Mobilization of extracellular Ca^{2+} by prostaglandin $\text{F}_{2\alpha}$ can be modulated by fluoride in 3T3-L1 fibroblasts

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Changes in the intracellular concentration of calcium ($[\text{Ca}^{2+}]_i$) have been shown to mediate the physiological effects of certain agonists. Ca^{2+} mobilization occurs through multiple mechanisms which involve both influx and internal release of Ca^{2+} . Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) caused a transient mobilization of intracellular Ca^{2+} in 3T3-L1 fibroblasts. This effect was characterized by fluorescence measurements of trypsin-treated cells loaded with fura-2/AM. In the absence of extracellular Ca^{2+} , the peak amount of Ca^{2+} mobilized by $\text{PGF}_{2\alpha}$ was decreased by 70 %, a lag time before the onset of $[\text{Ca}^{2+}]_i$ increase was observed, and the rate of rise of $[\text{Ca}^{2+}]_i$ was slowed. Addition of NaF (10 mM) to fura-2-loaded 3T3-L1 cells caused a dose-dependent increase in $[\text{Ca}^{2+}]_i$ after a brief (~ 10 s) lag. Maximal effects (~ 300 nM) were observed at 5–10 mM-NaF. This effect was dependent on the presence of extracellular Ca^{2+} and appeared to be independent of inositol phosphate production. After reaching a peak at around 40 s after fluoride addition, $[\text{Ca}^{2+}]_i$ returned to near-baseline within 120 s. This return of $[\text{Ca}^{2+}]_i$ to near-baseline after fluoride stimulation and the inability of the cells to respond to a subsequent addition of fluoride indicated that the response to fluoride underwent desensitization. Similarly, the pathway used by $\text{PGF}_{2\alpha}$ to mobilize Ca^{2+} underwent desensitization. Exposure of the cells to a maximally effective concentration of fluoride and subsequent addition of $\text{PGF}_{2\alpha}$ produced a $[\text{Ca}^{2+}]_i$ response to $\text{PGF}_{2\alpha}$ which was similar in magnitude and kinetics to that seen for $\text{PGF}_{2\alpha}$ in the absence of extracellular Ca^{2+} . Conversely, prior exposure of cells to $\text{PGF}_{2\alpha}$ diminished the ability of fluoride to mobilize Ca^{2+} . $\text{PGF}_{2\alpha}$ also increased inositol phosphate formation, with a time course and dose-response consistent with its ability to increase $[\text{Ca}^{2+}]_i$. Prior exposure of cells to fluoride did not change the time course or dose-response characteristics of $\text{PGF}_{2\alpha}$ -induced generation of inositol phosphates. These data suggest that $\text{PGF}_{2\alpha}$ and fluoride share a common mechanism of activating Ca^{2+} influx in 3T3-L1 cells.

INTRODUCTION

Receptor-mediated increases in intracellular levels of calcium ($[\text{Ca}^{2+}]_i$) play a vital role in signal transduction for a variety of hormones, neurotransmitters and growth factors. Comparison of the magnitude and kinetics of agonist-induced Ca^{2+} mobilization in the absence and presence of external Ca^{2+} has revealed that both internal release of Ca^{2+} and influx of external Ca^{2+} play a role in Ca^{2+} -mediated responses (Merritt & Rink, 1987b; Davis *et al.*, 1987).

Internal release of Ca^{2+} in response to agonists is currently believed to occur through an agonist-induced activation of polyphosphoinositide-specific phospholipase C (PLC) which specifically cleaves phosphatidylinositol 4,5-bisphosphate found in the plasma membrane to generate inositol 1,4,5-trisphosphate (Berridge & Irvine, 1984). This water-soluble metabolite acts as a second messenger, diffusing to its site of action, possibly the endoplasmic reticulum, to ultimately cause the discharge of internal stores of Ca^{2+} into the cytoplasm.

Ca^{2+} influx appears to play a role both in the early phase of Ca^{2+} mobilization and in the later sustained phase of the Ca^{2+} response to some agonists (Hallam & Rink, 1985; Merritt & Rink, 1987b; Davis *et al.*, 1987). Unlike increases in $[\text{Ca}^{2+}]_i$ observed in the presence of extracellular Ca^{2+} , responses in the absence of extracellular Ca^{2+} often appear to occur more slowly,

reach a smaller peak, and fail to maintain a sustained plateau above the original baseline (Merritt & Rink, 1987b). These differences are attributed to agonist-induced Ca^{2+} influx. Although the mechanism regulating this influx has not been identified, it is attractive to speculate that it involves activation of a cation-specific channel.

Ca^{2+} channels can be classified into at least two major categories, i.e. voltage-operated and voltage-independent. Voltage-operated Ca^{2+} channels (VOC) open upon cell depolarization to allow Ca^{2+} entry. The action of a VOC cannot explain receptor-mediated Ca^{2+} entry in non-excitable cells. Non-voltage-operated Ca^{2+} channels appear to be regulated by intracellular second messengers such as Ca^{2+} and cyclic AMP, possibly through the activation of specific protein kinases (Kaczmarek, 1988) or more directly by agonist-receptor interactions (Meldolesi & Pozzan, 1987; Zschauer *et al.*, 1988). Recently, evidence in support of receptor-operated channels (ROC) comes from the observations that members of the signal-transducing family of GTP-binding proteins (G-proteins) can regulate channel activity. Coupling of receptors and channels by G-proteins is believed to occur for Ca^{2+} (Yatani *et al.*, 1988) and K^{+} channels (Pfaffinger *et al.*, 1985; VanDongen *et al.*, 1988; Logothetis *et al.*, 1988).

Fluoride activates G-proteins and can be used as a tool to study G-protein-mediated processes. In intact cells, fluoride causes, among other things, Ca^{2+} mobilization (Strnad & Wong,

Abbreviations used: G-protein, heterotrimeric signal-transducing GTP-binding protein; $\text{PGF}_{2\alpha}$, prostaglandin $\text{F}_{2\alpha}$; PLC, phosphoinositide-specific phospholipase C; GPI, glycerol-phosphatidylinositol; IP_1 , inositol monophosphate; IP_2 , inositol bisphosphate; IP_3 , inositol trisphosphate; IP_4 , inositol tetrakisphosphate; VOC, voltage-operated calcium channel; ROC, receptor-operated calcium channel; DMEM, Dulbecco's modified Eagle's medium; KRH, Krebs-Ringer buffer containing Hepes; PAF, platelet-activating factor; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; EC_{50} , concn. causing half-maximal effect.

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1985; Blackmore *et al.*, 1985; O'Shea *et al.*, 1987; Shoback & McGhee, 1988), the generation of inositol phosphates (Blackmore *et al.*, 1985; Strnad *et al.*, 1986; O'Shea *et al.*, 1987; Shoback & McGhee, 1988), and the inhibition of cyclic AMP accumulation (Blackmore *et al.*, 1985; Shoback & McGhee, 1988).

In the present study, Ca^{2+} mobilization was studied using the mouse 3T3-L1 fibroblast cell line. In intact 3T3-L1 fibroblasts, we have found that the agonist $\text{PGF}_{2\alpha}$ mobilizes Ca^{2+} from internal and external sources and also causes the formation of inositol phosphates. These effects of $\text{PGF}_{2\alpha}$ have also been observed in other systems (Sasaki, 1985; Davis *et al.*, 1987). In 3T3-L1 cells we have also found that fluoride causes a transient increase in $[\text{Ca}^{2+}]_i$ which is dependent on the presence of external Ca^{2+} and appears to be independent of inositol phosphate generation. The pathways used by both $\text{PGF}_{2\alpha}$ and fluoride to increase $[\text{Ca}^{2+}]_i$ undergo desensitization. This property has allowed us to use fluoride as a tool to study the multiple mechanisms used by $\text{PGF}_{2\alpha}$ to mobilize $[\text{Ca}^{2+}]_i$.

MATERIALS AND METHODS

Materials

Serum for cell culture was obtained from Hyclone (Logan, UT, U.S.A.) and other cell culture reagents were from Gibco (Grand Island, NY, U.S.A.). Fura-2/AM (the penta-acetoxymethyl ester of fura-2) was obtained from Behring Diagnostics (San Diego, CA, U.S.A.). $\text{PGF}_{2\alpha}$ and NaF were from Sigma (St. Louis, MO, U.S.A.). Dowex AG 1-X8 100–200 mesh, formate form, was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

3T3-L1 cell culture

3T3-L1 cells, generously donated by Dr. Charles Rubin (Albert Einstein College of Medicine, Bronx, NY, U.S.A.), were cultured at 37 °C in a humidified atmosphere with 10% CO_2 in complete media consisting of Dulbecco's modification of Eagle's medium (DMEM) with 4.5 g of glucose/l, supplemented with 10% fetal bovine serum and non-essential amino acids. Cells were utilized immediately upon reaching confluence.

Fura-2 loading and Ca^{2+} measurement

Cells were grown to confluence in 150 cm^2 tissue culture flasks. The cells were treated with trypsin for 3 min at 37 °C and collected in DMEM containing 10% fetal bovine serum. The cells were washed with a Krebs–Ringer buffer containing Hepes (KRH buffer) consisting of 118 mM-NaCl, 4.6 mM-KCl, 24.9 mM- NaHCO_3 , 1.0 mM- KH_2PO_4 , 11.1 mM-glucose, 1.1 mM- MgSO_4 , 1.0 mM- CaCl_2 , 5 mM-Hepes, pH 7.4, and 0.1% BSA. The cells were resuspended at 2×10^6 cells/ml in KRH buffer and incubated in the dark for 30 min at 37 °C with 2 μM -fura-2/AM. The cells were centrifuged and resuspended in fresh KRH buffer and incubated for another 20 min at 37 °C to allow hydrolysis of the intracellular fura-2 ester. The cells were stored at 5×10^6 cells/ml on ice until used. For $[\text{Ca}^{2+}]_i$ measurements in the absence of extracellular Ca^{2+} , cells were resuspended in KRH buffer without Ca^{2+} and stored on ice at 5×10^6 cells/ml until used (10–120 min). These cells were assayed in KRH buffer without Ca^{2+} , in the presence of 1 mM-EGTA to chelate any residual Ca^{2+} . Fluorescence (F) was measured using a spectrofluorimeter designed by the Johnson Biomedical Instrumentation Group (Philadelphia, PA, U.S.A.) equipped with a 75 W xenon lamp. The excitation beam was set at 340 nm and the emission detector was set to 510 nm. A quartz cuvette with a 1 cm path length was secured in a holder which held constant temperature and was equipped with magnetic stirring. Cells were assayed at 10^6 cells/ml

in 2 ml of KRH buffer. At the end of each transient, extracellular fura-2 was determined with 25 mM-Tris and 2.5 mM-EGTA, F_{min} was determined with 0.05% Triton X-100 and F_{max} was determined with 5 mM- CaCl_2 . $[\text{Ca}^{2+}]_i$ was calculated using a Ca^{2+} binding constant of 224 nM, as indicated by Grynkiewicz *et al.* (1985). The relatively high (300–400 nM) baseline levels of $[\text{Ca}^{2+}]_i$ in these experiments were presumably due to trypsin treatment of the cells, which may have allowed extracellular Ca^{2+} to leak into the cells. Although baseline $[\text{Ca}^{2+}]_i$ was somewhat variable from experiment to experiment, qualitatively similar results were consistently obtained. Unless indicated otherwise, the data shown are from representative experiments which were repeated at least twice with comparable results.

Measurement of inositol phosphates

3T3-L1 cells were seeded into 150 cm^2 tissue culture flasks (250 000/cell). After 2 days the media were removed and replaced with inositol-free DMEM supplemented with 10% dialysed fetal bovine serum (Gibco) and 5 μCi of myo - $[\text{H}]$ -inositol/ml (Amersham, Chicago, IL, U.S.A.) and incubated for 48 h. Labelled cells were trypsin-treated for 3 min at 37 °C and collected in DMEM containing 10% fetal bovine serum. The cells were centrifuged and resuspended at 5×10^5 cells/ml and distributed into test tubes containing 1 ml each. The tubes were placed in a 37 °C waterbath and the reaction begun by the addition of 100 μl of a $10 \times$ stock of either NaF or $\text{PGF}_{2\alpha}$. The reaction was stopped by addition of 100 μl of 100% trichloroacetic acid on ice. After 20 min, the tubes were centrifuged at 400 g for 10 min. The supernatant was extracted with 3×5 ml of water-saturated ether and then neutralized with 10 μl of 1 M-Tris base. Residual ether was blown off with argon. Samples were diluted with 4 ml of a 5 mM-sodium tetraborate/0.5 mM-EDTA solution and applied to Dowex columns (1 ml of a 60% Dowex solution/column). After 2×4 ml washes, inositol phosphates were eluted with 8 ml of each of the following buffers: glycerol-phosphatidylinositol (GPI), 20 mM-ammonium formate; inositol monophosphate (IP_1), 0.2 M-ammonium formate/0.1 M-formic acid; inositol bisphosphate (IP_2), 0.4 M-ammonium formate/0.1 M-formic acid; inositol trisphosphate (IP_3), 0.8 M-ammonium formate/0.1 M-formic acid; inositol tetrakisphosphate (IP_4), 1.0 M-ammonium formate/0.1 M-formic acid. To each 8 ml sample, 10 ml of high-salt-capacity Tru Count scintillation cocktail (IN/US Service Corp., Fairfield, NJ, U.S.A.) was added, and radioactivity in the samples was determined in a Beckman liquid scintillation counter. Recovery of inositol phosphates by this procedure was 80–90%.

RESULTS

In 3T3-L1 cells, 1 μM - $\text{PGF}_{2\alpha}$ caused a transient rise in $[\text{Ca}^{2+}]_i$, as measured by the fluorescent indicator fura-2/AM (Fig. 1a). The response peaked by 10 s and declined to near-baseline levels by 50 s. In the absence of extracellular Ca^{2+} , the ability of $\text{PGF}_{2\alpha}$ to mobilize Ca^{2+} was decreased, suggesting that Ca^{2+} influx may be responsible for part of the increase in $[\text{Ca}^{2+}]_i$ in response to $\text{PGF}_{2\alpha}$.

Fluoride also increased $[\text{Ca}^{2+}]_i$ in 3T3-L1 cells. Upon addition of 10 mM-NaF to 3T3-L1 cells, $[\text{Ca}^{2+}]_i$ increased by approx. 300 nM within 40 s and declined to near-baseline within 120 s (Fig. 1b). A short (~ 10 s) lag between the time when fluoride was added to the cells and that when $[\text{Ca}^{2+}]_i$ began rising was observed. In the absence of extracellular Ca^{2+} and the presence of 1 mM-EGTA, fluoride did not increase $[\text{Ca}^{2+}]_i$ (Fig. 1b). Similar data were obtained when 2 mM-EGTA was used to chelate the 1 mM- CaCl_2 in the buffer immediately before the addition of fluoride. In the presence of extracellular Ca^{2+} , 1 mM- Ni^{2+} , which

Fig. 1. PGF_{2α}- and fluoride-induced changes in [Ca²⁺]_i in fura-2 loaded 3T3-L1 cells

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer with 1 mM-CaCl₂ (trace A) or buffer without 1 mM-CaCl₂ and with 2 mM-EGTA (trace B). At time 0 s, (a) 1 μM-PGF_{2α} or (b) 10 mM-fluoride was added to the cuvette, which was maintained at 37 °C, and fura-2 fluorescence was measured. [Ca²⁺]_i was calculated from fluorescence signals as described in the Materials and methods section. A tracing representative of 10 separate experiments is shown. [Ca²⁺]_i values for traces in the presence of 1 mM-CaCl₂ are shown on the left-hand axes, whereas values for traces in the absence of CaCl₂ are shown on the right-hand axes.

Fig. 2. Concentration-response curve of fluoride-induced [Ca²⁺]_i increase

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer containing 1 mM-CaCl₂. The peak [Ca²⁺]_i in response to various concentrations of fluoride was determined as described in the Materials and methods section. The data points shown are the means ± S.E.M. of three separate experiments.

blocks Ca²⁺ influx through cation channels (Hallam & Rink, 1985; Merritt & Rink, 1987b), blocked fluoride's ability to mobilize Ca²⁺ (results not shown). If Ca²⁺ influx was responsible for all or part of the observed increase in [Ca²⁺]_i, this influx probably did not occur through VOCs, since addition of 50 mM-KCl to the cell suspension did not cause an increase in [Ca²⁺]_i, suggesting that the clone of 3T3-L1 cells used in the present study had no detectable VOCs. Nifedipine and verapamil (100 μM), two different VOC blockers, had no effect on the ability of fluoride to

mobilize [Ca²⁺]_i (results not shown). The ability of fluoride to mobilize Ca²⁺ was dose-dependent, with maximal effects occurring at 5–10 mM-fluoride (Fig. 2). Activation of certain G-proteins by fluoride has been shown to require the presence of Al³⁺ (Sternweis & Gilman, 1982), which forms a complex with fluoride. In our studies, the addition of aluminium had no effect on fluoride-mediated effects (results not shown), but it is possible that trace amounts of aluminium from glassware were present even before aluminium was added. Deferoxamine, an aluminium chelator, also had no effect on fluoride-induced increases in [Ca²⁺]_i at concentrations as high as 50 μM (results not shown). We cannot rule out the possibility that an aluminium-fluoride complex is the active species, since work with intact cells may prevent effective chelating of trace metals within the cell.

The return of [Ca²⁺]_i to near-baseline after the PGF_{2α}- and fluoride-induced transients suggested that the Ca²⁺-mobilizing mechanisms had become refractory. Re-addition of PGF_{2α} (Fig. 3a) or fluoride (Fig. 3b) did not cause a further mobilization of [Ca²⁺]_i, supporting the notion that the pathways underwent desensitization. The small decline in baseline fluorescence after re-addition of fluoride is probably attributable to addition of the fluoride solution.

If fluoride and PGF_{2α} shared a common Ca²⁺-mobilizing mechanism, then a change in the magnitude and/or kinetics of their responses would be observed after prior exposure of the cells to the other activator, since both pathways underwent desensitization. Previous exposure to 10 mM-fluoride decreased a

Fig. 3. Effect of exposure of fura-2-loaded 3T3-L1 cells to fluoride or PGF_{2α} on subsequent [Ca²⁺]_i increases induced by fluoride or PGF_{2α}

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer containing 1 mM-CaCl₂ and fura-2 fluorescence was measured. Addition of 10 mM-NaF or 1 μM-PGF_{2α} to the cuvette is marked. A tracing representative of four different experiments is shown.

Fig. 4. Effect of fluoride pretreatment and removal of extracellular Ca^{2+} on $\text{PGF}_{2\alpha}$ -induced increases in $[\text{Ca}^{2+}]_i$ in fura-2-loaded 3T3-L1 cells

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer containing 1 mM- CaCl_2 . The indicated concentrations of $\text{PGF}_{2\alpha}$ were added to control cells (●), cells pretreated for 2 min with 10 mM-NaF (○) or cells in the absence of extracellular Ca^{2+} with 1 mM-EGTA (▲), and maximal fura-2 fluorescence was measured. $[\text{Ca}^{2+}]_i$ was calculated from fluorescence signals as described in the Materials and methods section. Peak $[\text{Ca}^{2+}]_i$ values are presented from one experiment that was repeated twice with similar results

subsequent response to $\text{PGF}_{2\alpha}$ (from 1128 nM- to 344 nM- $[\text{Ca}^{2+}]_i$ above baseline, Fig. 3c). Likewise, fluoride-induced Ca^{2+} mobilization was greatly diminished (from 233 nM- to 67 nM- $[\text{Ca}^{2+}]_i$ above baseline) by prior exposure to $\text{PGF}_{2\alpha}$ (Fig. 3d).

The ability of $\text{PGF}_{2\alpha}$ to increase $[\text{Ca}^{2+}]_i$ was dose-dependent (Fig. 4), and removal of extracellular Ca^{2+} revealed that the increase in $[\text{Ca}^{2+}]_i$ induced by $\text{PGF}_{2\alpha}$ comprised at least two components. In the absence of extracellular Ca^{2+} , only ~30% of $[\text{Ca}^{2+}]_i$ mobilization by $\text{PGF}_{2\alpha}$ seen in the presence of extracellular Ca^{2+} was induced (Fig. 4). This suggested that ~30% of the increase in $[\text{Ca}^{2+}]_i$ was attributable to internal release, presumably due to the generation of inositol phosphates. Conversely, 70% of Ca^{2+} mobilized was either primarily or secondarily caused by possible Ca^{2+} influx.

Fig. 5. Effect of $\text{PGF}_{2\alpha}$ pretreatment on fluoride-induced increases in $[\text{Ca}^{2+}]_i$ in fura-2-loaded 3T3-L1 cells

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer containing 1 mM- CaCl_2 . Cells were exposed to the indicated concentrations of $\text{PGF}_{2\alpha}$ for 2 min, after which the cells were stimulated with 10 mM-NaF and fura-2 fluorescence was measured. $[\text{Ca}^{2+}]_i$ was calculated from fluorescence signals. Peak $[\text{Ca}^{2+}]_i$ values are presented. The experiment was repeated twice with similar results.

Table 1. Effect of pretreating cells with fluoride on the subsequent ability of $\text{PGF}_{2\alpha}$ to mobilize Ca^{2+} in the presence or absence of extracellular Ca^{2+}

Cells were assayed with $\text{PGF}_{2\alpha}$ (1 μM) in the presence or absence of extracellular Ca^{2+} as indicated, either with or without pretreatment with 10 mM-fluoride for 2 min. Data are expressed as percentages of control, which was defined as the increase in $[\text{Ca}^{2+}]_i$ over baseline induced by $\text{PGF}_{2\alpha}$ in the presence of extracellular Ca^{2+} . Data are expressed as means $\pm$ S.E.M. ($n = 3$). $\text{PGF}_{2\alpha}$ increased $[\text{Ca}^{2+}]_i$ by 552 ± 75 nM ($n = 3$). *Significantly different from $\text{PGF}_{2\alpha}$ in the absence of extracellular Ca^{2+} ; $P < 0.01$.

Pretreatment of cells	Extracellular Ca^{2+} in assay	$\text{PGF}_{2\alpha}$ -mediated Ca^{2+} mobilization (% of control)
None	+	100
None	—	50.0 ± 3.7
NaF	+	42.3 ± 5.0
NaF	—	26.7 ± 0.3

Exposure of cells to fluoride for 2 min activated and desensitized the putative influx mechanism. Subsequent addition of $\text{PGF}_{2\alpha}$ resulted in only 30% of the Ca^{2+} mobilization seen without fluoride pretreatment at all concentrations of $\text{PGF}_{2\alpha}$ tested (Fig. 4). The 30% of the Ca^{2+} response remaining after fluoride pretreatment matched the response observed in the absence of extracellular Ca^{2+} . This mobilization was presumably due to inositol phosphate-mediated release from internal stores, which was unaffected by fluoride (see below). It is possible that exposure of the cells to fluoride activated and then desensitized an influx mechanism to further stimulation, diminishing the portion of the $\text{PGF}_{2\alpha}$ -induced Ca^{2+} response which was dependent on extracellular Ca^{2+} . Conversely, pretreatment of cells with $\text{PGF}_{2\alpha}$ decreased the response to fluoride in a dose-dependent manner (Fig. 5). $\text{PGF}_{2\alpha}$ at concentrations ≥ 1 μM had maximal effects on the inhibition of Ca^{2+} mobilization by a subsequent challenge with 10 mM-fluoride.

Fig. 6. Kinetics of $\text{PGF}_{2\alpha}$ -induced changes in $[\text{Ca}^{2+}]_i$ in fura-2-loaded 3T3-L1 cells

3T3-L1 cells were trypsin-treated and loaded with fura-2/AM as described in the Materials and methods section. Cells were then resuspended in 2 ml of KRH buffer containing 1 mM- CaCl_2 . At 0 s, 1 μM - $\text{PGF}_{2\alpha}$ was added to the cuvette, and fura-2 fluorescence was then measured. Control cells and cells pretreated for 2 min with 10 mM-NaF (designated 'After NaF') were assayed in the presence of 1 mM- CaCl_2 . Cells assayed in the absence of extracellular Ca^{2+} with 1 mM-EGTA are designated '- Ca^{2+} '. The experiment was repeated twice with similar results.

Fig. 7. Effect of fluoride pretreatment and removal of extracellular Ca^{2+} on the dose-response curve of $PGF_{2\alpha}$ -induced inositol phosphate formation in 3T3-L1 cells

$[^3H]$ Inositol-labelled cells were incubated for 15 s in KRH buffer with the indicated concentrations of $PGF_{2\alpha}$. Control cells (■) and cells pretreated for 2 min with 10 mM-NaF (▲) were assayed in the presence of 1 mM extracellular Ca^{2+} . Cells assayed in the absence of extracellular Ca^{2+} with 1 mM-EGTA are designated by ●. The reaction was stopped with trichloroacetic acid and inositol phosphates were extracted and separated on Dowex AG 1-X8 columns as described in the Materials and methods section. (a) IP_1 ; (b) IP_2 ; (c) IP_3 ; (d) IP_4 . The radioactivity of each inositol phosphate is expressed as the mean $\pm$ S.E.M. of three separate experiments.

If fluoride only stimulated and desensitized Ca^{2+} influx, then pretreating cells with fluoride should not affect the subsequent increase in $[Ca^{2+}]_i$ by $PGF_{2\alpha}$ in the absence of extracellular Ca^{2+} . Table 1 shows that fluoride pretreatment of the cells decreased the $PGF_{2\alpha}$ -mediated increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} , suggesting that fluoride may also deplete internal stores of Ca^{2+} (see the Discussion section).

Close examination of the kinetics of Ca^{2+} mobilization by $PGF_{2\alpha}$ revealed changes upon removal of extracellular Ca^{2+} (Fig. 6). These changes were more apparent with submaximal concentrations of $PGF_{2\alpha}$. With Ca^{2+} in the external medium, 0.1 μM - $PGF_{2\alpha}$ caused a rapid increase in $[Ca^{2+}]_i$ with no apparent lag. Removal of Ca^{2+} from the buffer resulted in a 1–2 s lag and a decrease in the rate of rise of $[Ca^{2+}]_i$, as well as a decrease in the peak. Prior exposure of the cells to 10 mM-fluoride also decreased the rate of rise of $[Ca^{2+}]_i$ but there was no significant lag period before the Ca^{2+} response began.

$PGF_{2\alpha}$ increased the formation of inositol phosphates in a dose- (Fig. 7) and time- (Fig. 8) dependent manner. The dose-response for Ca^{2+} mobilization [EC_{50} (concn. causing 50% of maximal stimulation) $\approx$ 0.25 μM ; Fig. 4] and the generation of IP_3 ($EC_{50} \approx$ 0.25 μM ; Fig. 7c) correlated well, as did the time course for Ca^{2+} mobilization ($t_{1/2} \approx$ 3 s; Fig. 6) and the generation of IP_3 ($t_{1/2} \approx$ 3 s; Fig. 8c). Although in some systems fluoride can activate PLC to cause the generation of inositol phosphates (Blackmore *et al.*, 1985; Strnad *et al.*, 1986), in 3T3-L1 cells no increases in IP_1 , IP_2 , IP_3 or IP_4 were detected within the time course required for Ca^{2+} mobilization (Fig. 9).

The effects of fluoride on $PGF_{2\alpha}$ -induced Ca^{2+} mobilization were not due to a detectable change in the dose-response (Fig. 7) or time course (Fig. 8) of $PGF_{2\alpha}$ -induced generation of inositol phosphates. The absence of extracellular Ca^{2+} did not change the increase in inositol phosphates compared with controls, but did decrease the basal value of all inositol phosphates measured (Figs. 7 and 8). Previous studies have also observed a decrease in inositol phosphates in the absence of extracellular Ca^{2+} (Litosch *et al.*, 1985; Pribluda & Metzger, 1987).

In conclusion, $PGF_{2\alpha}$ and fluoride both appear to activate Ca^{2+} influx. Increases in $[Ca^{2+}]_i$ induced by fluoride appear to be dependent on influx and independent of inositol phosphate metabolism, whereas for $PGF_{2\alpha}$ influx and inositol phosphate-mediated intracellular Ca^{2+} mobilization both seem to contribute to the observed increase in $[Ca^{2+}]_i$. Responses to both $PGF_{2\alpha}$ and fluoride appear to undergo desensitization, and prior exposure to one attenuates subsequent response to the other.

DISCUSSION

In 3T3-L1 fibroblasts, $PGF_{2\alpha}$ caused an increase in $[Ca^{2+}]_i$ and the generation of inositol phosphates. $PGF_{2\alpha}$ elicited a large and consistent Ca^{2+} response. We found in 3T3-L1 cells that no other agonists tested (including vasopressin, bombesin, thrombin, somatostatin, bradykinin, PGI_2 , PGE_2 and PGE_1) induced a consistent Ca^{2+} -mobilizing response. $PGF_{2\alpha}$ caused a rapid dose-dependent increase in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+} , the response was decreased by $\sim 70\%$, a lag time was

Fig. 8. Effect of fluoride pretreatment and removal of extracellular Ca^{2+} on the time course of $\text{PGF}_{2\alpha}$ -induced inositol phosphate formation in 3T3-L1 cells

$[\text{^3H}]$ Inositol-labelled cells were incubated for the indicated times in KRH buffer with $1 \mu\text{M-PGF}_{2\alpha}$. Control cells ($\blacksquare$) and cells pretreated for 2 min with 10 mM-NaF ($\blacktriangle$) were assayed in the presence of 1 mM extracellular Ca^{2+} . Cells assayed in the absence of extracellular Ca^{2+} with 1 mM-EGTA are designated by $\bullet$. The reaction was stopped with trichloroacetic acid and inositol phosphates were extracted and separated on Dowex AG 1-X8 columns as described in the Materials and methods section. (a) IP_1 ; (b) IP_2 ; (c) IP_3 ; (d) IP_4 . The radioactivity of each inositol phosphate is expressed as the mean $\pm$ S.E.M. of three separate experiments.

Fig. 9. Time course for inositol phosphate formation in 3T3-L1 cells stimulated with 10 mM-NaF

$[\text{^3H}]$ Inositol-labelled cells were incubated for the indicated times in KRH buffer with 10 mM-NaF . The reaction was stopped with trichloroacetic acid and inositol phosphates were extracted and separated on Dowex AG 1-X8 columns as described in the Materials and methods section. $\blacksquare$, IP_1 ; $\blacktriangle$, IP_2 ; $\bullet$, IP_3 ; $\triangle$, IP_4 . The radioactivity of each inositol phosphate is expressed as the mean $\pm$ S.E.M. of three separate experiments.

observed before the increase in $[\text{Ca}^{2+}]_i$, and the rate of rise of $[\text{Ca}^{2+}]_i$ was decreased. This suggests that although internal release of Ca^{2+} by inositol phosphates was probably responsible for some of the Ca^{2+} mobilized, the majority of the increase in $[\text{Ca}^{2+}]_i$ observed in response to $\text{PGF}_{2\alpha}$ was presumably due to Ca^{2+} influx. In these cells, only a slight sustained increase in $[\text{Ca}^{2+}]_i$ was observed after the initial $\text{PGF}_{2\alpha}$ transient; therefore no conclusions can be drawn on the role of Ca^{2+} influx in this sustained Ca^{2+} response.

In the 3T3-L1 cells, fluoride caused a brief ($< 120 \text{ s}$) increase in $[\text{Ca}^{2+}]_i$, followed by a return to near-baseline. The kinetics of this response differed slightly from those observed with $\text{PGF}_{2\alpha}$. The fluoride-induced transient began after a 10 s lag, not observed with $\text{PGF}_{2\alpha}$, rose at a slower rate than for $\text{PGF}_{2\alpha}$, and required a longer time to return to near-baseline levels. The relatively rapid and transient nature of the Ca^{2+} response caused by fluoride in 3T3-L1 cells was different from the Ca^{2+} -mobilizing effects reported in other studies, where fluoride-induced $[\text{Ca}^{2+}]_i$ increases were slower (occurring over 10 min) and there was no decline to baseline levels (Strnad & Wong, 1985; O'Shea *et al.*, 1987; Shoback & McGhee, 1988).

The effects of fluoride were dependent on the presence of external Ca^{2+} and were blocked by the inorganic Ca^{2+} channel blocker Ni^{2+} . Both types of data suggested that fluoride increased $[\text{Ca}^{2+}]_i$ by allowing external Ca^{2+} to enter the cell through specific

channels and not through a general increase in cell permeability. Although VOCs have been identified in Swiss 3T3 cells by the voltage clamp technique (Chen *et al.*, 1988), in our clone of 3T3-L1 cells no detectable Ca^{2+} mobilization was observed upon depolarization of fura-2-loaded cells with 50 mM-KCl. In addition, two different kinds of VOC blockers, nifedipine and verapamil, did not block Ca^{2+} mobilization by fluoride. These data suggested that it was unlikely that the fluoride-induced Ca^{2+} transient was due to activation of VOCs. In addition, the mechanism used by fluoride to mobilize Ca^{2+} did not appear to involve inositol phosphate generation, since no increase in inositol phosphates was detected during the time course of Ca^{2+} mobilization induced by fluoride.

Because fluoride is known to activate G-proteins, it is tempting to propose that its effects are due to activation of a G-protein coupled in an ROC. This G-protein might normally be activated by a receptor upon binding of an agonist which, among other things, activates Ca^{2+} influx. How fluoride selectively activates the G-protein coupled to Ca^{2+} influx, without activating other G-proteins (such as the G-proteins coupled to the stimulation of adenylate cyclase or to the activation of PLC) is not clear, but this type of selective activation has been observed in other intact cell systems in which, for example, the G-protein coupled to PLC is activated by fluoride without a concomitant activation of G_s and adenylate cyclase (Blackmore *et al.*, 1985; Hughes & Barritt, 1987; Shoback & McGhee, 1988).

Activation of G-proteins is not the only known mechanism by which fluoride regulates cellular functions. Fluoride can decrease levels of cellular ATP (Clarke & Weigel, 1985; Okada & Brown, 1988) and has been shown to inhibit ATPase (Penzotti & Titus, 1972). An alternative mechanism by which fluoride increases $[Ca^{2+}]_i$ is by inhibiting the ATP-dependent Ca^{2+} pump which is responsible for maintaining low levels of Ca^{2+} inside the cell. The ability of Ni^{2+} to block fluoride-induced Ca^{2+} mobilization does not support a role for fluoride in regulating a Ca^{2+} pump, since external Ni^{2+} would not affect a decrease in the extrusion of internal Ca^{2+} . Additionally, fluoride can inhibit phosphatases (Storey *et al.*, 1984), and inhibition of a Ca^{2+} channel phosphatase could cause an increase in $[Ca^{2+}]_i$, since regulation of channel activity can occur through phosphorylation/dephosphorylation of the channel protein (Kaczmarek, 1988). Fluoride's effects do not appear to be caused by cytotoxicity, since during the relatively short time (2–4 min) that the cells were exposed to fluoride there was no loss in cell viability, as assessed by Trypan Blue dye exclusion. Levels of ATP also did not change during exposure to fluoride in these studies (results not shown). It is unlikely that the effects of fluoride described in the present study are the result of an interaction of fluoride with calcium to form insoluble CaF_2 . This phenomenon could not explain a fluoride-induced Ca^{2+} transient, or an effect of $PGF_{2\alpha}$ on fluoride's ability to increase $[Ca^{2+}]_i$. Preliminary data on the recovery of the $PGF_{2\alpha}$ -mediated $[Ca^{2+}]_i$ increase after fluoride pretreatment of the cells indicate that removal of fluoride does not immediately result in the recovery of full responsiveness to $PGF_{2\alpha}$ in the presence of external Ca^{2+} . These data suggest that fluoride is not acting simply as a chelator of external Ca^{2+} .

It appears that a putative influx mechanism for Ca^{2+} used by $PGF_{2\alpha}$ is shared by fluoride, since desensitization of the fluoride mechanism by prior exposure of the 3T3-L1 cells to fluoride also resulted in a decrease in the response to $PGF_{2\alpha}$. A reciprocal pattern of Ca^{2+} responses was observed if cells were exposed to $PGF_{2\alpha}$ and subsequently challenged with either $PGF_{2\alpha}$ or fluoride. However, the total mobilization of Ca^{2+} in response to either agent appears to involve multiple mechanisms. The Ca^{2+} response to $PGF_{2\alpha}$ appears to result from a combination of Ca^{2+} influx across the plasma membrane and mobilization of internal Ca^{2+}

stores as a result of enhanced production of inositol phosphates. This latter mechanism can be observed in the absence of Ca^{2+} in the extracellular medium. In contrast, the Ca^{2+} response to fluoride is strictly dependent on the presence of external Ca^{2+} , and could not be related to changes in phosphatidylinositol metabolism. However, the data do not completely rule out a role for inositol phosphates in the mobilization of Ca^{2+} by fluoride. At low $PGF_{2\alpha}$ concentrations (0.1 μM), which promote a Ca^{2+} transient that is similar in magnitude to the maximal response to fluoride, a significant $[Ca^{2+}]_i$ response can be observed in the absence of extracellular Ca^{2+} (Fig. 4), but no significant increases in soluble inositol phosphates were observed (Fig. 7c). This suggests that we cannot detect low levels of IP_3 which are sufficient to cause a measurable increase in $[Ca^{2+}]_i$. Thus it is possible that fluoride may also generate inositol phosphates, but, unlike the response to $PGF_{2\alpha}$, this pathway would be dependent on an initial Ca^{2+} influx. Experiments using fluoride pretreatment to alter the $PGF_{2\alpha}$ -mediated $[Ca^{2+}]_i$ increase suggest that fluoride affects other processes in addition to Ca^{2+} influx. Pretreatment of cells with fluoride (in the presence of extracellular Ca^{2+}) diminished the ability of $PGF_{2\alpha}$ to mobilize $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} . These data suggest that fluoride may partially deplete internal Ca^{2+} stores. A possible explanation for fluoride's effects is that an initial Ca^{2+} influx primes release of Ca^{2+} from internal stores. Without the initial influx, no Ca^{2+} mobilization is observed. Fluoride's ability to reduce $PGF_{2\alpha}$ -mediated Ca^{2+} mobilization is due to both a partial desensitization of a Ca^{2+} influx mechanism and a decrease in intracellular Ca^{2+} stores.

The kinetics of the Ca^{2+} transient induced by $PGF_{2\alpha}$ were altered by removal of Ca^{2+} from the extracellular medium. A lag time before $[Ca^{2+}]_i$ began rising appeared, and the rate of rise of $[Ca^{2+}]_i$ was slowed. This difference in the kinetics in the absence of extracellular Ca^{2+} was similar to that seen for Ca^{2+} mobilization in response to thrombin, vasopressin, ADP and platelet-activating factor (PAF) previously reported in platelets (Sage & Rink, 1987). The data suggest that Ca^{2+} influx in these instances precedes internal Ca^{2+} mobilization and also contributes to the rapid rise in $[Ca^{2+}]_i$. In other systems there is no apparent difference in the lag times before $[Ca^{2+}]_i$ rises (Merritt & Rink, 1987a), and there is a sustained increase in $[Ca^{2+}]_i$ above baseline after an initial transient. These data and other data regarding the role of inositol phosphates in Ca^{2+} mobilization have led to the proposal of several similar models (Putney, 1986; Merritt & Rink, 1987c; Gill *et al.*, 1988) in which agonist binding results in the activation of PLC, generating IP_3 which releases Ca^{2+} from internal stores. As this store of Ca^{2+} is depleted, it is refilled by Ca^{2+} entering from outside the cell. These models explain the sustained Ca^{2+} response, but questions still remain regarding the role of Ca^{2+} influx in the rapid initial response. In the 3T3-L1 cells, $PGF_{2\alpha}$ appears to cause Ca^{2+} influx before internal mobilization, suggesting possibly that agonist binding causes an immediate activation of ROCs.

Pre-exposure of the cells to fluoride followed by subsequent challenge with $PGF_{2\alpha}$ resulted in a slower rate of rise of $[Ca^{2+}]_i$ in response to $PGF_{2\alpha}$, which was similar to that seen for $PGF_{2\alpha}$ in the absence of extracellular Ca^{2+} . It appeared that the Ca^{2+} influx which was responsible for the rapid rate of rise in $[Ca^{2+}]_i$ in response to $PGF_{2\alpha}$ was blocked by desensitization to fluoride. Fluoride pretreatment did not dramatically increase the lag period before $[Ca^{2+}]_i$ began rising. If fluoride desensitized the Ca^{2+} influx mechanism which was responsible for the immediate increase in $[Ca^{2+}]_i$ in response to $PGF_{2\alpha}$, then the lag time before the Ca^{2+} response began should have been increased by fluoride pretreatment. It is possible, though, that desensitization of the fluoride mechanism may not completely inhibit the initial influx.

Stop-flow techniques may be necessary to make accurate rapid measurements of these kinetics.

In conclusion, $\text{PGF}_{2\alpha}$ apparently caused the mobilization of Ca^{2+} from internal and external stores in 3T3-L1 cells. Receptor-mediated activation of G-protein(s), coupled to PLC and possibly to an ROC, may be responsible for Ca^{2+} mobilization by $\text{PGF}_{2\alpha}$. Fluoride appears to cause a $[\text{Ca}^{2+}]_i$ increase in this system through a mechanism which is rapid, dependent on external Ca^{2+} , undergoes desensitization and appears to be independent of phosphoinositide metabolism. Ca^{2+} mobilization by $\text{PGF}_{2\alpha}$ can be decreased by fluoride, presumably by the latter's ability to partially desensitize a priming Ca^{2+} -influx mechanism and to partially deplete internal Ca^{2+} stores. Conversely, desensitization of the mechanisms used by $\text{PGF}_{2\alpha}$ caused an attenuation of fluoride's ability to mobilize Ca^{2+} . These data support the hypothesis that, in 3T3-L1 cells, $\text{PGF}_{2\alpha}$ and fluoride stimulate a common Ca^{2+} -influx mechanism.

We thank Dr. Timothy Rink, Dr. Henry Sarau and Dr. Glenn Hoke for their helpful comments.

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Received 29 May 1990; accepted 26 June 1990