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Phosphatidic Acid Induces Calcium Influx in Neutrophils via Verapamil-Sensitive Calcium Channels

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Abstract Phosphatidic acid (PA) induces a biphasic Ca^{2+} mobilization response in human neutrophils. The initial increase is due to the mobilization of Ca^{2+} from intracellular stores, whereas the secondary increase is due to the influx of Ca^{2+} from extracellular sources. The present investigation characterizes PA-induced Ca^{2+} influx in neutrophils. Depolarization of neutrophils by 50 mM KCl enhanced PA-induced Ca^{2+} influx, whereas verapamil, a Ca^{2+} channel blocker, attenuated this response in a dose-dependent manner. These observations suggest that PA-induced Ca^{2+} influx is mediated via verapamil-sensitive Ca^{2+} channels. Stimulation of neutrophils with exogenous PA results in accumulation of endogenously generated PA with a time course similar to the effects of exogenous PA on Ca^{2+} influx. Ethanol inhibited the accumulation of endogenous PA and calcium mobilization, indicating that activation of membrane phospholipase D plays a role in PA-mediated Ca^{2+} influx. The results of this study suggest that exogenously added PA stimulates the generation of intracellular PA, which then mediates Ca^{2+} influx through verapamil-sensitive Ca^{2+} channels. J. Cell. Biochem. 78:297–304, 2000.

Key words: phosphatidic acid; neutrophils; calcium influx; verapamil

Exogenous phosphatidic acid (PA) has been shown to transport calcium in a variety of cellular systems including smooth muscle [Salmon and Honeyman, 1980], parotid gland [Putney et al., 1980], platelets [Gerrard et al., 1978], synaptosomes [Harris et al., 1981], hepatocytes [Barritt et al., 1981], mast cells [Pearce and Messis, 1982], neuroblastoma cells [Ohsako and Deguchi, 1981], and cultured heart cells [Ohsako and Deguchi, 1981]. Earlier studies demonstrated that PA acts as either a Ca^{2+} ionophore [Putney et al., 1980; Tyson et al., 1976] or opens a specific Ca^{2+} gate in various membranes [Ohsako and Deguchi, 1981]. Studies characterizing PA as a Ca^{2+} transporter or ionophore in an artificial membrane system have ruled out PA as a Ca^{2+} ionophore [Holmes and Yoss, 1983]; however, it is possible that PA binds to cellular membranes and alters calcium channel activity.

In neutrophilic leukocytes, PA induces calcium mobilization in a biphasic manner. Stimulation of neutrophils with PA induces an initial increase in cytosolic Ca^{2+}, which occurs within seconds, followed by a more persistent secondary increase after several minutes. The biphasic nature of agonist-activated calcium mobilization has previously been described [Putney, 1978; Putney et al., 1981; Droogmans et al., 1985; Putney, 1986; Parekh et al., 1993]. Putney [1986] proposed a capacitative Ca^{2+} entry theory explaining the biphasic nature of agonist-activated Ca^{2+} mobilization. Agonists induce the initial increase by mobilizing calcium from intracellular stores through an inositol 1,4,5 trisphosphate-mediated process. This mobilization of calcium results in emptying of the IP_3-sensitive intracellular Ca^{2+} pool. This decrease in Ca^{2+} content of the intracellular pool relieves an inhibitory constraint and subsequently provokes an influx of calcium from extracellular sources [Putney, 1986]. Our previous studies have also demonstrated that the PA-induced initial increase in cytoplasmic

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Ca^{2+} is a result of the mobilization of calcium from intracellular stores, whereas the secondary increase is caused by an influx from the extracellular source [Siddiqui and English, 1997]. We have described the regulation of PA-induced intracellular Ca^{2+} mobilization in neutrophils. This process appears to be mediated by tyrosine phosphorylation [Siddiqui and English, 1997]. However, it is interesting to note that inhibition of tyrosine kinase activity by herbimycin-A [Siddiqui and English, 1997] or phosphatidylinositol 3′-kinase (PI 3′-kinase) activity by LY294002 or Wortmannin [Siddiqui and English, 1999] has no effect on PA-induced Ca^{2+} influx. These observations suggest that PA-induced Ca^{2+} influx does not depend on the initial Ca^{2+} mobilization from intracellular stores, but appears to be an independent process. This indicates that PA acts differently than other agonists [Putney, 1986] and has independent effects on Ca^{2+} mobilization from intra- and extracellular sources. The present study was undertaken to describe PA-induced calcium influx in neutrophils. The results of this study suggest that exogenous PA activates phospholipase D, which results in intracellular generation of PA and causes Ca^{2+} influx through a verapamil-sensitive Ca^{2+} channel.

MATERIALS AND METHODS

Reagents

Dicapryl PA (DiC10-PA) was obtained from Avanti Polar Lipids, Alabaster, AL. Fura 2-acetoxymethyl ester (fura-2AM) and verapamil came from Calbiochem, San Diego, CA. [^{32}P] Pi (8,500–9,120 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Other chemicals and reagents came from Sigma Chemical Co., St Louis, MO.

Isolation of Neutrophils

Neutrophils were isolated from the blood of healthy donors using ficoll-Hypaque density gradient centrifugation as previously described [English et al., 1981]. After enhanced erythrocyte sedimentation at room temperature, buffy coats were layered on a 3-ml cushion layer of ficoll-Hypaque and centrifuged at 800 g for 20 min. The pellets were washed once with Hanks' balanced salt solution (HBSS), and contaminating erythrocytes were removed by isotonic ammonium chloride lysis. Neutrophils were washed and suspended at a final concentration of 1.0×10^7/ml in HBSS unless otherwise indicated.

Determination of Intracellular Free Calcium

Neutrophils were loaded with fura-2AM as described previously [English et al., 1987]. Briefly, cells (1×10^7) were incubated in HBSS in the presence of 5 μM fura-2AM for 15 min at 37°C. After incubation, the cells were washed twice with modified (Ca^{2+}- and Mg^{2+}-free) HEPES buffered (1 mg/ml) HBSS (pH 7.2) and finally resuspended in the same buffer prewarmed at 37°C. Cells (1×10^6) were preincubated for 1 min in modified HEPES buffered HBSS containing approximately 120 μM Ca^{2+} at 37°C in a temperature-controlled, Perkin-Elmer LS50B luminescence spectrofluorometer (Perkin-Elmer Ltd., Buckinghamshire, England) to determine baseline fluorescence at 340 nm excitation and 510 nm emission. After stimulation under the conditions listed below, fluorescence emission was continuously monitored for 5 min to determine relative alterations in intensity. The changes in fluorescence intensities at 340 nm are related to changes in intracellular calcium concentrations as determined previously [Siddiqui and English, 1997]. The concentration of free Ca^{2+} was maintained at 120 ± 20 μM by using 2 mM EGTA and 1.25 mM $CaCl_2$ in incubation mixtures with neutrophils. The concentration of Ca^{2+} in the buffer was routinely monitored by aequorin chemiluminescence as described previously [Siddiqui and English, 1997].

Demonstration of Intracellular PA Generation

Neutrophils (1×10^7/ml) suspended in low Ca^{2+} (100–120 μM) HBSS were incubated with [^{32}P] inorganic phosphate (10 μCi/ml) for about 3 h in a humidified CO_2 incubator at 37°C. The cells were washed four times with low Ca^{2+} HBSS and then suspended in the same buffer. Cells (1×10^6) were stimulated with DiC10-PA (30 μM) for the time periods indicated in the figure legends in a total volume of 300 μl. The reaction was stopped by adding 1 ml of chloroform:methanol:HCl (1:2:0.03), and the phospholipids were extracted by adding 500 ml of chloroform and distilled water. The extracts, along with different phospholipid standards, were dried and spotted on 20 × 20-cm silica gel thin-layer chromatography plates (K6F, Whatman, NJ). Plates were devel-

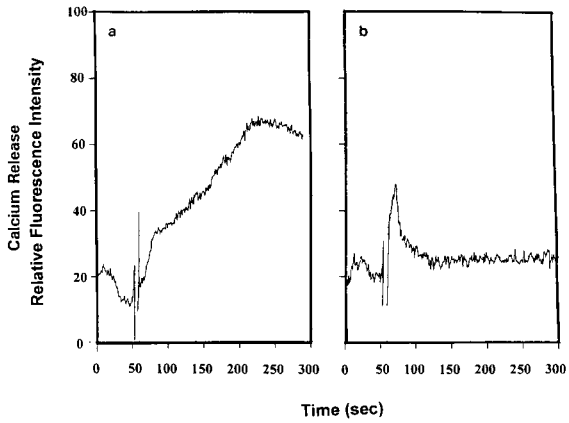


Fig. 1. Stimulation of calcium influx by phosphatidic acid. Neutrophils (10^7/ml) were labeled with fura 2-acetoxymethyl ester (5 μM) for 15 min at 37°C in Hanks Balanced Salt Solution (HBSS), then washed twice and resuspended in modified HBSS containing approximately 100 μM free Ca^{2+}. Cells (10^6) were briefly incubated (approximately 1 min) in a thermocontrolled (37°C) spectrofluorometer and then stimulated with 30 μM dicapryl PA in the absence (**a**) or presence (**b**) of 2 mM EGTA. Assays of calcium mobilization were carried out using an excitation wave-length of 340 nm, and fluorescence emission was quantified as relative intensities at 510 nm. The results shown represent at least three experiments.

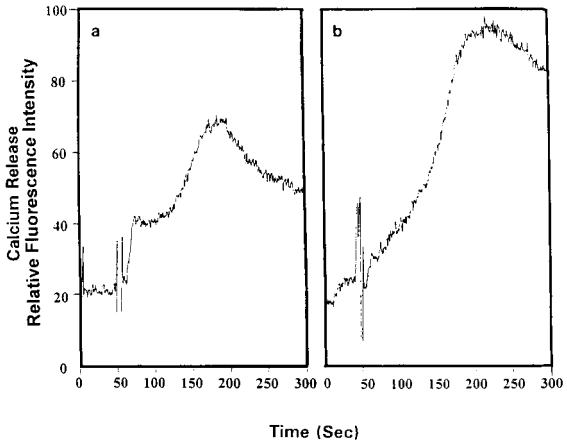


Fig. 2. Effect of membrane depolarization on phosphatidic acid-induced calcium influx. Cells were labeled with fura 2-acetoxymethyl ester as described in the legend for Fig. 1. Cells were stimulated in the absence (**a**) or presence (**b**) of 50 mM KCl. Assays of calcium mobilization were carried out using an excitation wave-length of 340 nm, and fluorescence emission was quantified as relative intensities at 510 nm. The results shown represent at least three experiments.

oped using a one-dimensional solvent system consisting of chloroform:methanol:20% aqueous methylamine (60:35:10). The plates were then dried and autoradiographed. The phospholipid standards were developed by exposing the plates to iodine.

Phospholipase D Assay

Neutrophils were stimulated with DiC10-PA in the presence or absence of 0.05% ethanol as described above. The phospholipids were extracted and separated by thin-layer chromatography as described above. The separated phospholipids were detected by charring (190°C for 15 min) after saturation with 5% cupric sulfate [Siddiqui and Exton, 1992]. The spots for PA were identified based on the authentic phospholipid standards.

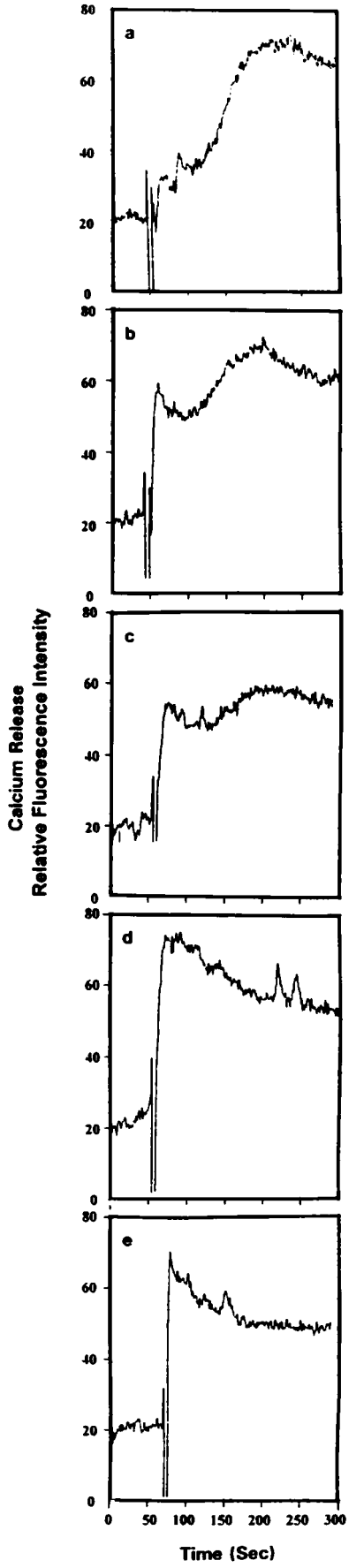
RESULTS

Calcium Mobilization in Neutrophils by Phosphatidic Acid

The effects of PA on calcium mobilization in neutrophils are shown in Fig. 1. PA induced a biphasic response of calcium mobilization in neutrophils. EGTA, an extracellular Ca^{2+} chelator, did not inhibit the initial increase of PA-induced cytoplasmic calcium, but completely attenuated the secondary cytoplasmic Ca^{2+} increase. These results clearly indicate that the initial increase in cytoplasmic Ca^{2+} resulted from the mobilization of calcium from intracellular stores; however, the subsequent and sustained increase in cytosolic calcium resulted from an influx of calcium from the extracellular stores.

Effect of Membrane Depolarization on Phosphatidic Acid-Induced Calcium Influx

The results from previous experiments indicate that exogenously applied PA induces an influx of Ca^{2+} in neutrophils. Therefore, we examined the hypothesis that PA-induced secondary increases in cytoplasmic Ca^{2+} are mediated through a Ca^{2+} channel. Neutrophils were incubated in the presence of 50 mM KCl to depolarize the plasma membranes. Membrane depolarization increases the open state probability of voltage-sensitive Ca^{2+} channels. The degree of membrane depolarization, as predicted from the Nernst equation, appears to be 24.4 mV. The results shown in Fig. 2 demonstrate that PA-induced calcium influx increased greatly in the presence of KCl. This observation suggests that PA-induced calcium influx is mediated through a voltage-dependent process.



Effects of Verapamil on Phosphatidic Acid-Induced Calcium Influx

In a subsequent experiment, we described PA activation of a calcium channel. The results shown in Fig. 3 demonstrate that verapamil, which blocks voltage-sensitive Ca^{2+} channels, caused a dose-dependent (10–100 μM) inhibition of PA-induced calcium influx. These results suggest that PA-induced calcium influx is mediated through a verapamil-sensitive calcium channel in human neutrophils.

Stimulation of Endogenous PA Generation by Exogenous PA

In preliminary experiments we investigated the formation of various second messengers in neutrophils stimulated with PA. The results shown in Fig. 4 demonstrate that stimulating neutrophils with exogenous PA causes enhanced formation of endogenous PA with a time course similar to its effects on stimulation of Ca^{2+} influx. Because the phospholipid pool inside the cells was prelabeled with radioactive phosphate and the exogenous PA was not radioactive, any radioactive PA generated from stimulated neutrophils must have come exclusively from intracellular degradation of phospholipids. These results suggest that exogenous PA stimulated the generation of intracellular PA.

Activation of Phospholipase D by Phosphatidic Acid

Phosphatidic acid in cells is derived either from the breakdown of phospholipids through direct-action phospholipase D (PLD) activity or indirectly through phosphorylation of diacylglycerol with ATP through diacylglycerol kinase activity. Because it is known that PA can stimulate PLD activity (see Discussion section), we initially measured its direct synthesis. Ethanol reacts only with intracellularly generated PA from PLD activation and converts it into phosphatidylethanol (Peth). Diversion of PA into Peth results in decreased accu-

Fig. 3. Effect of verapamil on phosphatidic acid-induced calcium influx. Cells were labeled with fura 2-acetoxymethyl ester as described in the legend for Fig. 1. Cells were stimulated in the presence of verapamil **a:** 0 μM; **b:** 10 μM; **c:** 25 μM; **d:** 50 μM; **e:** 100 μM. Assays of calcium mobilization were carried out using an excitation wave-length of 340 nm, and fluorescence emission was quantified as relative intensities at 510 nm. The results shown represent at least three experiments.

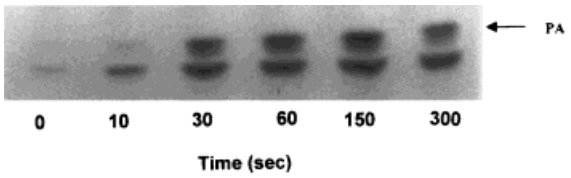


Fig. 4. Generation of endogenous [^{32}P] phosphatidic acid formation by exogenous phosphatidic acid. Neutrophils (1×10^7/ml) were labeled with [^{32}P] inorganic phosphate (10 μCi/ml) for 3 h in a humidified CO_2 incubator at 37°C as described in the text. The labeled cells (1×10^6) were stimulated with dicapryl PA (30 μM) and the phospholipids were extracted as described in the text. The lipids were separated on 20 × 20-cm silica gel thin-layer chromatography plates (K6F, Whatman, NJ), along with different phospholipid standards, using a one-dimensional solvent system [chloroform:methanol:20% aqueous methylamine (60:35:10)]. The separated phospholipids were autoradiographed and identified by comparing to authentic phospholipid standards.

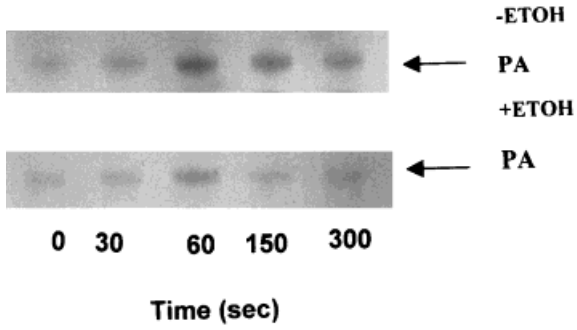


Fig. 5. Effect of phosphatidic acid (PA) on phospholipase D activation. Neutrophils were stimulated with dicapryl PA in the presence or absence of 0.025% ethanol as described above. The phospholipids were extracted and separated by thin-layer chromatography as described in the legend for Fig. 5. The separated phospholipids were detected by charring (190°C, 15 min), after saturation with 5% cupric sulfate, as described in the text. The spots for PA were identified based upon the authentic phospholipid standards.

mulation of PA in cells. Although this assay is less sensitive in measuring accumulation of PA, it is a specific assay for PLD activation. Results shown in Fig. 5 demonstrate that in the presence of ethanol, accumulation of PA was substantially reduced in neutrophils upon stimulation with exogenously added PA. Accumulation of Peth was also observed in PA-stimulated cells in the presence of ethanol on thin-layer chromatographs (data not shown). These observations demonstrate that extracellular PA stimulates the generation of intracellular PA, and in the presence of ethanol, attenuates the accumulation of intracellularly generated PA. These results clearly suggest that most intracellular generation of PA is derived from PLD actions on phospholipids. As most of the PA accumulation in neutrophils is blocked by ethanol, these results also indicate that generation of PA indirectly from phosphorylation of diacylglycerol with ATP through diacylglycerol kinase may be negligible.

Effect of Ethanol on PA-Induced Calcium Influx

We then investigated the possibility that the inhibition of intracellular generation of PA by ethanol has an effect on PA-induced calcium mobilization. The results presented in Fig. 6 show that ethanol has no effect on PA-induced calcium mobilization from intracellular stores (first peak), but inhibits the secondary calcium influx, presumably due to inhibition of the intracellular accumulation of PA.

DISCUSSION

Phosphatidic acid induces a classic agonist-mediated biphasic response of calcium mobilization in human neutrophils. Our results demonstrate that the initial increase is a result of calcium mobilization from intracellular stores, whereas the subsequent secondary increase is caused by influx of calcium from extracellular sources. We have previously demonstrated that inhibition of PA-induced calcium mobilization from intracellular stores by protein tyrosine kinase inhibitors [Siddiqui and English, 1997] or PI 3′-kinase inhibitors [Siddiqui and English, 2000] has no effect on PA-induced Ca^{2+} influx. These observations suggest that the calcium influx is not regulated through a capacitative Ca^{2+} entry mechanism [Putney, 1986]. Therefore, this investigation was undertaken to describe PA-induced Ca^{2+} influx in human neutrophils.

The results of our investigation clearly suggest that PA-induced Ca^{2+} influx is mediated through a Ca^{2+} channel, because the depolarization of membranes by KCl resulted in the enhancement of PA-induced Ca^{2+} influx. Neutrophils are nonexcitable tissues, and it is not clear if these cells exhibit time-dependent closing of calcium channels on depolarization. Further electrophysiology experiments using patch clamp technique are required to demonstrate this fact. However, during depolarization (Vm = 24.4 mV), mobilization of Ca^{2+} from intracellular stores (initial peak) was still

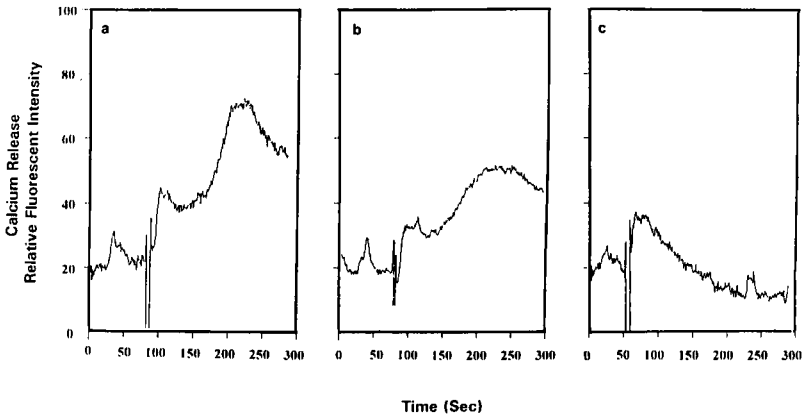


Fig. 6. Effect of ethanol on phosphatidic acid-induced calcium influx. Cells were labeled with fura 2-acetoxymethyl ester as described in the legend for Fig. 1. Cells were stimulated in the absence (**a**) or presence of 0.025% (**b**) or 0.05% (**c**) ethanol. Assays of calcium mobilization were carried out using an excitation wave-length of 340 nm, and fluorescence emissions were quantified as relative intensities at 510 nm. The results shown represent at least three experiments.

present but it was relatively smaller than in the absence of KCl. This effect could be a result of depolarization-mediated increased probability of Ca^{2+} channel opening, causing enhanced Ca^{2+} influx when cells are stimulated with PA. Verapamil, a Ca^{2+} channel blocker, inhibited PA-stimulated Ca^{2+} influx in a dose-dependent manner. This observation further suggests that PA-induced Ca^{2+} influx is mediated through a Ca^{2+} channel. However, we noted that verapamil caused stimulation of PA-induced Ca^{2+} mobilization from intracellular stores (the first peaks shown in Fig. 3), but had inhibitory effects on PA-induced Ca^{2+} influx (the second peaks shown in Fig. 3). The reasons verapamil stimulates PA-induced intracellular calcium mobilization are not clear. It is possible that a complete inhibition of the verapamil-sensitive Ca^{2+} channel may have resulted in a feedback mechanism, causing excessive Ca^{2+} mobilization from intracellular stores. It is also possible that, in some way, verapamil influenced the restraint mechanism that regulates Ca^{2+} mobilization from the intracellular stores. Alternatively, verapamil may have caused mobilization of Ca^{2+} from a different intracellular source, probably through an IP_3-insensitive mechanism or through a ryanodine-sensitive calcium release mechanism.

Verapamil, in addition to inhibiting calcium agonist effects, also interferes with the effects of other agonists. For example, verapamil inhibits responses elicited by platelet activating factor through a mechanism other than the inhibition of Ca^{2+} influx in neutrophils [Filep and Foldes-Filep, 1990]. Furthermore, verapamil inhibits dofetilide binding to leukocytes and thus alters dofetilide effects through a Ca^{2+}-activated K^+ channel [Geonzon et al., 1998]. The mechanisms of verapamil-induced stimulation of Ca^{2+} mobilization from intracellular stores by PA were not explored during the present investigation. However, it is clear from these observations that PA-stimulated Ca^{2+} influx is mediated through a verapamil-sensitive Ca^{2+} channel. We realize that verapamil is a rather nonspecific calcium channel blocker, and this inhibitor was used as a screening tool during the present study to demonstrate the fact that PA-induced calcium influx is regulated through a calcium channel. Clearly, additional experiments are needed to identify the nature and type of calcium channels on neutrophils using specific inhibitors.

The existence of verapamil-sensitive Ca^{2+} channels on human neutrophils and the important role they play in the functional activation of neutrophils have previously been reported [Horl et al., 1995; Weisdorf and Thayer, 1989; Khalfi et al., 1998; Ishizaki et al., 1997]. However, not much is known about the mechanism of neutrophil activation and regulation. This study demonstrates that verapamil-sensitive Ca^{2+} channels appear to be activated by endogenously generated PA. Inhibition of endogenous PA formation in the presence of ethanol suggests that PLD is activated [Kanfer, 1980; Exton, 1997] by exogenously added PA. In addition, PA-induced Ca^{2+} influx was also inhibited in the presence of ethanol, suggesting that the inhibition of endogenous PA generation may be involved in the regulation of Ca^{2+} influx. The results of this study (results not shown) and of studies reported by others [Patel et al., 1996; Nilsson et al., 1995] have shown that ethanol itself does not have any effect on

the baseline Ca^{2+} levels in neutrophils. Furthermore, Ca^{2+} influx induced by other agonists through PLD activation is also attenuated by ethanol [Brandiolini et al., 1996]. Moreover, enhanced formation of PA in neutrophils [Perry et al., 1993] and other cells [Bursten et al., 1992; Bursten and Harris, 1994], upon stimulation with exogenous PA, has previously been reported. Consistent with these observations, a recent study has shown that PA can stimulate PLD by interacting at an allosteric site in the phospholipid bilayer [Geng et al., 1998]. Therefore, it appears that exogenous PA causes activation of PLD and results in the generation of endogenous PA, which subsequently mediates Ca^{2+} influx through a verapamil-sensitive pathway.

Results from other investigations [Murayama and Ui, 1987] and from our laboratory (unpublished observation) have demonstrated that PA can be released into the extracellular medium from cells undergoing hormone stimulation. This allows PA action at the cell surface in both an autocrine and paracrine mechanism. These observations suggest that PA can act as an extracellular agonist and mediate its effects through a specific receptor [Pearce et al., 1994]. In addition, PA also induces its own generation and performs its role as an intracellular messenger. As previously suggested [Ohsako and Deguchi, 1981], it is possible that the intracellularly generated PA binds to a channel in the lipid bilayer and induces Ca^{2+} influx. Further experimentation is required to explore this possibility. In conclusion, our investigation indicates that PA causes calcium influx in neutrophils via verapamil-sensitive calcium channels.

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