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Halothane, an inhalation anesthetic, activates protein kinase C and superoxide generation by neutrophils

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The rate of superoxide generation of guinea pig intraperitoneal neutrophils by a chemotactic peptide or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was increased by 2-bromo-2-chloro-1,1,1,-trifluoroethane (halothane), an inhalation anesthetic. This increase was inhibited by 1-(5-isoquinolinesulfonyl)methylpiperazine dihydrochloride (H-7), a specific inhibitor of Ca^{2+}- and phospholipid-dependent protein kinase C (PKC). Halothane was found to significantly activate partially purified PKC. The activation required phosphatidylserine (PS) and Ca^{2+}. Dioleoylglycerol- or TPA-activated PKC activity was further increased by halothane. The cytoplasmic proteins of guinea pig neutrophils phosphorylated by halothane-activated PKC were similar to those phosphorylated by PMA-activated PKC. The phosphorylation of a 48 kDa protein, a phosphorylated protein required for NADPH oxidase activation, was also increased by halothane. These data suggest that the increase of superoxide production by halothane is correlated with its activation of PKC.

Halothane; Anesthetic; Protein kinase C; Superoxide generation; (Neutrophil)

1. INTRODUCTION

It is generally accepted that inhalation anesthetics such as halothane cause a perturbation of neuronal membrane structure through a nonspecific interaction with membrane lipids or affect the activity of cellular enzymes by binding to an appropriate site of membrane bound proteins [1]. The physical state of the phospholipids in biological membranes is important in determining their susceptibility to activation of lipid requiring enzyme. Therefore, it could be expected that halothane may induce a change of membrane functions by nonspecific interactions with membrane lipids. Recently, it has been reported that halothane decreases neutrophil functions such as chemotaxis, phagocytosis, bactericidal activity and superoxide production [2,3]. It also prevents fatty acid release in different tissues and inhibits leukotriene formation in human leukocytes suggesting inhibition of PLA_2 [4,5]: Vigo et al. [4] reported that halothane interacts with membrane phospholipids and modifies membrane fluidity resulting in inhibition of PLA_2.

In contrast to these findings, we found that halothane increases the superoxide generation of neutrophils induced by FMLP or TPA.

Several investigators reported that the biochemical sequence of the activation pathway of the NADPH oxidase is likely to involve the activation of PKC [6–8]. Chloroform, a tumor promoter and an inhalation anesthetic, also activates the PKC by the modification of the physical or chemical structure of phospholipid [9]. These

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Abbreviations: Cyt. c, cytochrome *c*; DE-52, DEAE-cellulose; FMLP, *N*-formylmethionylleucylphenylalanine; KRP, Krebs-Ringer-phosphate solution; halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane; H-7, 1-(5-isoquinolinesulfonyl)methylpiperazine dihydrochloride; PS, phosphatidylserine; PLA_2, phospholipase A_2; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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results suggest that halothane may act as an activator of the PKC. We have therefore investigated the effect of halothane on PKC activity and found that the drug activates the partially purified enzyme and increases the phosphorylation of cytoplasmic proteins.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Aprotinin, Cyt.c, dioleoylglycerol, calf thymus H1 histone (type IIIs), FMLP, PMSF and TPA were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane) was from Hoechst Japan Co. (Tokyo, Japan). PS (bovine brain), Sephadex G25 and Superose 12 were obtained from Serdary Research Lab. Inc. (Canada) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. Nutrose was from Eastman Kodak Co. (Rochester, NY). [γ-^{32}P]ATP was obtained from ICN Radiochemicals (Irvine, CA). DEAE-cellulose (DE-52) and glass filter (GF/C) were from Whatman Ltd (Maidstone, England). Leupeptin and other chemicals were from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of reagent grade. In most experiments, halothane was added directly into the reaction ixture in liquid phase after dilution with ethanol. In some experiments, halothane was equilibrated by bubbling the gas phase halothane for 10 min.

2.2. *Preparation of neutrophils and cytoplasmic proteins*

Guinea pig neutrophils were obtained from the peritoneal cavity after intraperitoneal injection of 2% nutrose as described [10]. The cells were suspended in a medium containing 0.15 M KCl, 20 mM Hepes-K buffer (pH 7.4), 4 mM iodoacetate, 1 mM vanadate, 10 mM β-glycerophosphate, 1 mM *p*-nitrophenylphosphate, 1 mM PMSF, 1 μg/ml aprotinin, 0.01% leupeptin, and 1 mM EGTA at 4°C and homogenized with glass-Teflon homogenizer with 50 strokes up and down at 2000 rpm. Homogenate was centrifuged for 60 min at 100000 × *g* at 4°C and the supernatant retained [11].

2.3. *Preparation of PKC from rat brain*

Partially purified PKC from cerebral tissues of male Dawley rats, weighing 200–300 g, was obtained by the method of Kikkawa et al. [12].

2.4. *Superoxide production*

Superoxide production by cells was quantitated by the reduction of Cyt.c, monitored continuously as an increase in absorbance at 550 nm and an extinction coefficient of 19100 $M^{-1} \cdot cm^{-1}$ in a dual wavelength spectrophotometer, thermostated at 37°C [13].

2.5. *Assay of PKC*

The activity of PKC was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus H1 histone (type IIIs) or cytoplasmic proteins of neutrophils at 30°C for 3 to 10 min by the method of Boni and Rondo [14] as described [15]. For the phosphorylation of cytoplasmic proteins by PKC, cytoplasmic supernatant was treated at 55°C for 3 min to inactivate endogenous protein kinases [11]. Phospholipid and dioleoylglycerol were first mixed in a small volume of chloroform and evaporated in vacuo. The residue was suspended in 20 mM Tris-HCl (pH 7.5) by sonication at 4°C for 30 min under nitrogen gas. Various Ca^{2+} concentrations of incubation mixture were prepared by EGTA-Ca^{2+} buffer. A final concentration of Ca^{2+} was measured by the fluorescence intensity of quin-2 [16]. SDS-PAGE [17] of the reaction mixture was employed. The gels were stained with Coomassie brilliant blue R250 then autoradiograms of the ^{32}P-labelled proteins were made as reported [11,15].

3. RESULTS AND DISCUSSION

3.1. *Stimulatory effect of halothane on FMLP-dependent superoxide generation of guinea pig neutrophils*

Superoxide generation of guinea pig neutrophils was increased by the treatment of guinea pig neutrophils with a high concentration of halothane. This stimulatory effect was markedly enhanced in the presence of other agents (fig.1). In the presence of TPA or FMLP, maximum stimulation was observed by 1 mM of halothane and the K_a value was about 0.3 mM halothane. Furthermore, the increased TPA-induced superoxide generation by halothane was inhibited by H-7, a specific inhibitor of PKC [18] (fig.2). Stimulation by halothane was also observed in the presence of dioleoylglycerol (not shown) as in the case of arachidonate [19], indicating synergism between halothane and other stimuli in stimulating the superoxide generation. A similar effect of halothane was also observed in the medium equilibrated by bubbling with the gas phase halothane. These data, however, are in disagreement with the results of other investigators [1–5]. The reason for this is not clear. One possibility is the type of neutrophils used, which in our experiments were primed by nutrose. In connection with this, chloroform also stimulated the superoxide generation of guinea pig neutrophils (not shown).

3.2. *Stimulation by halothane of H1 histone phosphorylation by rat brain PKC*

PKC, a key enzyme in membrane signal transduction and tumor promotion, is defined by its requirement for phospholipid and calcium and/or its activation by diacylglycerols or phorbol ester tumor promoters [20]. Fig.3 shows the phosphorylation of H1 histone by rat brain PKC in

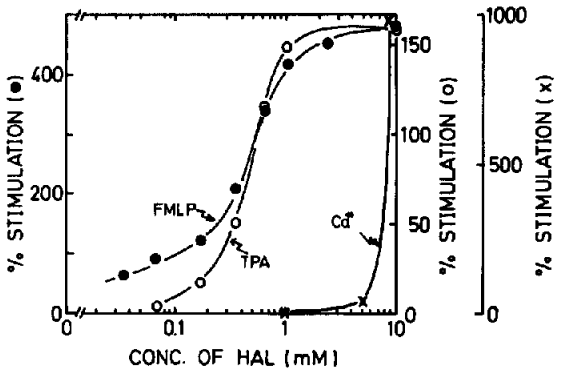

Fig.1. Effect of halothane (HAL) on the superoxide generation by guinea pig neutrophils. Guinea pig peritoneal neutrophils (2×10^6 cells/ml) were incubated in a medium of Krebs-Ringer-phosphate solution (KRP) containing 1 mM Ca^{2+}, 10 mM glucose, 25 μM Cyt.c and 0.1 mM NaN_3 at 37°C. The concentrations of FMLP and TPA were 3.8 nM and 0.4 nM, respectively.

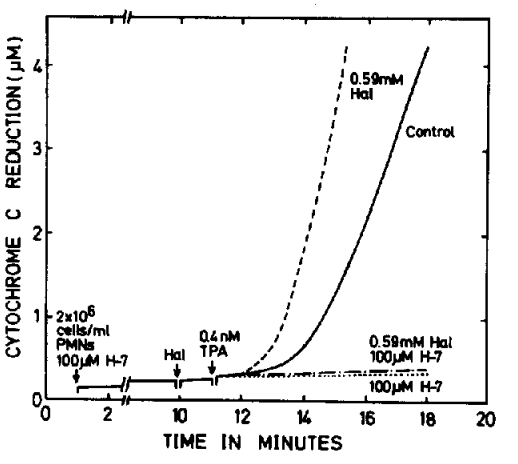

Fig.2. Effect of H-7 on the halothane-stimulated superoxide generation of guinea pig neutrophils. Experimental conditions were as described in fig.1. Added concentrations are given in the figure. Hal, halothane.

the presence of 0.3 mM Ca^{2+}/100 μM phospholipid (egg PC/PS, 4:1 in molar ratio), or 1 μM Ca^{2+}/100 μM phospholipid/5 μM dioleoylglycerol, and/or 1 μM Ca^{2+}/100 μM phospholipid/10 nM TPA. In every case, halothane stimulated the phosphorylation of H1 histone in a concentration-dependent manner (fig.4). The rates of stimulation were however different in each case (fig.3), and Ca^{2+} was the most susceptible requirement for the stimulation of the enzyme activity: the reaction was strongly stimulated by the increase in concentration of Ca^{2+}.

3.3. *Ca^{2+}-dependency on the halothane-induced PKC stimulation*

Fig.5 shows that in the presence of 39 mM halothane and 0.1 mM phospholipid, PKC activity was enhanced by Ca^{2+} in a concentration-dependent manner. A similar Ca^{2+} dependency was also observed in the medium containing phospholipid and TPA or dioleoylglycerol (not shown).

3.4. *Effect of H-7 on the halothane-activated PKC*

To confirm the increased phosphorylation of the protein by halothane-activated PKC, the effect of H-7 was tested. Fig.6 shows that H-7 inhibits the halothane-activated PKC. The concentration of H-7 required for half inhibition of the enzyme activity was about 30 μM, which is higher than that required for the inhibition of TPA-activated PKC, 7 μM [18]; maximum inhibition was about 80%.

3.5. *Effect of halothane on the phosphorylation of cytoplasmic proteins by PKC*

As in the case of histone phosphorylation,

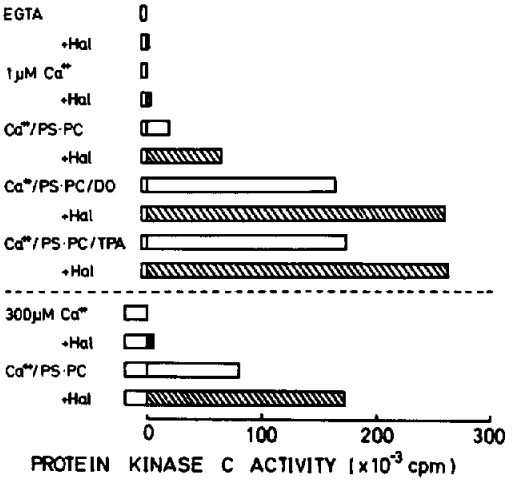

Fig.3. Effect of halothane on the activity of rat brain PKC in the presence or absence of various activators. Rat brain PKC was partially purified and enzyme activity was assayed as described in text in the presence of H1 histone. The concentrations of phospholipid (egg PS/PC, 1:4 in molar ratio), dioleoylglycerol (DO), TPA and halothane (Hal) were 0.1 mM, 5 μM, 10 nM, and 38 mM, respectively.

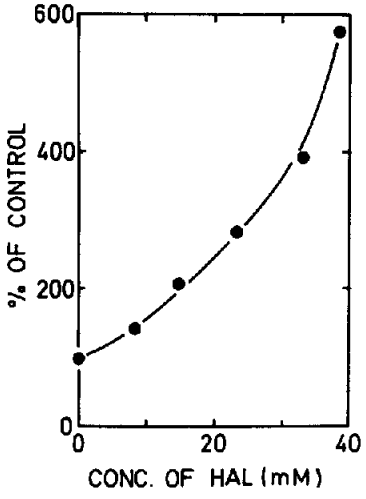

Fig.4. Effect of halothane concentration on the % stimulation of PKC. Experimental conditions were as described in fig.3. The rat brain PKC was incubated in the medium containing 8 × 10^{-6} M Ca^{2+}, 0.1 mM phospholipids (egg PS/egg PC, 1:4 in molar ratio).

cytoplasmic proteins of neutrophils were phosphorylated by the halothane-activated PKC. The cytoplasmic supernatant of neutrophils was treated at 55°C for 3 min for inactivation of endogenous protein kinases [11], and the proteins

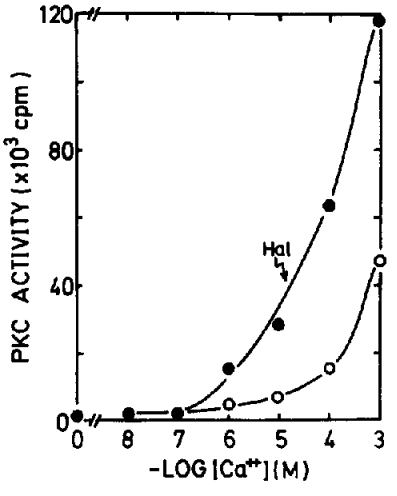

Fig.5. Effect of calcium ion concentration on the halothane-activated PKC activity. Experimental conditions were as described in fig.4, except that the halothane concentration was 37.9 mM. Hal, halothane.

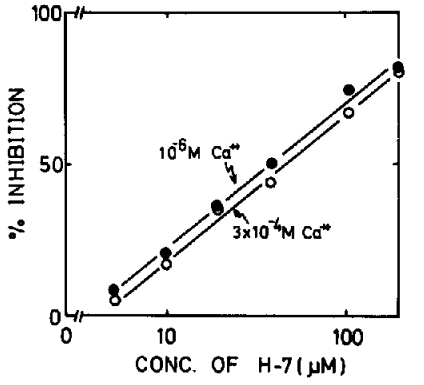

Fig.6. Effect of H-7 on the halothane-activated PKC activity in the presence of low and high concentrations of Ca^{2+}. Experimental conditions were as described in fig.4. The concentrations of Ca^{2+} were 0.3 mM and 1 μM.

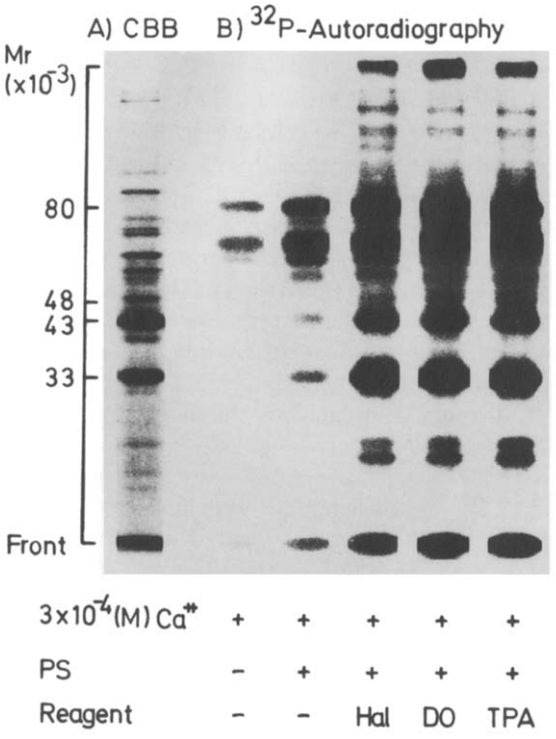

Fig.7. Autoradiogram of endogenous proteins of guinea pig neutrophils phosphorylated by partially purified rat brain PKC. Supernatant proteins of guinea pig neutrophils were treated for 3 min at 55°C for the inactivation of endogenous protein kinases. The proteins were incubated in the medium described in the text containing 0.1 mM phospholipid (egg PS/egg PC, 1:4 in molar ratio) and 0.3 mM Ca^{2+} in the presence of various activators. The concentrations of dioleoylglycerol (DO), TPA, and halothane (Hal) were 5 μM, 10 nM and 37.9 mM, respectively. CBB, Coomassie blue staining; ^{32}P-autoradiography, autoradiography of phosphorylated proteins.

were phosphorylated by addition of partially purified rat brain PKC. Fig.7 shows the effect of halothane on the phosphorylation of cytoplasmic proteins by PKC. The autoradiographic pattern of the phosphorylated proteins by the halothane-activated PKC was essentially similar to that of dioleoylglycerol- or TPA-activated PKC: many cytoplasmic proteins of neutrophils, including the 48 kDa protein, were phosphorylated by the PKC in the medium of Ca^{2+}-PS, TPA and halothane.

From the deficiency of 48 kDa protein phosphorylation in neutrophils of patients with the autosomal recessive form of the chronic granulomatous disease, Kramer et al. [21] and Caldwell et al. [22] suggest that the cytosolic factor is required for activation of NADPH oxidase, which regulates phosphorylation of a specific protein such as the 47 or 48 kDa one [19]. In the present experiments, it was also found that the increase in both superoxide generation and PKC activity were sensitive to H-7. Taken together with these findings, the present data suggest that the increase in superoxide production by halothane is correlated with its activation of the PKC.

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