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CALCIUM CHANNEL ANTAGONIST INDUCED INHIBITION OF SUPEROXIDE PRODUCTION IN HUMAN NEUTROPHILS

MECHANISMS INDEPENDENT OF ANTAGONIZING CALCIUM INFLUX

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Abstract—Three calcium channel antagonists, verapamil, diltiazem and nisoldipine, inhibited superoxide production in human neutrophils that were stimulated by phorbol 12-myristate 13-acetate (PMA) in a buffered saline lacking calcium. Concentrations of these drugs giving 50% control activity (IC_{50}) were 0.3, 0.45 and 0.01 mM respectively. This inhibition was also observed in the presence of ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) and was not reversed by the addition of calcium. This suggests that calcium channel antagonists inhibited superoxide production independently of extracellular calcium. These calcium channel antagonists inhibited the mobilization of membrane-associated calcium, and protein phosphorylation probably catalyzed by C-kinase, both of which are thought to be involved in the signal transmission for the induction of superoxide production. Calcium channel antagonists also inhibited NADPH oxidase, responsible for superoxide production, with IC_{50} = 0.5, 3 and more than 0.08 mM, respectively, for verapamil, diltiazem and nisoldipine. The results indicate that calcium channel antagonists inhibit superoxide production by affecting not only the catalytic activity by also the activation of NADPH oxidase. Inhibition of superoxide production by calcium channel antagonists suggests that these antagonists do not affect cell functions merely by affecting calcium influx.

Calcium channel antagonists act on the cardiovascular system, providing antianginal, antiarrhythmic, antihypertensive and cardiovascular protective effects [1]. They are also used for protection from or treatment of brain ischemic injury [2]. These antagonists also interfere with cell functions in mast cells [3] and platelets [4]. The mechanisms of their action have not been clearly elucidated. Although these antagonists appear to inhibit the entry of calcium across or through cell membranes [5, 6], they may act by blocking calcium efflux or release of calcium from intracellular stores, by facilitating calcium efflux or sequestration, or by preventing calcium interaction with intracellular target proteins [5, 6]. It is also clear that these antagonists can inhibit other membrane-mediated events including ionic currents and receptor processes [7].

Neutrophils produce superoxide, which plays important roles in microbial killing and pathogenesis of adult respiratory distress syndrome [8, 9]. The need of extracellular calcium for superoxide production in human neutrophils is dependent on the stimuli. Leukotriene B_4 does not induce superoxide production in the absence of extracellular calcium, but a chemotactic peptide does induce production in the absence of calcium. The superoxide-producing activity of the cells that are stimulated by a chemotactic peptide in the absence of calcium is about one-fourth the activity in the presence of calcium. The production induced by PMA is essentially independent of extracellular calcium [10]. Among many stimuli which induce superoxide production, phorbol 12-myristate 13-acetate (PMA) and 1-oleoyl-2-acetyl-glycerol (OAG) are characteristic ones, because both of them have been shown to activate calcium-activated phospholipid-dependent protein kinase (C-kinase) in the absence of diacylglycerol *in vitro* [11, 12]. In neutrophils these stimuli induce neither phosphatidylinositol turnover [13] nor a rise in the cytosolic free calcium concentration [14, 15]. We have shown previously that protein phosphorylation is associated with the activation of pig neutrophils stimulated by PMA [16] and that OAG, a direct activator of C-kinase, induces not only protein phosphorylation but also superoxide production in human neutrophils [14]. It has been suggested that either PMA or OAG bypasses the calcium-requiring segment of the activation sequence by direct activation of C-kinase followed by protein phosphorylation and then by activation of NADPH oxidase. The proposed mechanism for the induction of superoxide-producing activity in neutrophils stimulated by PMA or OAG is illustrated in Fig. 1.

Verapamil has been reported to inhibit chemotaxis [17], phagocytosis [18], aggregation [19], degranulation [18] and superoxide production [20, 21] in neutrophils that are stimulated by a chemotactic peptide or opsonized zymosan. The authors of these papers concluded that the inhibition of neutrophil functions by verapamil was due to the inhibition of calcium entry. Verapamil has also been shown to inhibit C-kinase *in vitro* [22].

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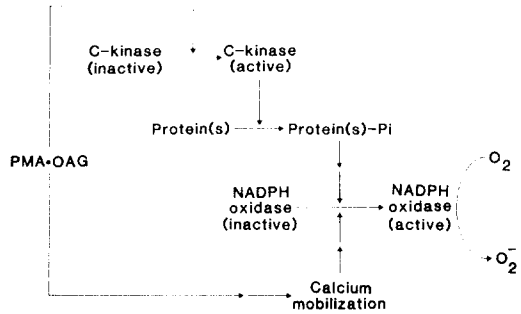

Fig. 1. Proposed mechanism for the induction of NADPH oxidase in neutrophils stimulated by PMA or OAG. See details in the text.

We re-examined the effect of calcium channel antagonists on superoxide production in human neutrophils that were stimulated by PMA. We further examined their effects on mobilization of membrane-associated calcium, protein phosphorylation and NADPH oxidase activity. It should be noticed that calcium channel antagonists could affect human neutrophils at rather high concentrations.

MATERIALS AND METHODS

Materials. Verapamil (a gift from the Eisai Pharmaceutical Co., Tokyo, Japan) was dissolved in dimethyl sulfoxide (Me_2SO) at a concentration of 100 nM. Diltiazem (a gift from the Tanabe Pharmaceutical Co., Tokyo, Japan) was dissolved in a buffered saline at a concentration of 50 mM. Nisoldipine (a gift from Dr. Kanmura, Department of Pharmacology, Kyushu University School of Medicine) was dissolved in 15% (w/v) ethanol and 15% (w/v) polyethylene glycol at a concentration of 0.65 mM. Other materials were obtained as described elsewhere [16, 23, 24].

Cell preparation. Human neutrophils were isolated from ACD-venous blood taken from healthy volunteers as described previously [10] and suspended in a buffered saline consisting of 135 mM NaCl, 5 mM KCl, 2 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-NaOH (pH 7.4).

Assay of superoxide production. Cellular activity for production of superoxide was monitored by assay for superoxide using the reduction of cytochrome *c* in a buffered saline as descibed previously [10].

Mobilization of membrane-associated calcium. We examined mobilization of membrane-associated calcium by the change of fluorescence of chlortetracyline (CTC)-loaded neutrophils [23]. Neutrophils (1×10^7 cells/ml) were incubated for 20 min at 37° in a buffered saline containing 0.1 mM CTC and 1 mM $CaCl_2$, washed once, resuspended in the same buffered saline, and transferred to a Shimazu spectrofluorophotometer RF 500 with a constant temperature cuvette holder (37°).

Protein phosphorylation. Protein phosphorylation in intact cells was determined by two-dimensional gel electrophoresis followed by autoradiography [14, 16].

Assay of NADPH oxidase activity. The activity of NADPH oxidase was measured simultaneously with superoxide production induced by 100 ng/ml PMA [24]. Cellular activity that produced superoxide (3×10^5 cells/ml) was monitored at 25° in a buffered saline containing 1 mM $MgCl_2$. When this activity reached a maximum, Renex 30 and NaN_3 were added to the mixture at a final concentration of 0.05%

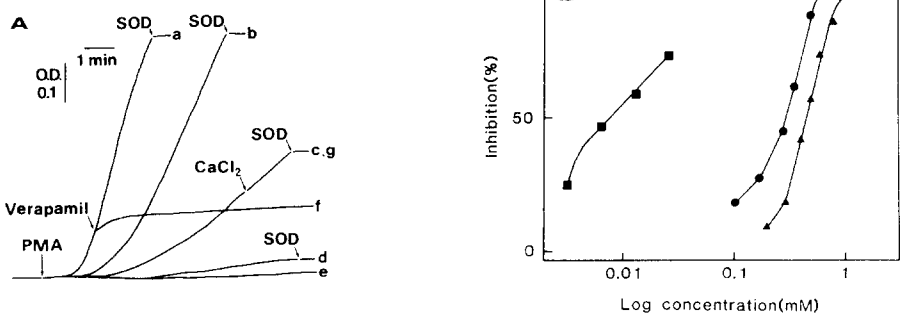

Fig. 2. Effects of calcium channel antagonists on superoxide production in human neutrophils stimulated by PMA. (A) Time course of superoxide production. The assay mixture consisted of 75 μM ferricytochrome *c* and 1×10^6 cells in a buffered saline. The cells were preincubated for 5 min at 37° in a thermostatically controlled cuvette which was placed in a Hitachi 556 dual-wavelength spectrophotometer. The reaction was started by the addition of PMA on a final concentration of 100 ng/ml. The absorbency change at 550 nm in reference to 540 nm was followed on a recorder. Key: (a) control; (b, c, d and e) with 0.3, 0.5, 0.7 and 1.0 mM verapamil, respectively, added 5 min before the addition of PMA; (f) with 1.0 mM verapamil added at the point indicated by an arrow; and (g) with 10 mM $CaCl_2$ added at the point indicated by an arrow during the active production of superoxide in the presence of 0.5 mM verapamil. The reduction of cytochrome *c* was inhibited completely by SOD. (B) Inhibition of superoxide production by verapamil, diltiazem or nisoldipine. The conditions were the same as described for panel A. The rate of superoxide production was calculated from the linear portion of cytochrome *c* reduction using a molar absorption coefficient of $19.1 \times 10^3\ M^{-1}\ cm^{-1}$. The control rate for this experiment was 9.5 nmoles superoxide/10^6 cells per min. Key: (●) verapamil, (▲) diltiazem and (■) nisoldipine.

Table 1. Effects of verapamil on superoxide production in the presence of EGTA or $CaCl_2$

	Control	EGTA (2 mM)	$CaCl_2$ (10 mM)
None	9.7 ± 1.0 (6)	10.2 ± 0.6 (4)	10.8 ± 0.8 (8)
Verapamil			
0.35 mM	3.8 ± 0.4 (6)	3.3 ± 0.3 (5)	ND
0.70 mM	0.1 ± 0.0 (3)	ND	0.5 ± 0.1 (3)

Cells were preincubated with verapamil in the presence or the absence of 2 mM EGTA or 10 mM $CaCl_2$ for 5 min at 37°, and exposed to 100 ng/ml PMA. The maximal rates of superoxide production are given and the values represent the mean ± S.E. with the numbers of experiments in parentheses. ND = not determined.

(w/v) and 10 mM respectively. The activity of the oxidase was measured by adding NADPH 50 sec after the addition of Renex 30 at a final concentration of 0.15 mM. The activity was assessed by superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction.

RESULTS

Superoxide production. Verapamil inhibited superoxide production in human neutrophils stimulated by PMA, whether it was added before stimulation or during active production (Fig. 2A). It caused a decrease in the rate and a prolongation of lag time. Figure 2B shows the inhibition of superoxide production induced by various concentrations of verapamil, diltiazem and nisoldipine. Concentrations of these drugs giving 50% control activity (IC_{50}) were 0.3, 0.45 and 0.01 mM respectively. These antagonists affect superoxide production neither by scavenging radicals [20, 21] nor by destruction of the cells [17]. The highest concentrations of Me_2SO, ethanol and polyethylene glycol in reaction mixtures were 1.51% (w/v), 0.6% (w/v) and 0.6% (w/v), respectively, which did not inhibit superoxide production. The order of potency for inhibiting superoxide production is the same as that for vasodilation [1]. To confirm that calcium channel antagonists did not act by inhibiting a calcium influx, we carried out the following experiments (Table 1). PMA was able to induce superoxide production in neutrophils whether in a medium without calcium or in the presence of a calcium chelator, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA). The addition of EGTA to a reaction mixture did not affect the inhibitory effect of verapamil. Furthermore, a high concentration of extracellular calcium did not reverse the inhibition induced by verapamil (Fig. 2, trace g). These results suggested that calcium channel antagonists inhibited superoxide production independently of extracellular calcium in neutrophils that were stimulated by PMA.

Mobilization of intracellular calcium. The observed change related to calcium metabolism in neutrophils that are stimulated by PMA is the mobilization of membrane-associated calcium. To examine the effect of calcium channel antagonists on mobilization of membrane-associated calcium, we utilized CTC as a probe for intracellular membrane-associated calcium and its mobilization. PMA induced a decrease of CTC-fluorescence due to the mobilization of calcium from an intracellular hydrophobic environment (Fig. 3, trace a); this decrease was inhibited by verapamil at concentrations which inhibited superoxide production (Fig. 3, traces c and d). Verapamil also inhibited a spontaneous decrease of the fluorescence in a dose-dependent manner, suggesting that verapamil stabilized or fixed membrane-associated calcium. Diltiazem and nisoldipine inhibited the fluorescence change in the same manner (data not shown). Calcium channel antagonists did not inhibit the fluorescence spectra of the CTC–calcium complex.

Protein phosphorylation. We examined the effect of calcium channel antagonists on phosphorylation of proteins in intact cells that were preloaded with radioactive phosphate and stimulated by PMA. Figure 4, panels A and B, show that, upon exposure of cells to PMA, the incorporation of ^{32}P began to increase in at least ten proteins, relative to resting cells. Spot No. 9 is probably a light chain of myosin [25]. Among the proteins numbered in Fig. 4, phosphorylation of M_r = 49,000, 19,500 and 19,000 proteins (spots No. 4, 8 and 9) correlated well with superoxide production with respect to the time–courses, the dose–responses and the inhibition by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), a relatively specific inhibitor of C-kinase*. Calcium channel antagonists inhibited phosphorylation of

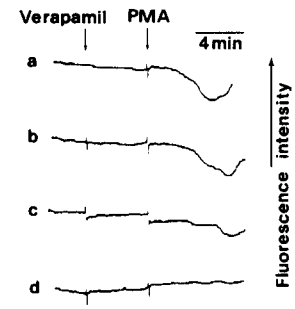

Fig. 3. Fluorescence change in the CTC-loaded neutrophils. The fluorescence was monitored at 514 nm with the excitation wavelength at 410 nm. Verapamil and PMA (100 ng/ml) were added sequentially at the points indicated. Key: (a) control cells; and (b, c and d) with 0.01, 0.3 and 0.7 mM verapamil respectively.

* Fujita, manuscript submitted for publication.

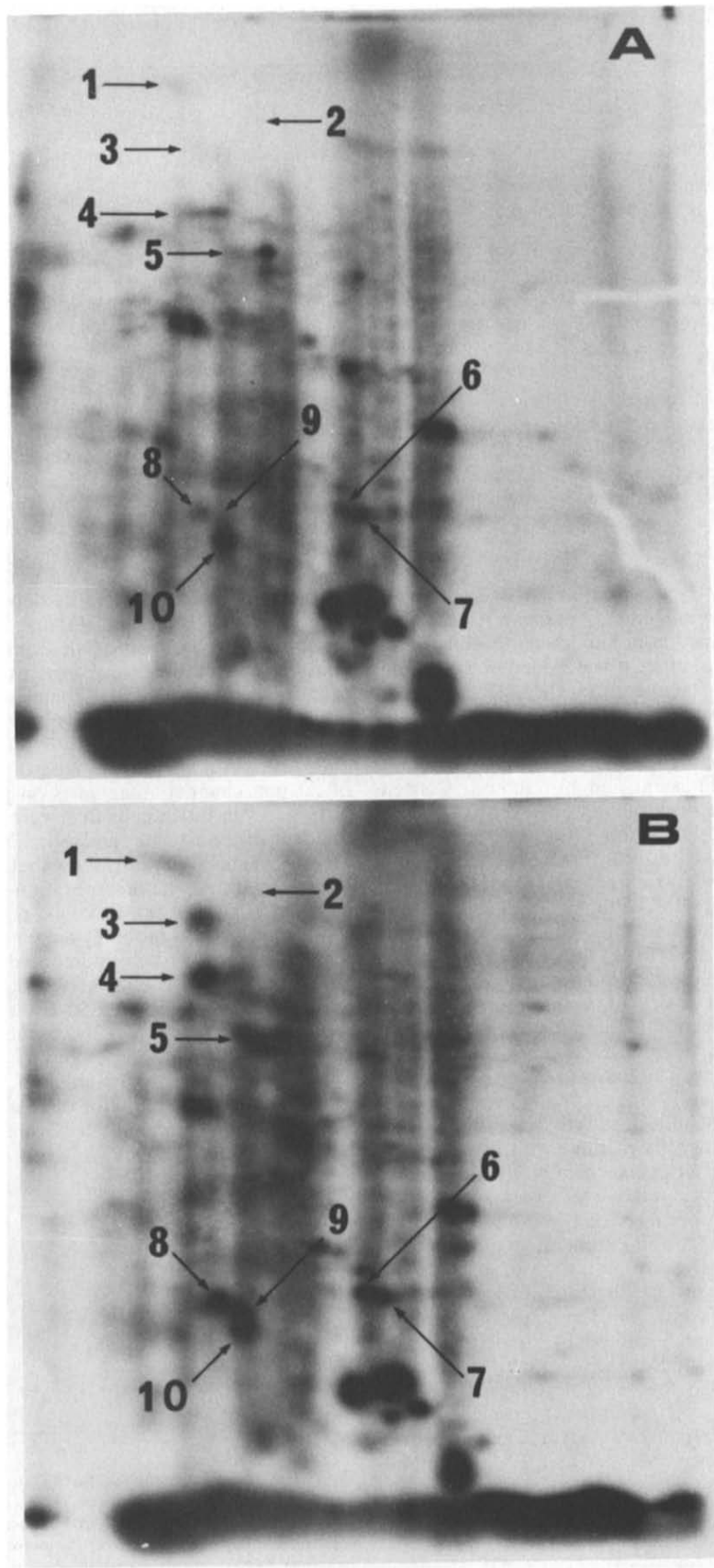

Fig. 4(A) and (B).

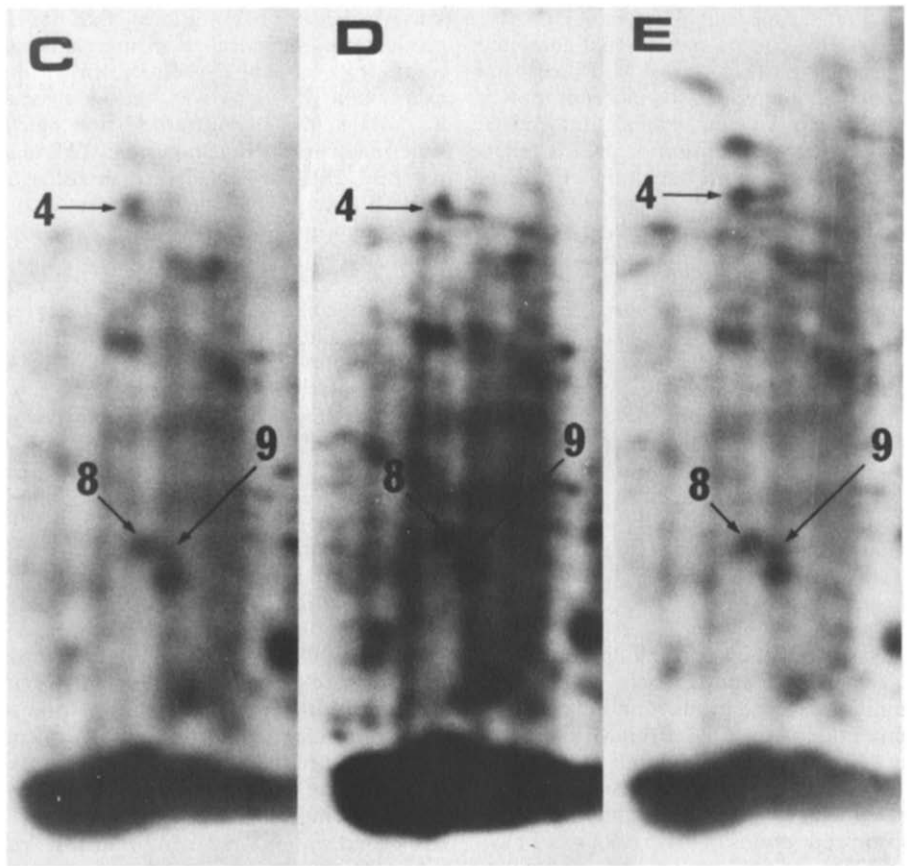

Fig. 4. Effects of calcium channel antagonists on protein phosphorylation in intact neutrophils stimulated by PMA. The cells loaded with ^{32}P were incubated with or without 1000 ng/ml PMA for 10 min at 37° and extracted. The extracts (0.3 mg protein) were electrophoresed on isoelectric focusing gels containing Ampholine with a pH range 3.5 to 10 (from right to left), then separated on 13.5% sodium dodecyl sulfate-polyacrylamide gels (from top to bottom). The gels were fixed, stained, dried and then autoradiographed at −70° for 7 days. Autoradiograms are shown. Proteins that were phosphorylated after the stimulation of cells are indicated by arrows and numbers in panels A and B. Only spots No. 4, 8 and 9 are shown in panels C–E. Fig. 4A: resting cells; Fig. 4B: control cells; Fig. 4C: with 0.7 mM verapamil; Fig. 4D: with 1.0 mM diltiazem; and Fig. 4E: with 0.026 mM nisoldipine.

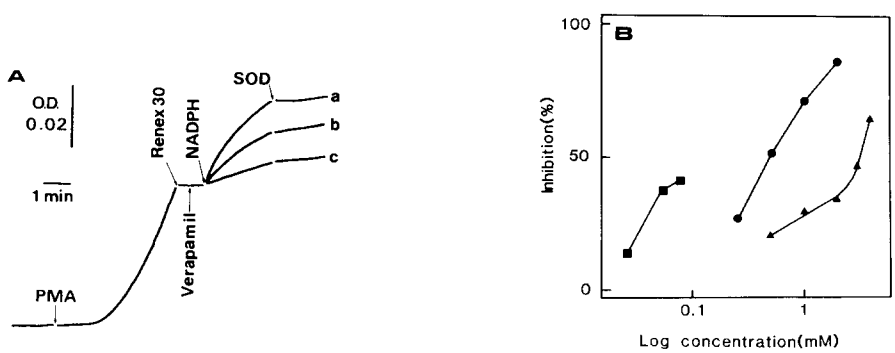

Fig. 5. Effects of calcium channel antagonists on NADPH oxidase. (A) Simultaneous demonstration of superoxide production and the corresponding NADPH oxidase activity. PMA, Renex 30, verapamil, NADPH and SOD were added sequentially. The absorbency change was followed on a recorder. Key: (a) control cells; and (b and c) with 0.5 and 1.0 mM verapamil added 30 sec before the addition of NADPH respectively. (B) Inhibition of NADPH oxidase activity by verapamil, diltiazem or nisoldipine. The conditions were the same as described in panel A. NADPH oxidase activity was calculated from the maximal rate of cytochrome *c* reduction after the addition of NADPH. The control rate for this experiment was 5.6 nmoles superoxide/10^6 cells per min. Key: (●) verapamil, (▲) diltiazem, and (■) nisoldipine.

these three proteins, especially M_r = 49,000 protein (spot No. 4), at the concentrations that inhibited superoxide production (Fig. 4,C–E). There were proteins in which an increase in the incorporation of ^{32}P was not affected by calcium channel antagonists, thus indicating that the inhibition of protein phosphorylation was not due to a depletion of cellular ATP.

NADPH oxidase activity. We finally examined whether calcium channel antagonists affected NADPH oxidase activity itself. Verapamil, added 20 sec after the destruction of cells with Renex 30 or 30 sec before the addition of the electron donor, NADPH, inhibited NADPH oxidase activity (Fig. 5A). Figure 5B shows the inhibition of oxidase activity by various concentrations of verapamil, diltiazem and nisoldipine (IC_{50} = 0.5, 3 and more than 0.08 mM respectively). The inhibition of NADPH oxidase by calcium channel antagonists seems not to be related to calcium metabolism, because calcium is not essential to oxidase activity itself [26].

DISCUSSION

Verapamil has been shown to inhibit superoxide production. Simchowitz and Spilberg [20] showed that verapamil inhibits the production induced by a chemotactic peptide with IC_{50} = 0.1 mM. Steiner *et al.* [21] showed that verapamil inhibits the increase in oxygen consumption in human neutrophils that were pretreated with cytochalasin B and then stimulated by opsonized zymosan with IC_{50} = 0.1 mM. These researchers used a reaction mixture containing 1 mM $CaCl_2$.

We showed that calcium channel antagonists inhibited superoxide production independently of extracellular calcium. PMA induced superoxide production in human neutrophils whether in a calcium-free medium or in the presence of a calcium chelator, EGTA. This means that extracellular calcium or a calcium influx was not involved in the induction. We conclude that the calcium channel antagonists inhibited superoxide production by mechanisms other than the blocking of a calcium influx. The direct inhibition of oxidase by calcium channel antagonists does not fully explain the inhibition of superoxide production observed in intact cells, because (a) IC_{50} values for oxidase were higher than those for intact cells, and (b) 1 mM verapamil and 2 mM diltiazem, which inhibited superoxide production in intact cells completely, produced only 70 and 35% inhibition, respectively, with respect to oxidase activity. The inhibition of superoxide production by calcium channel antagonists seems to be due not only to the direct inhibition of oxidase activity, but also to the inhibition of the induction. Both calcium mobilization without a rise in the cytosolic free calcium concentration, and activation of C-kinase followed by protein phosphorylation are thought to play pivotal roles in the induction of superoxide production in neutrophils that are stimulated by PMA or OAG, although the exact sequence of these biochemical events in signal transmission is not understood. Calcium channel antagonists inhibit both of these events.

Verapamil was reported to inhibit C-kinase isolated from rat brains with IC_{50} = 0.6 mM [22], the same order of magnitude as that for superoxide production. Calcium channel antagonists at high concentrations have also been shown to interact with calmodulin [27]; however, the involvement of calmodulin in the signal-transmission mechanism for superoxide production induced by PMA has not been resolved [28]. The light chain of myosin isolated from smooth muscle has been reported to be phosphorylated instantaneously by calmodulin-dependent myosin light chain kinase and C-kinase, although the functional role of myosin phosphorylation catalyzed by each kinase is unclear [29]. Adelstein and co-workers [30] showed that verapamil inhibits calmodulin-dependent myosin light chain kinase isolated from turkey gizzards with IC_{50} = 0.1 mM. Although it is not established which kinases are involved in phosphorylation of neutrophil myosin, calcium channel antagonists might inhibit it by affecting either C-kinase, calmodulin-dependent myosin light chain kinase, or both. These considerations and our results support the hypothesis that calcium channel antagonists act at intracellular sites [6]. It is also reported that, at high concentrations, some calcium channel antagonists affect calcium-ATPase and calcium uptake in skeletal and cardiac muscle sarcoplasmic reticulum [31]. Another possibility is that these antagonists act nonspecifically on plasma membrane, because (a) calcium storage sites in neutrophils are thought to be plasma membrane itself [32], (b) active C-kinase is located in plasma membrane [33], and (c) NADPH oxidase is associated with plasma membrane [8]. We must take into consideration that small amphipathic molecules such as anesthetics and tranquilizers induce structural rearrangements in the membranes resulting in extractions of lipids and proteins [34]. Anesthetics and tranquilizers have been shown to inhibit superoxide production in neutrophils [35–37]. Because of the high concentrations of the antagonists needed to produce the above effects, the relevance of these findings to the therapeutic effects remains questionable (the concentration of the antagonists observed clinically in the plasma is about 10^{-7} M). However, calcium channel antagonists might facilitate inhibition of neutrophil functions that have already been induced by other causes.

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