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CONTRIBUTION OF EXTERNAL AND INTERNAL Ca^{2+} TO CHANGES IN INTRACELLULAR FREE Ca^{2+} PRODUCED BY MITOGENS IN SWISS 3T3 FIBROBLASTS: THE ROLE OF DIHYDROPYRIDINE SENSITIVE Ca^{2+} CHANNELS

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Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by growth factors and mitogens have been studied using aequorin-loaded Swiss 3T3 cells. Decreasing free Ca^{2+} in the external medium by using EGTA had no significant effect on the increase in $[Ca^{2+}]_i$ produced by vasopresin, bradykinin, bombesin or prostaglandin E_2, but reduced the increase in $[Ca^{2+}]_i$ produced by platelet derived growth factor (PDGF) by 58%, by prostaglandin E_1 44% and by prostaglandin $F_{2\alpha}$ 47%. The dihydropyridine Ca^{2+}-channel antagonist nifedipine at 10 μM inhibited the $[Ca^{2+}]_i$ response to PDGF by 41% in both the presence of and in the absence of external Ca^{2+}. Methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (BAY K8644), a Ca^{2+}-channel agonist, at 10 μM produced an increase in $[Ca^{2+}]_i$ and decreased the $[Ca^{2+}]_i$ response to PDGF by 39%. Nifedipine did not block $^{45}Ca^{2+}$ uptake or release by inositol 1,4,5-trisphosphate in saponin-permeabilized Swiss 3T3 fibroblasts but BAY K8644 inhibited $^{45}Ca^{2+}$ release by inositol 1,4,5-trisphosphate. The results suggest that the increase in $[Ca^{2+}]_i$ caused by PDGF in Swiss 3T3 fibroblasts is due to the influx of external Ca^{2+} through dihydropyridine sensitive Ca^{2+} channels, as well as release of internal Ca^{2+}.

Eukaryotic cells maintain resting cytoplasmic free calcium concentrations ($[Ca^{2+}]_i$) at very low levels, around 10^{-7} M, while the extracellular Ca^{2+} concentration is around 10^{-3} M (1). Transient increases in $[Ca^{2+}]_i$ appear to be important as intracellular signals mediating the effects of a variety of hormones, neurotransmitters, growth factors and mitogens (1-3). An increase in $[Ca^{2+}]_i$ can be brought about either by the release of Ca^{2+} from specialized stores in the endoplasmic reticulum (4) by intracellular second messengers such as inositiol 1,4,5-trisphosphate (5) or arachidonic acid (6), or by the influx of external Ca^{2+} through plasma membrane Ca^{2+} channels (1,3).

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Abbreviations

$[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PDGF, platelet derived growth factor; DMEM, Dulbecco's modified Eagle's medium; PG, prostaglandin; EGF, epidermal growth factor; BAY K8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate.

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There are two reported types of plasma membrane Ca^{2+} channels, voltage-gated Ca^{2+} channels and receptor-operated Ca^{2+} channels. Voltage-gated Ca^{2+} channels are similar in structure and function to other voltage gated ion channels (7) and are frequently associated with GTP-binding proteins which may modulate their activity (8). Voltage-gated Ca^{2+} channel activity can be subject to regulation by phosphorylation by 3',5'-cyclic AMP-dependent protein kinase (7). Less is known about receptor-operated Ca^{2+} channels. Their activity may be modulated by inositol phosphates acting on the internal surface of the plasma membrane (9,10) or by phosphorylation by receptor-dependent protein tyrosine kinases (11).

Growth factors and mitogens have been reported to increase $[Ca^{2+}]_i$ in a variety of cell types (12-17), including Swiss mouse 3T3 fibroblasts (19-26). The purpose of the present investigation was to identify the source of Ca^{2+} for changes in $[Ca^{2+}]_i$ caused by a number of growth factors and mitogens in Swiss 3T3 fibroblasts. The study has shown that agents acting on voltage-gated Ca^{2+} channels can affect $[Ca^{2+}]_i$ signalling by some growth factors.

MATERIALS AND METHODS

Vasopressin-^{8}Arg, bradykinin, prostaglandins E_1, E_2, and $F_{2\alpha}$, and nifedipine were purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin, bombesin and myo-inositol 1,4,5-trisphosphate were obtained from Calbiochem (San Diego, CA). Platelet derived growth factor (PDGF) as the B chain homodimer was obtained from Bachem Inc. (Torrance, CA). Bay K8644 was a gift from Miles Laboratories (New Haven, CT). Aequorin was obtained from Dr. John Blinks, Mayo Clinic. Swiss 3T3 fibroblasts were provided by Dr. H.R. Herschmann, University of California, Los Angeles, CA. The cells were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum and harvested at each passage with 0.5% trypsin and 0.5 mM EDTA before becoming confluent. Studies were conducted on cells between passages 37 and 58. Aequorin loading of the Swiss 3T3 fibroblasts employed a low Ca^{2+}-centrifugation method as previously described (25). The loaded cells were plated at 10^6 cells/35 mm culture dish (Falcon, Becton Dickinson, Lincoln Park, NJ) in DMEM containing 10% fetal calf serum and incubated 18 hr at 37°C, at which time the medium was replaced with DMEM without fetal calf serum for 2 hr. Light emitted from the aequorin loaded cells was measured as previously described (25) and a quantitative estimate of $[Ca^{2+}]_i$ obtained employing the calibration method for aequorin described by Allen and Blinks (27). In some studies cells were exposed to Ca^{2+}-free DMEM with 0.5 mM EGTA for 5 min before adding growth factors. The growth factors, mitogens, and other agents were added to the culture dish dissolved in 0.2 to 1 ml of warm DMEM with or without Ca^{2+}, as appropriate. The uptake and release of $^{45}Ca^{2+}$ by saponin-permeabilized Swiss 3T3 cells was measured by the method of Gill and Cheu (28). Statistical analysis of the results used Students t-test and $p < 0.05$ was considered significant.

RESULTS

The increases in $[Ca^{2+}]_i$ caused by PDGF and some mitogens in Swiss 3T3 cells are shown in Figure 1. The concentration of the agents was chosen to give the maximal increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase with the Ca^{2+} ionophore ionomycin (30) was employed as a positive control. The concentration of ionomycin chosen, 2×10^{-6} M, gave a submaximal and transient increase in $[Ca^{2+}]_i$. Higher concentrations of ionomycin produced larger and sustained increases in $[Ca^{2+}]_i$ (results not shown). Values for the peak increase in $[Ca^{2+}]_i$ are

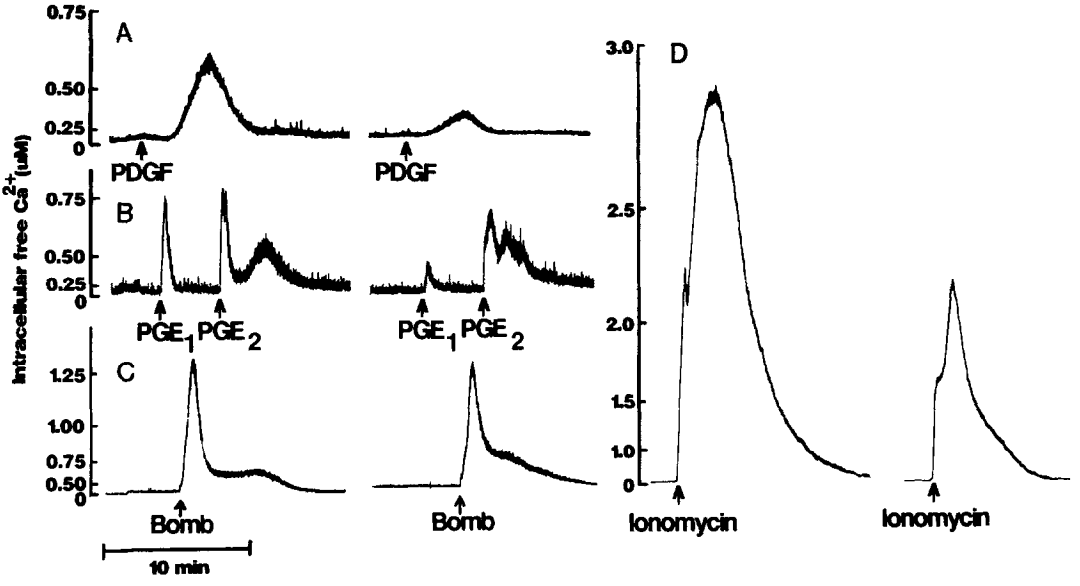

Figure 1. $[Ca^{2+}]_i$ responses of Swiss 3T3 cells in the presence and absence of external Ca^{2+}. Cells were exposed to **A**, PDGF, 3.3 x 10^{-9} M; **B**, PGE_1, 2 x 10^{-5} M, and PGE_2, 2 x 10^{-5} M; **C**, bombesin, 2 x 10^{-7} and **D**, ionomycin, 2 x 10^{-6} M, at the arrows. The left panel of each pair is with 1.8 mM Ca^{2+} in the medium and the right panel is 5 min after exposure to medium without Ca^{2+} containing 0.5 mM EGTA (free Ca^{2+} concentration < 10^{-8} calculated by the method of Fabiato (29).

given in Table 2. PDGF produced a slow rise in $[Ca^{2+}]_i$ with a delay of 1 min, that peaked at 3 min and lasted 6 min. Vasopressin, bombesin, PGE_1, PGE_2, and $PGF_{2\alpha}$ gave an immediate increase in $[Ca^{2+}]_i$ that lasted 4 to 5 min. With bradykinin, PGE_1, $PGF_{2\alpha}$, and sometimes bombesin a secondary smaller increase in $[Ca^{2+}]_i$ with a peak at 3 min was also seen. Insulin and epidermal growth factor did not given an increase in $[Ca^{2+}]_i$ in the serum free Swiss 3T3 cells (results not shown).

Exposure of the cells to Ca^{2+} free medium containing 0.5 mM EGTA for 5 min had no significant effect on the $[Ca^{2+}]_i$ increase caused by vasopressin, bradykinin, bombesin or PGE_2 but reduced the $[Ca^{2+}]_i$ increase caused by PDGF, PGE_1, and $PGF_{2\alpha}$ (Figure 1 and Table 1). The $[Ca^{2+}]_i$ response to 2 x 10^{-6} M ionomycin was inhibited 49% by the Ca^{2+} free medium with EGTA.

Ca^{2+} Channel Ligands and PDGF Induced $[Ca^{2+}]_i$ Response

The dihydropyridine Ca^{2+} channel antagonist nifedipine (31) had no effect on resting $[Ca^{2+}]_i$ but produced a decrease in the $[Ca^{2+}]_i$ response to PDGF (Figure 2). With Ca^{2+} in the external medium the $[Ca^{2+}]_i$ response to 3.3 x 10^{-7} M PDGF was decreased from 0.93 ± 0.31 μM (± S.D., n = 12) to 0.55 ± 0.03 μM (n = 4, P < 0.01) by 10^{-5} M nifedipine. In the absence of external Ca^{2+} the $[Ca^{2+}]_i$ response to PDGF was decreased from 0.39 ± 0.11 μM (n

Table 1. **Effect of Removing External Ca^{2+} on the Change in $[Ca^{2+}]_i$ Caused by Various Agents in Swiss 3T3 Cells**

	With Ca^{2+} $[Ca^{2+}]_i$, μM	Without Ca^{2+} $[Ca^{2+}]_i$, μM	
PDGF, 3.3 x 10^{-9} M	0.93 ± 0.31	0.39 ± 0.11[a]	(58)
Vasopressin, 10^{-7} M	0.84 ± 0.22	0.64 ± 0.08	(24)
Bradykinin, 2 x 10^{-7} M	1.23 ± 0.16	1.23 ± 0.29	(0)
Bombesin, 2 x 10^{-7} M	1.49 ± 0.18	1.32 ± 0.13	(11)
PGE_1, 2 x 10^{-5} M	0.80 ± 0.16	0.45 ± 0.09[a]	(44)
PGE_2, 10^{-5} M	1.15 ± 0.27	1.08 ± 0.34	(6)
$PGF_{2\alpha}$, 10^{-5} M	1.54 ± 0.35	0.82 ± 0.08[a]	(47)
Ionomycin, 2 x 10^{-6} M	2.91 ± 0.54	1.49 ± 0.20[a]	(49)

[a] $p < 0.05$ compared to control value with Ca^{2+}.
Swiss 3T3 fibroblasts were exposed to the agents at the concentrations shown, either in DMEM containing 1.8 mM Ca^{2+} (with Ca^{2+}) or after 5 min exposure to Ca^{2+} free DMEM with 0.5 mM EGTA (without Ca^{2+}). Values are mean ± S.D. of 6 to 12 preparations. Values in parenthesis are the percent decrease without Ca^{2+} compared to the control value with Ca^{2+}.

= 12) to 0.23 ± 0.06 μM (n = 5; P < 0.05) by nifedipine. Nifedipine did not affect the $[Ca^{2+}]_i$ response to vasopressin or bradykinin in the presence of external Ca^{2+} (results not shown). The dihydropyridine Ca^{2+} channel agonist BAY K8644 (32) by itself increased $[Ca^{2+}]_i$ to 0.50 ± 0.06 μM (n = 4) in the presence of external Ca^{2+} and to 0.34 ± 0.04 (n = 5) in the absence of external Ca^{2+}. Surprisingly, BAY K8644 decreased the $[Ca^{2+}]_i$ response to PDGF in the presence of external Ca^{2+} to 0.57 ± 0.09 μM (n = 4, P < 0.01 compared to PDGF alone), but had no effect on the $[Ca^{2+}]_i$ response to PDGF in the absence of external Ca^{2+} with a value of 0.38 ± 0.06 μM (n = 5, P > 0.05 compared to PDGF alone).

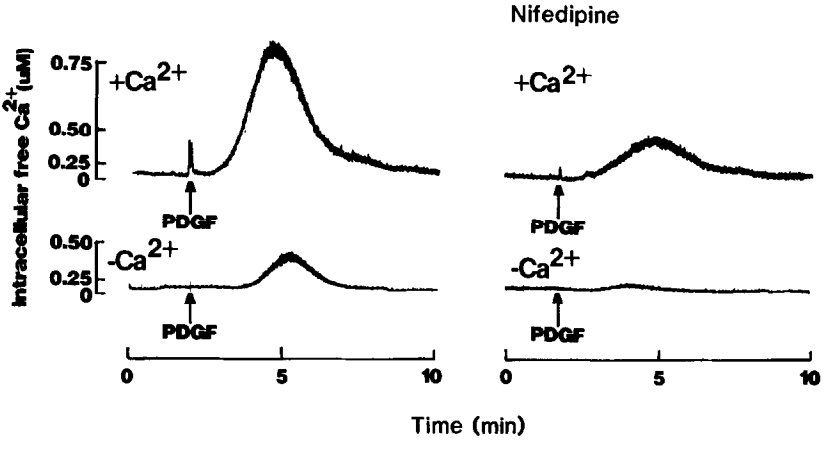

Figure 2. Effect of nifedipine on the PDGF-induced increase in $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts. Cells were exposed to 3.3 x 10^{-9} M PDGF at the arrows. The upper panels show the $[Ca^{2+}]_i$ response in medium containing 1.8 mM Ca^{2+} and the lower panels the response in Ca^{2+} free medium with 0.5 mM EGTA. The right panels of each pair show the responses after 2 min exposure to 10^{-5} M nifedipine.

Table 2. **Effect of Ca^{2+} Channel Ligand on $^{45}Ca^{2+}$ Uptake and Release in Permeabilized Swiss 3T3 Cells**

Addition	6 min $^{45}Ca^{2+}$ pmol/10^6 cells	7 min $^{45}Ca^{2+}$ pmol/10^6 cells	Release %
None	179 ± 8	164 ± 9	8
IP_3 at 6 min			
Control	167 ± 6	85 ± 5[a]	49
Nifedipine, 10 μM	188 ± 4	98 ± 24[a]	48
BAY K8644, 10 μM	158 ± 2[b]	123 ± 2[a]	22
$LaCl_3$, 1 mM	424 ± 36[b]	410 ± 37	3

[a] $p < 0.05$ compared to 6 min value before IP_3.
[b] $p < 0.05$ compared to 6 min value with no additions.
Saponin permeabilized cells, 0.5 x 10^6/ml, were incubated at 37°C in medium containing 50 μM total $^{45}Ca^{2+}$ buffered with EGTA to a free concentration of 100 nM, a 1 mM ATP generating system, and 0.5 mM 2,4-dinitrophenol, 10 μM antimycin A and 2 μg/ml oligomycin to inhibit mitochondrial function. Cellular $^{45}Ca^{2+}$ uptake was measured by collecting the cells on a filter and was shown to be maximal by 6 min. Inositol-1,4,5-trisphosphate (IP_3), 10 μM, was added at 6.25 min and $^{45}Ca^{2+}$ remaining in the cells measured at 7 min. Nifedipine, BAY K8644 and $LaCl_3$ were added at 0 min. Values are mean ± S.D. of 5 determinations each.

Ca^{2+} Channel Ligands and $^{45}Ca^{2+}$ Uptake and Release

The uptake and release of $^{45}Ca^{2+}$ was measured in saponin-permeabilized Swiss 3T3 fibroblasts (Table 2). Inositol 1,4,5-trisphosphate, 10 μM, was used to release the stored $^{45}Ca^{2+}$. Nifedipine, 10 μM, had no significant effect on the uptake or release of $^{45}Ca^{2+}$ in this system. BAY K8644, 10 μM, produced a small decrease in $^{45}Ca^{2+}$ uptake, and partly blocked the release of $^{45}Ca^{2+}$ by inositol 1,4,5-trisphosphate. $LaCl_3$, 1 mM, increased the uptake of $^{45}Ca^{2+}$ and completely blocked $^{45}Ca^{2+}$ release by inositol 1,4,5-trisphosphate.

DISCUSSION

We have investigated a number of agents reported to be growth factors or mitogens for Swiss 3T3 fibroblasts (25,33-36). Many of these agents have been found to increase $[Ca^{2+}]_i$ including PDGF (24,26), EGF (21), bombesin (26,37), prostaglandin $F_{2\alpha}$ (21,22), prostaglandin E_1 (36), and vasopressin (21,26,37). Hesketh et al. (21) have reported that Ca^{2+} free medium and EGTA produces a decrease in the $[Ca^{2+}]_i$ response to vasopressin in Swiss 3T3 fibroblasts. In our study the use of Ca^{2+}-free medium and EGTA had no effect on the increase in $[Ca^{2+}]_i$ produced by vasopressin. A lack of an effect of EGTA on the $[Ca^{2+}]_i$ response to vasopressin in Swiss 3T3 fibroblasts has also been reported by Yamashita and Takai (36). We also found that external Ca^{2+} made no contribution to the increase in $[Ca^{2+}]_i$ produced by bradykinin in Swiss 3T3 fibroblasts, confirming our previous observations (25), or to the increase in $[Ca^{2+}]_i$ produced by bombesin and prostaglandin E_2. The $[Ca^{2+}]_i$ response to bombesin has been found not to be noticeably decreased by EGTA in human small cell lung cancer cell lines (38).

A number of other growth factors and mitogens we studied appeared able to cause both the release of internal Ca^{2+} and the influx of external Ca^{2+}, thus increasing $[Ca^{2+}]_i$. PDGF has been shown to cause the release of $^{45}Ca^{2+}$ from internal stores in Balb/c-3T3 fibroblasts (20). Moolneaar et al. (14) has reported that EGTA has no effect on the increase in $[Ca^{2+}]_i$ caused by PDGF in human fibroblasts. We found that in Swiss 3T3 fibroblasts EGTA decreased by more than half the $[Ca^{2+}]_i$ increase with PDGF. We also found that prostaglandins E_1 and $F_{2\alpha}$ caused an increase in $[Ca^{2+}]_i$ that was dependent in part upon the presence of external Ca^{2+}. Yamashita and Takai (36) have reported that EGTA causes a marked inhibition of the increase in $[Ca^{2+}]_i$ caused by prostaglandin $F_{2\alpha}$ in Swiss 3T3 fibroblasts. Hesketh et al. (22), however, reported no effect of EGTA on the increase in $[Ca^{2+}]_i$ caused by prostaglandin E_1 in Swiss 3T3 fibroblasts.

Swiss 3T3 fibroblasts are known to possess two types of voltage-gated Ca^{2+} channels, classified as L and T (39). We used the specific L type Ca^{2+} channel antagonist nifedipine (40) and the agonist BAY K8644 (31) to study the role of voltage-gated Ca^{2+} channels in PDGF action. Nifedipine was found to significantly decrease the $[Ca^{2+}]_i$ response to PDGF by Swiss 3T3 fibroblasts in Ca^{2+} containing medium, suggesting that Ca^{2+} entry may occur through voltage-gated Ca^{2+} channels activated by PDGF. BAY K8644, but not nifedipine, produce an increase in $[Ca^{2+}]_i$ by itself. This was presumably by increasing the influx of Ca^{2+} through the same channels that were activated by PDGF and blocked by nifedipine. A small increase in $[Ca^{2+}]_i$ by BAY K8644 was also seen in the absence of external Ca^{2+} suggesting that BAY K8644 can also mobilize internal Ca^{2+}. It is noteworthy that the increase in $[Ca^{2+}]_i$ produced by BAY K8644 was relatively slow and exhibited a similar time course to the increase in $[Ca^{2+}]_i$ caused by PDGF. It was different from the rapid increase in $[Ca^{2+}]_i$ caused by, for example, bradykinin that increased $[Ca^{2+}]_i$ exclusively by release by internal Ca^{2+}. Although believed to act on voltage-gated Ca^{2+} channels there are reports that both nifedipine (41) and BAY K8644 (42) can act on mitogen activated voltage-insensitive Ca^{2+} channels in T lymphocytes.

Nifedipine decreased the $[Ca^{2+}]_i$ response to PDGF caused by the release of Ca^{2+} from internal stores when Ca^{2+} was absent from the external medium. An increased synthesis of inositol 1,4,5-trisphosphate has been suggested to be the mechanism by which PDGF produces a release of internal Ca^{2+} (43), although recent work has cast doubt on this mechanism (44,45). Nifedipine did not block the release of Ca^{2+} by inositol 1,4,5-trisphosphate in permeabilized Swiss 3T3 fibroblasts. Nifedipine has been reported not to block inositol-1,4,5-trisphosphate induced Ca^{2+} release in other cell types (43). Whether nifedipine could be having a direct effect on the binding of PDGF to its receptor, or on the coupling of the PDGF receptor to its effector mechanisms is not known. The weight of the evidence suggests, however, that the main action of nifedipine in preventing the $[Ca^{2+}]_i$ increase with PDGF is to block a plasma membrane Ca^{2+} channel responsible for Ca^{2+} entry.

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