrane potential can be qualitatively indicated by specific fluorescent probes, such as bis(oxonol) (23). When individual LC loaded with this probe were treated with dopamine in the microspectrofluorometer, their fluorescence decreased markedly, indicating hyperpolarization (Fig. 6*A*). Such an effect was reversible, as washing of the dopamine-hyperpolarized cells resulted in the return of fluorescence to the resting level.

*Dopamine Effects on $[Ca^{2+}]_i$ Are Mediated by the D2 Receptor*—Extensive evidence indicates that LC cells are endowed with a dopamine receptor of the D2 type (1) which is responsible for all the effects of catecholamine reported up to now. In order to establish whether the dopamine effects on $[Ca^{2+}]_i$ homeostasis now identified are also mediated by this receptor, pharmacological experiments were carried out by the use of selective D2 antagonists, l-sulpiride and domperidone (25, 26). Sulpiride (Table I) and domperidone (not shown) administered at concentrations as low as 0.1 and 1 μM, respectively, nearly completely abolished the inhibitory effects of dopamine on both the TRH-induced $Ca^{2+}$ redistribution and the $K^+$-induced $Ca^{2+}$ influx.

*Involvement of PTx-sensitive G Protein(s) in Dopamine Action*—The responses elicited by the activation of various types of inhibitory receptors, including somatostatin receptors in $GH_3$ cells (15, 27), have been reported to be blocked by pretreatment of the cells with PTx, a toxin that causes the ADP-ribosylation of various G proteins, such as $G_i$ and $G_o$ (28).

In rat LC, treatment with PTx (1 μg/ml for 4 h) was unable to affect the $[Ca^{2+}]_i$ transients induced by TRH (Fig. 7) or $K^+$ (not shown), as previously observed in $GH_3$ cells (15). In contrast, the dopamine-induced inhibition of the TRH peak, the TRH plateau, and the $K^+$ transient were all severely curtailed (Fig. 7*A* and Table I). Likewise, PTx caused the disappearance of the plasma membrane hyperpolarization induced by dopamine (Fig. 6*B*). When the concentration dependence of the effect on dopamine inhibition was investigated, the toxin proved to be approximately equipotent in releasing the dopamine inhibition of either type of $[Ca^{2+}]_i$ transient: by TRH in the $Ca^{2+}$-free/EGTA medium and by

TABLE I

*Dopamine inhibition of TRH- and $K^+$-induced $[Ca^{2+}]_i$ transients in single LC*

$[Ca^{2+}]_i$ was monitored from single LC as described in the legend to Fig. 2. The concentrations used were 100 nM TRH, 1 μM dopamine, 55 mM $K^+$, 1 μg/ml cholera toxin (4 h), 1 μg/ml PTx (4 h), 10 μM forskolin (5 min), and 0.1 μM l-sulpiride. Values shown are means, with ranges shown in parentheses.

| Treatment | Inhibition with respect to a previous transient | | |
|---|---|---|---|
| | TRH-induced $[Ca^{2+}]_i$ transients | | Peak of $K^+$-induced $[Ca^{2+}]_i$ transients[c] |
| | Peak[a] | Plateau[b] | |
| | % | | |
| Dopamine | 71 (60–85; $n = 20$) | 95 (80–100; $n = 10$) | 65 (60–100; $n = 15$) |
| Dopamine + cholera toxin | 10 (0–20; $n = 4$) | 88 (68–100; $n = 5$) | 60 (60–95; $n = 6$) |
| Dopamine + forskolin | 10 (0–20; $n = 4$) | 90 (80–100; $n = 3$) | |
| Dopamine + pertussis toxin | 22 (0–30; $n = 10$) | 30 (20–50; $n = 8$) | 24 (0–40; $n = 20$) |
| Dopamine + sulpiride | 10 (0–30; $n = 5$) | 30 (10–50; $n = 10$) | 17 (10–30; $n = 5$) |

[a] Dopamine followed by TRH in the presence of 3 mM EGTA.
[b] Dopamine after TRH in $Ca^{2+}$-containing medium.
[c] Dopamine followed by $K^+$ in $Ca^{2+}$-containing medium.

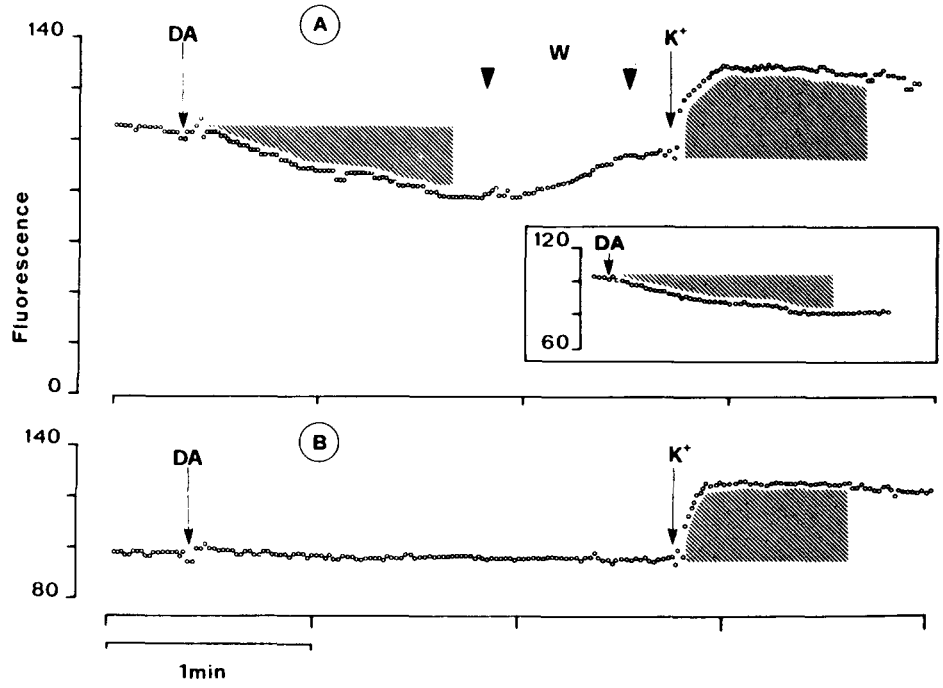


FIG. 6. **Changes of LC membrane potential by dopamine and high $[K^+]$.** Cells were loaded with bis(oxonol) as described under "Experimental Procedures." Fluorescence is expressed in arbitrary units. The cell shown in *A* was studied without prior treatment, that shown in the *inset* was pretreated with cholera toxin (1 μg/ml, 4 h), and that shown in *B* was pretreated with PTx (1 μg/ml, 4 h). Other details are as described in the legend to Fig. 5.

$K^+$ in the $Ca^{2+}$-containing medium (not shown).

*cAMP-elevating Agents Prevent Dopamine Inhibition of Triggered $Ca^{2+}$ Redistribution*—A well-known effect of D2 activation in pituitary LC is the inhibition of adenylate cyclase, with ensuing marked decrease of the cAMP concentration (2–4, 19). In order to establish whether and to what extent this latter effect is related to the inhibition of $[Ca^{2+}]_i$ transients we have observed, treatments that cause elevation of cAMP were given to cultured LC prior to exposure to dopamine, followed by TRH or $K^+$. The results obtained are illustrated in Fig. 7*B* and Table I.

Treatment with forskolin (10 μM) or preincubation of the cells with cholera toxin (1 μg/ml for 4 h) induced a clear dissociation of the inhibiting effects induced by dopamine on triggered $[Ca^{2+}]_i$ transients. The inhibition of the initial redistributive TRH peak was almost completely released by either one of the cAMP-elevating treatments. In contrast, the $[Ca^{2+}]_i$ increases sustained by stimulated influx, *i.e.* those induced by high $[K^+]$ and the TRH-induced plateaus, were almost entirely unaffected; and the same occurred with the hyperpolarization measured with bis(oxonol) (Fig. 6*A*, *inset*).

## DISCUSSION

Most of the studies on the intracellular signals generated in LC by D2 receptor activation were carried out so far on experimental preparations that suffer distinct limitations. These preparations include mixtures of cells dissociated from animal anterior pituitary tissue or human pituitary tumors (prolactinomas) and pituitary cell lines (*i.e.* various subclones of the rat tumoral GH lines). LC represent a considerable, but still a minor component of the anterior pituitary cell population. Therefore, any attempts made in tissue or cell mixtures to correlate PRL release with receptor-triggered intracellular signals is faced with the problem that release occurs from one cell type only (the LC), whereas signals can be generated in other types as well. In this respect, it should be emphasized that not only LC, but also thyrotroph and somatotroph cells, are endowed with dopamine receptors (1). Prolactinomas and tumor cell lines, on the other hand, although satisfactory from the point of view of homogeneity, might be inadequate because of their variable (if not lacking) sensitivity to dopamine (1, 29). These considerations emphasize the need to use bona fide LC to investigate the role of $[Ca^{2+}]_i$ in the inhibitory control of PRL release by dopamine.

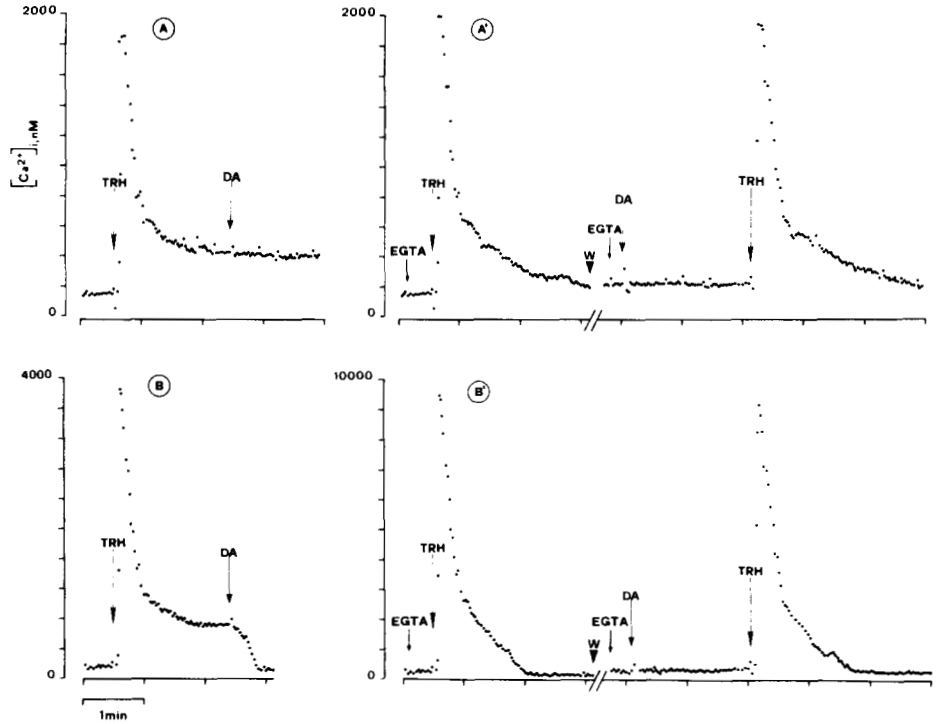


FIG. 7. **Effects of PTx (*A* and *A'*) and cholera toxin (*B* and *B'*) on dopamine inhibition of TRH-induced $[Ca^{2+}]_i$ transients.** Toxin treatment was carried out as described in the legend to Fig. 6. Fura-2-loaded LC were exposed to dopamine after the application of TRH in $Ca^{2+}$-containing medium (*A* and *B*) or before stimulation by TRH in the presence of EGTA (*A'* and *B'*). Other details are as described in the legends to Figs. 2 and 5.

This has been done for the first time in this work by a combination of two techniques: isolation of a cell preparation highly enriched in LC, followed by selection of the cells markedly responsive to TRH. Since the contaminants (<20% of the total) present in the cell preparation are somatotrophs, which are not responsive to TRH (Ref. 24 and the present results), this additional test makes sure that all cells we have investigated are true LC.

*Resting $[Ca^{2+}]_i$*—In the LC population we have analyzed, two groups were identified: one with fluctuating $[Ca^{2}]_i$ and the other with stable resting $[Ca^{2+}]_i$. Fluctuations were due to discrete oscillation of $[Ca^{2+}]_i$ that might be the consequence of spontaneous, $Ca^{2+}$-dependent action potentials. Spontaneous action potentials sustained in part by $Ca^{2+}$ were observed by electrophysiology first in the LC of a teleost fish (7) and recently in 40% of a population of bovine LC (30). Oscillations of $[Ca^{2+}]_i$ are not a property of LC only, but have been observed also in $GH_3$[2] and rat somatotroph cells (this work).[2] In these two cell types, the oscillations of $[Ca^{2+}]_i$ were found to subside after the application of somatostatin.[2] Spontaneous fluctuations in $[Ca^{2+}]_i$ appear to be, however, not general among neurosecretory cells; for example, in unstimulated bovine chromaffin cells and PC12 cells, stable $[Ca^{2+}]_i$ has been reported (31). The fluctuations we have observed in LC were of considerable size and might therefore be important to maintain the high rate of basal secretion typical of these cells. This would be consistent with recent studies (32) in which spontaneous PRL release was investigated at the single LC level by the hemolytic plaque assay and found to be quite variable, *i.e.* high in some cells (that might correspond to those with fluctuating $[Ca^{2+}]_i$) and low in others. The proportion of cells with stable resting $[Ca^{2+}]_i$ increased markedly from 1 to 3 days in culture, whereas the responses to TRH and $K^+$ were not modified. The reason(s) underlying the changed behavior of unstimulated LC during culture is not clear. In any case, such a change was convenient for our studies because the analysis of the triggered transients, their inhibition by dopamine, and the effect of the other treatments was much easier in the stable than in the fluctuating cell population.

*$[Ca^{2+}]_i$ Transients Induced by TRH and $K^+$*—Application of dopamine was found to affect markedly the $[Ca^{2+}]_i$ transients induced by the neurohormone TRH and by depolarizing concentration of $K^+$. As the effects of these two treatments have never been investigated in LC, they were characterized in some detail. Similar to the results previously reported in $GH_3$ cells (12, 17), the TRH-induced transients were found to be biphasic, with an initial peak predominantly due to redistribution from intracellular $Ca^{2+}$ stores and a secondary phase due to $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels. Although highly reproducible in individual cells, the two phases, and particularly the second, were variable in the cell population. Previous extensive studies (33–35) indicate that in $GH_3$ cells, the initial $[Ca^{2+}]_i$ peak is due to TRH-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate with generation of inositol-1,4,5-trisphosphate, an intracellular messenger that induces release of $Ca^{2+}$ from a "microsomal" store. Preliminary results[3] from our laboratory indicate that this sequence of events is induced by TRH in rat LC as well. As far as the second phase of the TRH-induced $[Ca^{2+}]_i$ transient, its variability has already been reported by Albert and Tashjian (13) when studying the populations of $GH_3$ cells endowed with different concentrations of TRH receptors. In addition, our present data revealed in a small proportion (10%) of the analyzed LC population the occurrence of $[Ca^{2+}]_i$ fluctuations. Such $[Ca^{2+}]_i$ fluctuations appear totally consistent with TRH-evoked action potentials described first in fish LC (7) and recently in a fraction of bovine LC studied by intracellular recording (30). At variance with our present results in LC, Albert and Tashjian (13), working on $GH_3$ cells, failed to observe fluctuations of $[Ca^{2+}]_i$ after administration of TRH. This observation might not be due to a real difference between $GH_3$ and LC, but to the greater resolution of the single cell technique used in the present work with respect to the con-

[2] W. Schlegel, personal communication.

[3] L. Vallar and J. Meldolesi, unpublished data.

ventional fluorometric technique used by Albert and Tashjian (13), in which the fluorescence signal is averaged over a large population of cells.

On the other hand, the $[Ca^{2+}]_i$ transients induced by high $[K^+]$ were found to be prevented by both withdrawal of $Ca^{2+}$ from the incubation medium and treatment with the $Ca^{2+}$ channel blocker verapamil. These transients appear therefore to be due to depolarization-induced activation of voltage-gated $Ca^{2+}$ channels.

*Effects of Dopamine on $[Ca^{2+}]_i$ Homeostasis*—Dopamine was found to have profound effects on $[Ca^{2+}]_i$ homeostasis. Two processes ($Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels and $Ca^{2+}$ redistribution from intracellular stores) were found to be inhibited by apparently independent mechanisms. Inhibition of $Ca^{2+}$ influx appears to be responsible for 1) the disappearance of spontaneous $Ca^{2+}$ fluctuations and 2) the inhibition of both the depolarization (high $[K^+]$)-induced transients and the TRH-induced plateaus. Our data with the membrane potential indicator bis(oxonol) as well as electrophysiological results (8, 30) suggest that the effects on $Ca^{2+}$ influx occur as a consequence of a dopamine-induced hyperpolarization, which in turn might depend on the activation of a $K^+$ conductance (8). A decrease in resting $[Ca^{2+}]_i$ induced by dopamine had already been reported by Schofield (10), who worked, however, on a mixed population of dissociated anterior pituitary cells. Our results demonstrate that this effect is due to the subpopulation of LC characterized by fluctuating $[Ca^{2+}]_i$. In this respect, it is interesting that the inhibition of spontaneous PRL release by dopamine has been found recently to be selective for a subpopulation of LC with high secretory activity (32). The effect of dopamine on $Ca^{2+}$ influx resembles markedly that of somatostatin, previously characterized in detail in $GH_3$ cells (15, 36).

Dopamine inhibition of $Ca^{2+}$ redistribution was demonstrated in the experiments with TRH applied in the $Ca^{2+}$-free medium. The site of action of catecholamine has not been identified yet. Dopamine could act by inhibiting the hydrolysis of polyphosphoinositides, in particular the generation of inositol 1,4,5-trisphosphate (37, 38); alternatively, the functioning of inositol 1,4,5-trisphosphate at the intracellular $Ca^{2+}$ store could be inhibited by dopamine.

The mechanism by which dopamine induced its inhibitory effects were investigated by employing PTx and the cAMP-elevating raising agents. The complete inhibition of dopamine effects brought about by PTx demonstrates the existence of G protein-mediated coupling(s) at the receptor level. $G_i$, the mediator of receptor-triggered inhibition of adenylate cyclase and a known substrate of PTx, could be indirectly responsible also for the inhibition of $Ca^{2+}$ redistribution induced by dopamine because this effect was no longer seen in the cells pretreated with either forskolin or cholera toxin. The precise site of interaction between the cAMP and the inositol 1,4,5-trisphosphate messenger systems (whether at the level of polyphosphoinositide hydrolysis or of $Ca^{2+}$ release from the intracellular store or other) remains to be elucidated. In contrast to the effects on $Ca^{2+}$ distribution, the effects of dopamine on membrane potential and $Ca^{2+}$ influx were found to be unaffected by cAMP-elevating agents. In this case, the possibility of a G protein-mediated coupling of the dopamine receptor to a $K^+$ channel, as has been suggested for other receptors (39–41), should be considered. We conclude that although the precise scenario of the events triggered by the activation of the D2 receptor in LC is still in the process of being unraveled, the available evidence already indicates not only the involvement of two second messengers ($Ca^{2+}$ and cAMP), but also the triggering of at least two apparently separate mechanisms: one dependent and the other independent of cAMP. The complexity of the signal array triggered by dopamine in LC appears to match important physiological needs. Secretion of PRL is known, in fact, to be stimulated by various messengers ($Ca^{2+}$, cAMP, diacylglycerol) acting not independently from one another, but in a concerted fashion to overcome the inhibitory dopaminergic tone (5). The multiple intracellular events elicited by the activation of the D2 receptor appear therefore as an efficient and economical way to keep PRL secretion under continuous and accurate control.

*Acknowledgments*—We are grateful to G. Giannattasio, M. Bassetti, and A. Pandiella for participating in part of this work and for helpful suggestions and discussions, to C. Denef and E. Wanke for advice, and to R. Rappuoli and R. Y. Tsien for the kind gifts of PTx and bis(oxonol), respectively.

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