Biochemical Pharmacology, Vol. 37, No. 17, pp. 3237–3243, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

EFFECTS OF ETHANOL ON MECHANISMS FOR SECRETORY AND AGGREGATORY RESPONSES OF HUMAN GRANULOCYTES

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(*Received* 29 *February* 1988; *accepted* 10 *March* 1988)

Abstract—Since the mechanisms for inhibitory effects of ethanol on polymorphonuclear (PMN) leukocyte function are largely unknown we studied secretory and aggregatory responses after *in vitro* treatment of PMN with ethanol. Oxidative metabolism (assessed by chemiluminescence, superoxide ion production and oxygen consumption) and aggregation were reduced dose-dependently in the presence of ethanol when elicited by stimuli binding to surface receptors or other structures (fMLP and ConA). Similar results were obtained with non-receptor-dependent stimuli, as NaF and the calcium ionophore A23187. In contrast, phorbol myristate acetate peak responses were not affected. FMLP induced changes in Fura-2AM fluorescence (as a probe for fast intracellular calcium concentration changes) were, however, not influenced by ethanol. Neither were membrane potential changes [assessed by di-O-C5(3) fluorescence]. Thus, it is suggested that ethanol may impair a step of the stimulus–response-coupling in PMNs distal to the initial intracellular calcium mobilization.

In clinical and experimental studies administration of ethanol has conferred increased morbidity or mortality in infections. One mechanism for this reduction of host defense was suggested to be decreased adherence of neutrophil polymorphonuclear (PMN)† granulocytes and delivery of these cells to the tissues [1–3]. Besides effects on adherence, ethanol may impair chemotaxis, phagocytosis and killing of microbes, all of which are fundamental functions of PMNs [4–5].

Although these studies have demonstrated impaired PMN function, little is known of mechanisms by which ethanol confers this change. Previous studies have shown that ethanol neither impairs specific binding of a chemotactic peptide (fMLP) to receptors on PMN surfaces, nor has a significant effect on microviscosity of the cell membranes [6].

The purpose of this study was to investigate effects of ethanol on various PMN functions stimulated by agents, which activate neutrophils in specific and different ways. We have found that ethanol impairs secretory and aggregatory functions in a stimulus-specific manner, indicating that ethanol effects can be localised to certain steps in the stimulus–response coupling.

MATERIAL AND METHODS

Chemicals. Chemicals obtained as follows were: Hanks' balanced salt solution (HBSS), SBL (Stockholm, Sweden); Percoll and superoxide dismutase (SOD) (Marklund), Pharmacia Fine Chemicals, (Uppsala, Sweden); Leukotriene B4 (LTB4) dissolved in ethanol, BioMol Research Lab (Philadelphia, PA); ionophore A23187 and Fura-2AM (dissolved in DMSO), Calbiochem (La Jolla, CA); hydrogen peroxide, Merck AG (Darmstadt, West Germany); 3-3'-dipentyloxacarbocyanine di-O-C5(3), Molecular Probes (Junction City, OR). All other chemicals were from Sigma Chemical Co. (St Louis, MO). The concentration of ethanol used here is given as v/v percentages. Thus, 1% ethanol corresponds to 7.9 mg ethanol/ml or 0.17 M.

Cell isolation. Heparinized blood samples were obtained from healthy members of the staff. None were on medication. Granulocytes were isolated by a one-step Percoll technique [7], followed by lysis of residual erythrocytes with 0.155 M ammonium chloride. With this technique, platelets were removed by an initial centrifugation step, and neutrophils comprised >95% of the cells, as determined from stained smears. Platelets represented approximately one in ten granulocytes [8]. Cells were suspended in HBSS at pH 7.45 and kept at +4° until use, followed by 15 min incubation at 37°. In some experiments, as indicated, cells were treated with cytochalasin B (5 μg/ml) for 3 min at +37°. Ethanol, in concentrations used here, did not influence the pH of suspensions.

Oxidative metabolism. The oxidative metabolism of granulocytes was assessed as luminol-enhanced chemiluminescence (LCL), as ferricytochrome *c* reduction and as oxygen consumption. Briefly, for LCL assays [9] purified granulocytes were mixed with luminol (0.17 mM) to a final concentration of 1.5×10^9 PMNs/l. The pH was kept at 7.6–8. After assessing the spontaneous chemiluminescence, reagents were added and chemiluminescence was followed continuously with a Luminometer 1050

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† Abbreviations: HBSS, Hank's balanced salt solution; ConA, Concanavalin A; LCL, luminol enhanced chemiluminescence; di-O-C5(3), dipentyloxacarbocyanine; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine. PMN, polymorphonuclear leukocyte.

(LKB, Bromma, Sweden). The results are given as net chemiluminescence (stimulated minus spontaneous light emission) in mV for (at least) triplicates. Only experiments in which the background LCL was <5 mV were used for calculations. Ethanol-treated PMNs had the same background LCL as untreated cells. Ethanol (0.2–5%) effects on the luminol system were also tested in cell-free chemiluminescence-generating systems consisting of hypoxanthine (20 mM) + xanthine oxidase (0.3 U) or H_2O_2 (20 mM). When ethanol was added to HBSS or to the standard luminol system in absence of other agents no chemiluminescence was produced.

Superoxide ion production was analyzed by the superoxide dismutase inhibitable cytochrome *c* reduction method [10] at +37° with continuous stirring of cytochalasin-treated granulocytes. The concentration of cytochrome *c* was 50 μM and of superoxide dismutase 3 μM. Absorbance was read at 550 nm and superoxide ion production was calculated as nmol reduced cytochrome *c* using an absorption coefficient of 21.1 mM^{-1} cm^{-1}.

Oxygen consumption was followed with a Clark electrode at 37° with continuous stirring, as described [9].

Granulocyte aggregation. Granulocyte aggregation was measured in a standard platelet aggregometer (Model 300 BD, Payton Associates, Buffalo, NY) [7, 8]. Stimuli were added to granulocyte suspensions containing 0.5% HSA. The resulting change in light transmission was recorded as ΔT. Results for fMLP and PMA are expressed as the maximal aggregation response (peak value), and for A23187 after 3 min, since this agent induces a continuous and irreversible aggregation [8].

Elastase release. Elastase release was monitored essentially as described previously [10]. After the addition of cytochalasin B to granulocytes, *n-t*-Boc-L-Ala-pNP (10 μM) was added to both reference and sample cuvettes in ethanol (0.1%) and baselines were recorded at 360–390 nm for approximately 1 min. All measurements were performed at 37° with continuous stirring. Stimuli were subsequently added and changes in absorbance were followed for at least 3 min. Since ethanol-treated samples exhibited slightly lower background absorbance, values are given as net increase of absorbance.

Intracellular Ca^{2+}. Intracellular Ca^{2+} was calculated from the change of Fura-2AM fluorescence [11, 12]. Neutrophils (5×10^6 cells/ml) in HBSS supplemented with 20 mM HEPES, pH 7.4, were incubated at 37° with 0.5 μM Fura-2AM for 30 min. Fura-2AM loaded cells were washed twice, reconstituted in HBSS (with Ca^{2+} at 1.27 mM) with 20 μM HEPES and stored on ice until use. Then, cells were warmed at 37° for 15 min and added to quartz cuvettes in a spectrofluorometer (Perkin–Elmer 650). Fluorescence was excited at 340 nm and emitted light at 510 nm. Measurements were made at 37° with continuous stirring of the cell suspension. After a stable baseline had been established, stimulus was added and emitted light recorded. The system was controlled by addition of EGTA, Tris buffer, Triton X-100 and $CaCl_2$ as described [13]. Calculations of calcium concentrations were performed according to Pollock and Rink [11] and Metcalf *et al.* [13].

Membrane potential. The cyanine dye di-O-C5(3) was added to the cells at a final concentration of 20 nM according to Metcalf *et al.* [13]. Excitation wavelength was 460 nM and emission wavelength was set at 510 nM. Measurements were made in a Perkin–Elmer spectrofluorometer, at 37° and with continuous stirring of cell suspensions.

Cell viability. This was assessed by exclusion of trypan blue, change of fluorescence of acridine orange and LDH-release [14].

Statistical calculations. Statistical calculations were performed with Student's *t*-test of paired samples.

RESULTS

The effect of the ethanol was first assessed by measuring chemiluminescence since this assay requires few cells and gives continuous information about the initial steps and the kinetics of neutrophil oxidative metabolism [9]. When using fMLP as stimulus we found that ethanol inhibited LCL in a dose-dependent manner (Fig. 1A). The inhibition was related to the fMLP concentration (Fig. 1B) with a most pronounced inhibition at low fMLP concentration. Inhibition occurred within 1 min of ethanol exposure (Fig. 1C). This inhibitory effect was not due to reduced cell viability since trypan blue was excluded, no change of acridine orange was noted in >95% of PMNs treated with 5% ethanol for 30 min and there was no difference in LDH-release between ethanol-treated and control PMN (0.07 and 0.08 μkat/l, respectively). The inhibitory effect was reversible since when neutrophils had been treated with 1% ethanol for 10 min and subsequently washed, no inhibition could be observed (110 ± 22% of the LCL response of HBSS-treated but otherwise similarly-handled cells).

The inhibitory effect could not be attributed to an interference with luminol because the cell-free systems were not affected by presence or addition of ethanol. Thus, LCL responses in presence of 0.2–5% ethanol were identical to HBSS-containing controls when LCL was initiated with the combination of hypoxanthine and xanthine oxidase system or with H_2O_2.

Based on these results and in order to enhance detection of effects we used treatment with 1% ethanol for 10 min in the following experiments, and, if effects were noted, clinically-relevant ethanol concentrations were also used.

In order to see whether the inhibitory effect of ethanol was specific for receptor-mediated stimuli we used a variety of agents acting independently of surface receptors or by defined receptors. Inhibitory effects were noted when A23187, sodium fluoride and Concanavalin A were employed (Fig. 2A). In contrast, phorbol myristate acetate (PMA)-induced LCL peaks were not altered by ethanol (Fig. 2A), regardless of ethanol concentrations (Fig. 1A).

Not only the peak values of LCL but also the kinetics of the response were influenced by ethanol. FMLP peak LCL reached its maximum faster when cells were treated with ethanol (Fig. 2B). The kinetics of A23187 responses were not clearly influenced by ethanol, whereas those of PMA responses were prolonged (Fig. 2B).

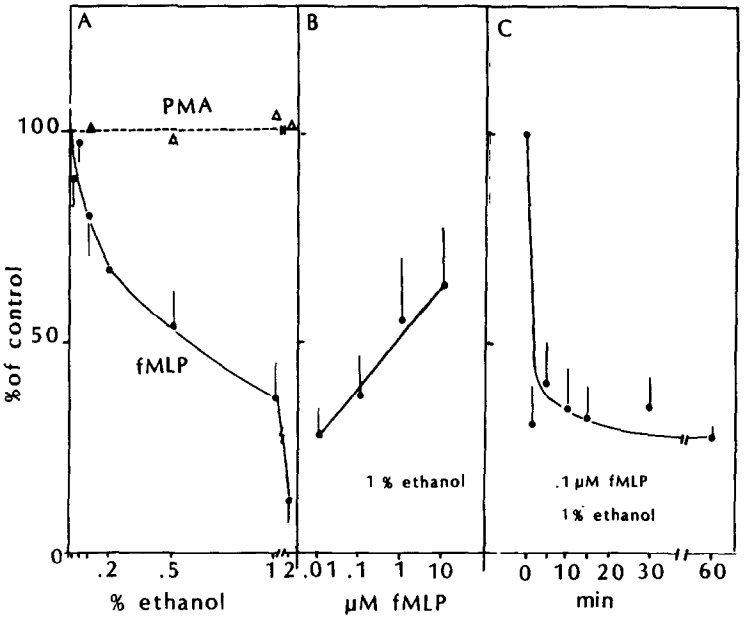

Fig. 1. Effect of ethanol on PMN chemiluminescence to fMLP and PMA.
A. Effect of varying the ethanol concentration. PMN were treated for 10 min before fMLP (●; 0.1 μM) or PMA (△; 7.5 μM) were added.
B. Effect of varying the fMLP concentration, when PMNs had been treated with 1% ethanol for 10 min.
C. Effect of varying the treatment time for PMNs exposed to 1% ethanol and 0.1 μM fMLP.
Results are given as percent of controls treated with HBSS alone. The chemiluminescence responses of those controls were, for fMLP (0.1 μM), 166 ± 16 mV, and for PMA (7.5 μM) 384 ± 87 mV. Mean and SE values for 3–10 experiments performed on cells from different donors.

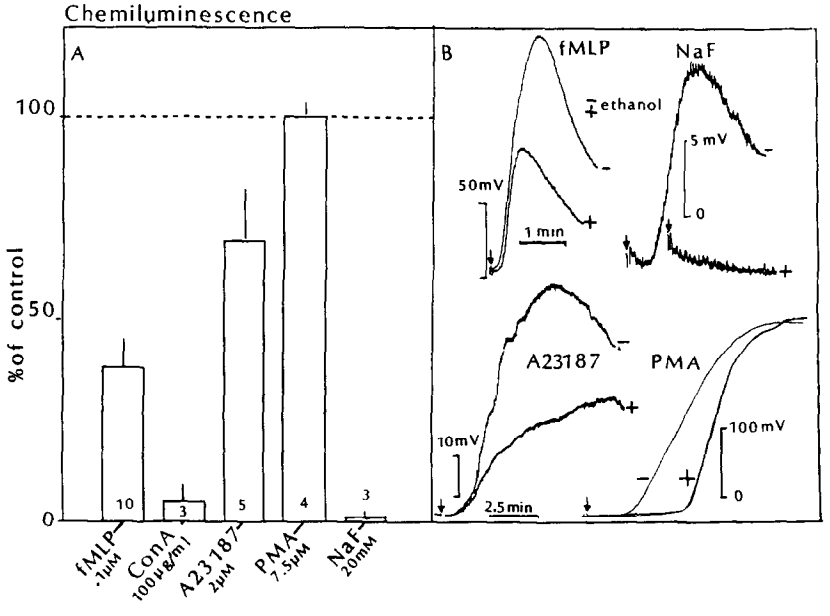

Fig. 2. Effect of treatment with 1% ethanol for 10 min on PMN chemiluminescence.
A. Effect on peak responses to various stimuli, at indicated final concentrations. Figures at bottom of bars indicate number of separate experiments. Mean and SE values for percentages of controls.
B. Typical chemiluminences tracings of samples treated with ethanol (+) or HBSS (−). Peak responses for controls were: fMLP 166 ± 16 mV, Con A 51 ± 2 mV, A23187 44 ± 4 mV, PMA 184 ± 87 mV, NaF 10 ± 2 mV.

Table 1. Effect of 1% ethanol on superoxide ion production, assessed by cytochrome *c* reduction

Stimulus	Concentration μM	Percent of controls	N
fMLP	0.1	74 ± 10	4
PMA	5	106 ± 1	3
A23187	5	62 ± 11	2

Superoxide production was for fMLP 0.76 ± 0.17 nM O_2, for PMA 2.5 ± 0.7 and for A23187 0.71 ± 0.01 nM O_2 (mean and SE value) to peak production was reached, which occurred at 2.1, 8.3, 3.9 min for fMLP, PMA and A23187, respectively.

To confirm that LCL responses were reflecting the oxidative metabolism of neutrophils we studied superoxide production and oxygen consumption. Results were consistent with those of LCL determinations. Superoxide ion production was reduced when fMLP and A23187 were stimuli, whereas PMA responses were unaffected by ethanol (Table 1). Likewise, oxygen consumption was reduced in the presence of ethanol; for fMLP it was 62 ± 12% (N = 3) of controls and the corresponding value for PMA was 101 ± 1% (N = 2).

To test whether these stimulus-specific effects could be observed on other functional responses of PMNs we studied release of elastase and aggregation. Ethanol was found to affect these responses in a manner compatible with that observed for oxidative metabolism (Figs 3 and 4). Thus, elastase release was inhibited in the presence of 1% ethanol when fMLP was used. However, it was similar to controls when A23187 was employed. The release was delayed in the presence of ethanol for both stimuli. PMA did not induce elastase release (Fig. 3).

FMLP-induced aggregation was modulated in a dose-dependent manner by ethanol. At very low ethanol concentrations an enhancement of aggregation was observed, whereas high concentrations conferred inhibition (Fig. 4A). However, the effect on aggregation was not dependent on fMLP concentrations between 0.1–1 μM (data not shown). As observed for previously-described functions, the peak of the PMA response was unaffected by ethanol (Fig. 4B). The A23187 response was, however, enhanced in the presence of ethanol (Fig. 4B). The

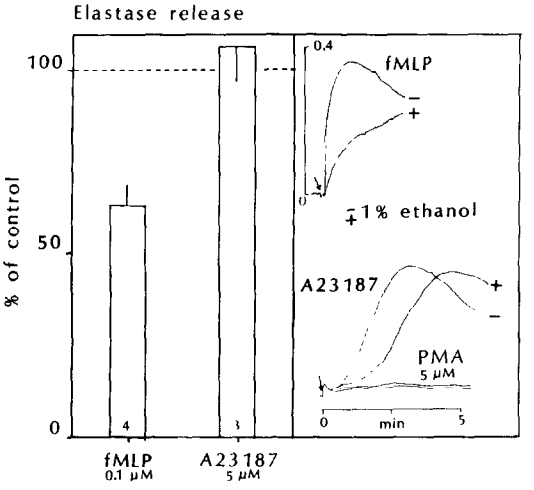

Fig. 3. Effect of ethanol on elastase release. A. Change of peak absorbance of *n-t*-Boc-L-Ala-pNP by PMNs treated with 1% ethanol for 10 min and stimulated with fMLP, PMA or A23187 at indicated concentrations. B. Typical tracings from ethanol (+) or HBSS (−) treated samples. The *y*-axes give absorbance units, corrected for a small decrease of absorbance (mean 0.05 units) for ethanol treated samples.

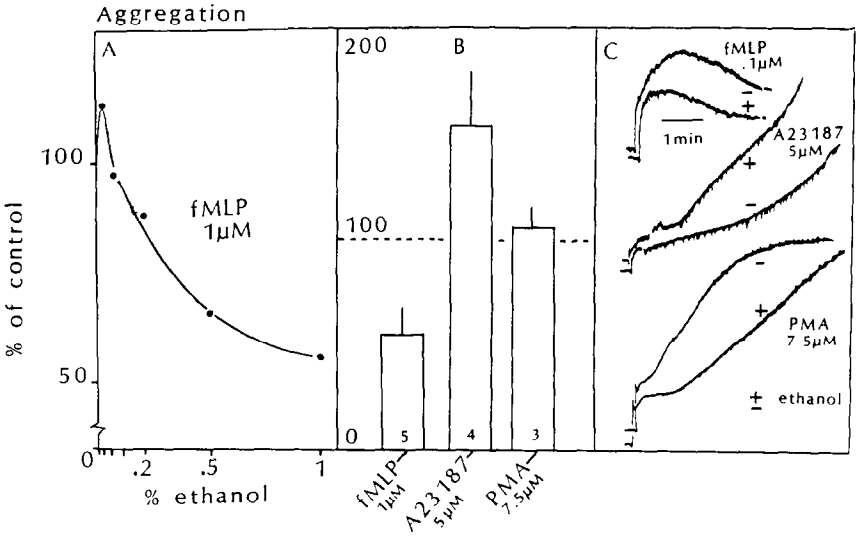

Fig. 4. Effect of ethanol on aggregation. A. Effect of various ethanol concentrations on response to 0.1 μM fMLP. B. Effect of 1% ethanol on aggregation induced by a variety of stimuli at indicated concentrations. C. Typical aggregation tracings in presence of ethanol (+) or HBSS (−). Mean and SE values for 3–5 experiments, as indicated at bottom of bars. ΔT for controls was, for fMLP 45 ± 8 mm, for A23187 82 ± 2 mm and for PMA 72 ± 22 mm.

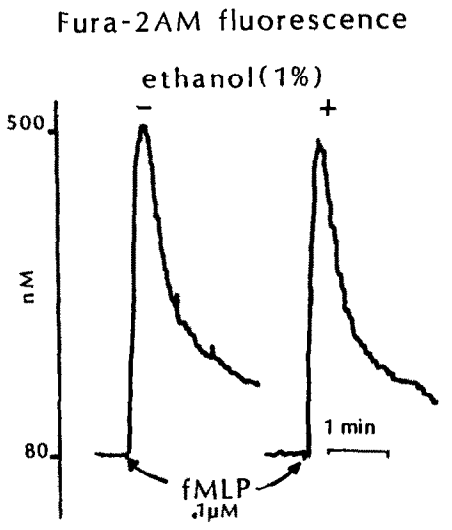

Fig. 5. Fura-2AM fluorescence in response to 0.1 μM fMLP in cells treated with ethanol (+) or HBSS (−). The mean basal level of fluorescence corresponded to $[Ca^{2+}]_i$ of 80 nM and peaks to 529 nM. The figure depicts a typical tracing from one of 4 experiments with similar results.

kinetics of the aggregation response were also affected by ethanol treatment. Both the fMLP and the A23187 responses were faster, whereas the PMA response was delayed (Fig. 4C).

These results suggest that ethanol does not exert a general toxic effect on functional responses of neutrophils. Rather, the effect appears to be stimulus-specific but not restricted to agents requiring a surface-receptor. We next tried to localize the effect of ethanol on the stimulus–response coupling by assessing intracellular calcium mobilisation and membrane potential changes. These reactions are among the first to be observed after activation of a granulocyte. We found that that addition of ethanol did not change the basal level of intracellular calcium in resting cells, as assessed by Fura-2AM-fluorescence. After addition of fMLP the fast, initial increment of calcium concentration was identical in ethanol-treated and control cells with regard both to peak heights (93% of controls) and time to peak (Fig. 5). PMA did not induce any rise of fluorescence. Likewise, ethanol did not affect membrane potential changes when activated by fMLP or A23187 (Fig. 6).

DISCUSSION

This study demonstrates that ethanol influences certain functional responses of human neutrophils *in vitro*. Chemiluminescence, superoxide ion production, oxygen consumption, aggregation and enzyme release were decreased, whereas membrane potential and initial intracellular calcium mobilization were not modified by ethanol when induced by a surface-receptor-dependent agent, as fMLP. Similar, but not completely identical results, were observed when the calcium ionophore A23187 was used. However, peak oxidative, secretory and aggregatory responses to PMA, which activates the intracellullarly-located protein kinase C, were not affected by ethanol treatment. Ethanol effects were reversible with washing of cells, which indicates that no permanent chemical alteration of structural elements of the cell was conferred. Inhibitory effects were observable within a very short time of treatment and did not change over 30 min. That the effects observed here on LCL reflect changes in the oxidative metabolism was confirmed by assessments of superoxide ion production and O_2 consumption.

The mechanisms by which ethanol confers impairment of functional responses of PMNs have not been clear. Explanations have included possible changes of membrane receptor function [6], which may or

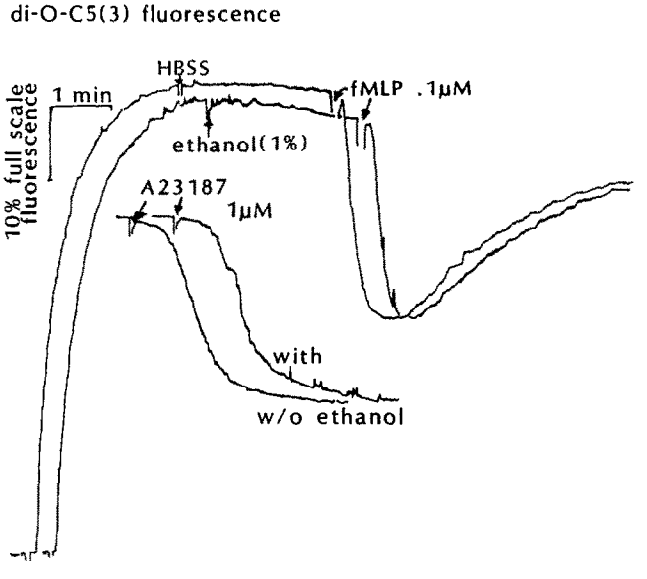

Fig. 6. Effect of ethanol on di-O-C5(3) fluorescence used as a probe for membrane depolarization. The tracings are taken from one experiment of four with similar results.

may not be related to increased fluidity of the cell membrane [6, 15] and fatty acid remodeling [16]. Also considerable interest has been paid to ethanol-enhanced calcium and sodium fluxes over cell membranes [17, 18]. Although ethanol appears not to inhibit the phosphorylative capacity of protein kinase C [19] it may hypothetically influence translocation of this enzyme from the cytosol to the membrane during cell activation [20].

Our results suggest that surface-receptors for fMLP, mediating oxidative, secretory and aggregatory responses, were largely intact since (i) other fMLP responses were not affected, i.e. membrane depolarization and calcium fluxes (which can be assumed to be mediated by both high- and low-affinity subsets of receptors [21, 22]), (ii) oxidative responses to non-receptor-dependent stimuli (A23187 and NaF) were equally inhibited, and (iii) Yuli *et al.* did not find any effect on fMLP receptor numbers with 2.5% ethanol [6]. However, a discrete effect of ethanol on low-affinity fMLP receptors cannot be completely ruled out at present. Such an effect would manifest itself as inhibition of secretory events, whereas effects on aggregatory events are unclear. That may be one explanation for the function that clearly did not fit the general response pattern, i.e. stimulatory effects of ethanol on A23187 aggregation.

Another possible explanation of ethanol effects is changes in microviscosity. Yuli *et al.* [6] demonstrated, however, only marginal effects of ethanol treatment on PMN steady-state fluorescence polarization of diphenylhexatriene, believed to reflect microviscosity changes. Nonetheless, such small changes have probably not affected signal transduction in our experiments in a general manner since PMA responses attained identical peak values regardless of ethanol treatment. Formation of ethyl ethers with cellular fatty acids is probably unlikely, since ethanol effects were readily reversible with washings [23].

Ethanol may hypothetically impair the guanine nucleotide system by which fMLP signals are transduced. The observation that sodium fluoride, which has been suggested to induce functional responses by a stimulatory effect on that nucleotide system [24, 25], did not elicit LCL in the presence of ethanol, can support that hypothesis. Nonetheless, the finding of ethanol inhibiting ConA and A23187 oxidative responses argues strongly against the hypothesis, since those agents have been shown to by-pass the nucleotide coupling system [26], which also appears to control conversion between the low- and high-affinity state of fMLP receptors. This finding may add to the evidence against the hypothesis that ethanol affected only low-affinity fMLP receptors.

The capacity of a PMN to mobilize calcium from internal (and external) stores is an important early step in the stimulus–response coupling for those stimuli whose effects were hampered by ethanol. Yet, ethanol did not affect either basal levels of Fura-2AM fluorescence of resting cells, or the fast peak response to fMLP. Assuming that these responses give information about the fast intracellular calcium mobilization, it is tempting to speculate that basal levels of this ion, as well as the preceding formation of inositol phosphate and diacylglycerols were unaffected by ethanol treatment. Ongoing research evaluates this possibility.

The present finding of a normal Fura-2AM signal indicates that the main inhibitory effect of ethanol is not on the afferent part of the stimulus–response coupling but distal to initial calcium mobilization. The exact localization of the effect remains unknown. Several possibilities exist. A change of cellular microviscosity may still be a possibility since it can be expected to modify the capacity of the cell to execute the actual response after having transduced the signal. The observed modulation of the kinetics of the functional responses to all stimuli may be expressed in such a way. This might also be true of changes in the translocation of protein kinase C, since this was observed to be inhibited by aliphatic alcohols [20].

The inhibitory effect of ethanol on superoxide anion production and lysosomal enzyme secretion resembles previous findings with *n*-butanol and *n*-pentanol on these PMN functions [6]. Also, Schopf *et al.* [27] noted that ethanol and its metabolite acetaldehyde suppressed generation of oxygen radicals from zymosan stimulated granulocytes. Unlike us, they did not document any effect on release of β-glucuronidase from these cells. This difference may be related to the choice of stimuli.

Effects of ethanol on neutrophil aggregation have not been reported previously. However, aggregation is a complex reaction in which adhesive phenomena may be important. In accordance with our aggregation findings, McGregor and others reported that ethanol hampers adhesion of neutrophils to nylon fiber columns [5] and hampers emigration of PMN to extravascular inflammatory foci [1–3].

Acknowledgements—This study was supported by grants from the Swedish Medical Research Council (19X-5991, 19P-7095), and from the Funds of P. & A. Hedlund, C. Bergh, S. Svensson, Södersjukhuset, and Karolinska Institutet. The skilful laboratory assistance of Mr. B. Cottell and typing by Mrs. S. Riddez is gratefully acknowledged.

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