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INHIBITION OF NEUTROPHIL SUPEROXIDE SECRETION BY THE PRESERVATIVE, METHYLHYDROXYBENZOATE: EFFECTS MEDIATED BY PERTURBATION OF INTRACELLULAR Ca^{2+}?

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The preservative, methylhydroxybenzoate inhibited O_2^- secretion from human neutrophils activated by both the chemotactic peptide fMet-Leu-Phe and phorbol myristate acetate (PMA): the low level of oxidant secretion activated by the ionophore A23187 was similarly reduced in preservative-treated suspensions. Oxidant secretion was similarly reduced in fMet-Leu-Phe and A23187 treated suspensions in which intracellular Ca^{2+} was buffered by loading with Quin-2, indicating that methylhydroxybenzoate may exert its effects by perturbation of intracellular Ca^{2+}-dependent processes. Methylhydroxybenzoate could mimic EGTA in preventing the Ca^{2+} dependent enhancement of trypsin activity and could also bind this cation in experiments using a Ca^{2+} electrode, although the preservative bound Ca^{2+} more slowly and had a lower affinity than EGTA. These data indicate that methylhydroxybenzoate may exert its effects on neutrophils by perturbation of Ca^{2+}-dependent activation pathways and this phenomenon may also explain its other known pharmacological effects. Furthermore, these observations provide an insight into the mechanisms by which intracellular Ca^{2+} may regulate oxidant secretion.

KEY WORDS: Neutrophils, oxidants, calcium, methylhydroxybenzoate, superoxide.

ABBREVIATIONS: PMA, phorbol myristate acetate; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; Pipes, piperazine-N,N′-bis[2-ethanesulphonic acid].

INTRODUCTION

The preservatives methyl and propyl hydroxybenzoate inhibit the growth of bacteria and fungi and thus are common additives to intravenous drug preparations such as gentamicin and Narcan® (the commercial preparation of naloxone). Naloxone is an opioid receptor antagonist, and apart from its use to manage opiate overdoses, it can also counteract some of the effects of ethanol intoxication.[1-4] Experiments designed to understand the biochemical basis of this latter phenomenon have revealed somewhat surprising results: whereas Narcan® could increase $^{14}CO_2$ production from ^{14}C-oleate in ethanol-inhibited hepatocytes[5] and could inhibit ethanol oxidation by

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purified liver alcohol dehydrogenase,[6] naloxone (the supposedly active drug) did not express these activities. The efficacy of Narcan® in these experiments was accounted for entirely by the preservatives methyl and propyl hydroxybenzoate.

Other independent experimentation has confirmed a pharmacological activity for these preservatives. For example, they have been shown to induce relaxation of tracheal[7] and other smooth muscle[8] and to interfere with receptor-mediated cytosolic free Ca^{2+} changes in individual isolated rat hepatocytes.[9,10] It has been postulated that some of these observations may be explained by effects of these preservatives upon transmembrane Ca^{2+} fluxes or else by stimulation of protein kinase C, two processes involved in cellular activation.

As polymorphonuclear leukocytes (neutrophils) can be activated to produce reactive oxidants via changes in intracellular free Ca^{2+} and/or activation of protein kinase C,[11] the aims of this work were twofold. Firstly, we wanted to establish if methylhydroxybenzoate had an effect on neutrophil function, as naloxone (or Narcan®) has been used in the treatment of septic shock[12-14] and also been shown to protect against pulmonary injury following experimental septicaemia:[15] this tissue-protective ability may be due to the fact that naloxone can inhibit O_2^- secretion from activated neutrophils.[16] Secondly, we wanted to identify the mechanisms by which methylhydroxybenzoate perturbed neutrophil function in order to propose a biochemical basis for the observed pharmacological actions of this compound.

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METHODS

Isolation and Preparation of Neutrophils

Neutrophils were prepared from 20 ml heparinized venous blood from healthy volunteers utilizing M-PRM (Flow Laboratories), exactly as described previously.[17] After purification they were suspended in a Krebs/hepes buffer containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; hepes (N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid), 25 (pH 7.4); 0.1% bovine serum albumin. Cells were counted after a suitable dilution in the above buffer using a Fuchs-Rosenthal haemocytomer slide and used within 4 h of preparation.

Analytical Methods

a) O_2^- generation

Superoxide generation was measured in a continuous assay by monitoring the rate of superoxide dismutase-inhibitable reduction of ferricytochrome c.[18] The assay (total volume of 1 ml) contained 75 μM cytochrome c plus 5 $\times$ 10^5 cells and was performed using a Perkin-Elmer Lambda 5 spectrophotometer.

b) Trypsin assays

Trypsin activity[19] was assayed by measuring the rate of hydrolysis of α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA). The assay contained 2 μg/ml trypsin in 0.1 M Tris/HCl buffer (pH 7.9) containing 0.3 mM BAPNA, and after incubation at 25° C for the times indicated, p-nitroanilide formation was measured at 405 nm using a Perkin-Elmer Lambda 5 spectrophotometer.

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c) Quin-2 loading

Neutrophils (suspended in Krebs/hepes buffer at 10^8 cells/ml) were incubated with gentle agitation at 37° C for 10 min with either 500 μM Quin-2-AM or 1% DMSO as solvent control. Samples were then diluted 10-fold with pre-warmed buffer and

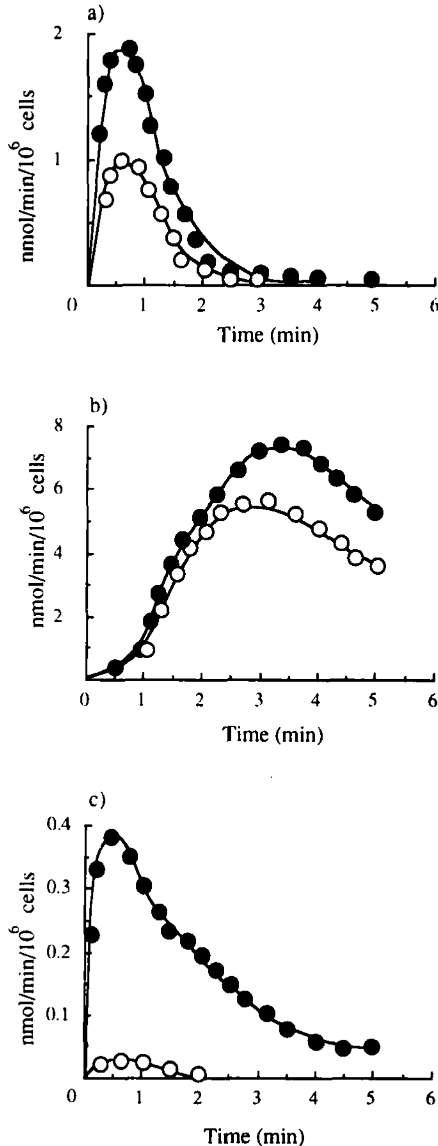

FIGURE 1. Effect of methylhydroxybenzoate on O_2^- secretion. Neutrophils (from a single donor) were suspended at 5×10^5 cells/ml in buffer containing 75 μM cytochrome c, in the absence (closed circle) and presence (open circles) of 5×10^{-4} M methylhydroxybenzoate. After a 2 min pre-incubation period, suspension were stimulated (time zero) by the addition of a) 1 μM fMet-Leu-Phe, b) 0.1 μg/ml PMA and c) 1 μM A23187.

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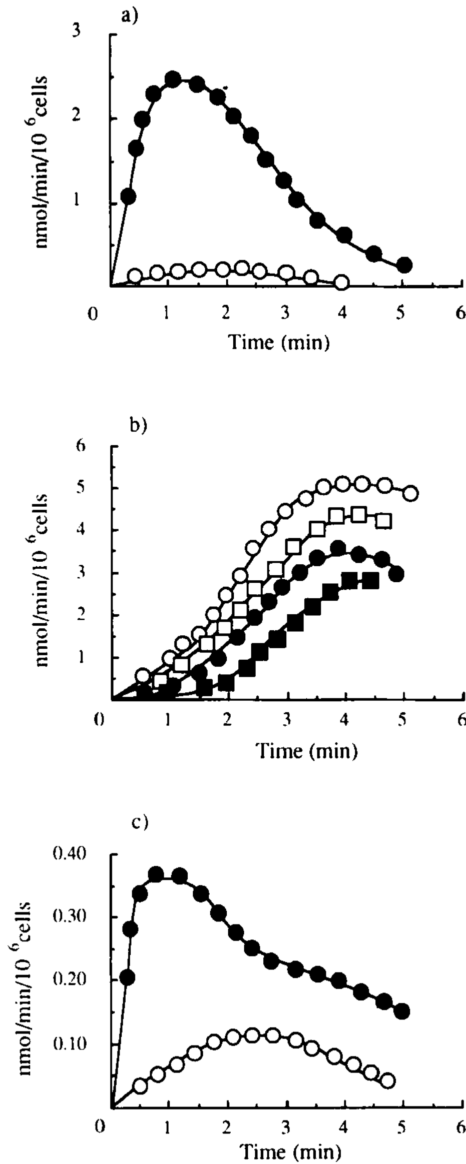

FIGURE 2. O_2^- secretion by Quin-2 loaded neutrophils. Neutrophil (from a single donor) were incubated in the absence (closed circles) or presence (open circles) of 500 μM Quin-2-AM, as described in Methods. After loading and washings, suspensions were incubated and stimulated as described in the legend to Figure 1. In b) EGTA was added to the suspensions immediately before stimulation of Quin-2 loaded (open squares) or control (closed squares) suspensions.

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incubated for a further 20 min, prior to centrifugation at 700 g for 3 min. Cell pellets were washed 3 times in Krebs/hepes buffer and finally suspended at 2×10^7 cells/ml.

d) Ca^{2+} electrode measurements

The chelation of Ca^{2+} by methylhydroxybenzoate and EGTA was measured using a Ca^{2+} selective electrode (model 93-3209) operating in conjuction with a single-junction reference electrode with 4M KCl (saturated with Ag^{2+}) filling solution (both from Russell pH Ltd., Auchtermuchty, Fife, Scotland). These were connected to a KENT/EIL pH/ion meter (Model 7055, EIL Analytical Instruments, Chertsey, Surrey, U.K.) and a potentiometric chart recorder for continuous readout. The reaction mixture comprised 5 mM Pipes (adjusted to pH 6.5 with solid tetramethylammonium hydroxide), 100 μM $CaCl_2$ and 20 mM KCl as ionic strength adjuster. Aliquots of EGTA or methylhydroxybenzoate were added to the mixture from concentrated stocks once a steady baseline had been attained on the chart recorder. Calibration was achieved by adding $CaCO_3$ of known concentration.

Statistical Analyses

Figure 1 and 2 show representative traces of neutrophils isolated from a single donor. Responses of neutrophils from different donors were compared by expressing the effects of methylhydroxybenzoate and Quin-2 as a percentage of untreated response which was taken as 100%: mean values of these effects are given ($\pm$ standard deviation) together with the ranges of values obtained.

Chemicals

fMet-Leu-Phe, A23187, PMA, Quin-2-AM, methylhydroxybenzoate and BAPNA were from Sigma. All other chemicals were of the highest purity available.

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RESULTS

Effect of methylhydroxybenzoate on O_2^- secretion

Reactive oxidant generation can be activated in neutrophils by a variety of chemical and particulate stimuli.[11] Figure 1 (closed symbols) shows the kinetics of O_2^- secretion stimulated by a) the chemotactic peptide fMet-Leu-Phe (which leads to an elevation of cytosolic free Ca^{2+}[20,21]), b) PMA (which activates protein kinase C in the absence of a free Ca^{2+} rise) and c) the ionophore A23187 (which raises cytosolic free Ca^{2+} levels directly): as previously-reported,[22] ionophore-stimulated oxidant secretion is considerably lower than that induced by receptor-mediated activation, being only 20% of activated by fMet-Leu-Phe. Initial experiments showed that at concentrations of 10^{-6} M and below, methylhydroxybenzoate had no effect on the magnitue and kinetics of activated neutrophil oxidant secretion. However, at concentrations greater than this, oxidant secretion was inhibited in a dose-dependent manner up to 10^{-3} M (the highest concentration tested). As the concentration of this preservative in drug preparations such as Narcan® is 0.5 mM, we decided to use this concentration in all subsequent experiments. Neutrophil suspensions incubated for 2 min in the presence of 0.5 mM methylhydroxybenzoate all exhibited reduced rates of O_2^- secretion after stimulation by the three agents in all experiments from all donors (Figure 1, open circles). The extent of inhibition observed, however, varied for the different stimuli.

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For example, the preservative inhibited the responses stimulated by A23187, fMet-Leu-Phe and PMA by approx 76% (±15%, range 90–53%, $n = 4$), 41% (±13%, range 17–60%, $n = 7$) and 23% (±6%, range 5–33%, $n = 7$), respectively. Experiments using a cell free xanthine/xanthine oxidase O_2^- generating system showed that methylhydroxybenzoate did not act as an oxidant scavenger (data not shown).

Effect of intracellular Ca^{2+} buffering on O_2^- secretion

As the extent of methylhydroxybenzoate inhibition in Figure 1 appeared to be related to the role played by intracellular free Ca^{2+} in activation of oxidant secretion, we decided to examine the effects of buffering intracellular Ca^{2+} changes on the kinetics of stimulated O_2^- secretion. The fluorescent Ca^{2+} indicator, Quin-2 can be used to monitor intracellular free Ca^{2+} changes,[23] but because it has a relatively high affinity for this cation it can alternatively act as an intracellular Ca^{2+} buffer when used at high concentrations to load cells. Thus, neutrophils were loaded with Quin-2 at concentrations sufficient to buffer intracellular Ca^{2+} prior to stimulation and measurement of O_2^- secretion. For the two stimuli known to mediate their effects via free Ca^{2+} increases, namely fMet-Leu-Phe (Figure 2a) and A23187 (Figure 2c), the rates of O_2^- secretion were reduced by 93% (±6%, range 98–86%, $n = 5$) and 65% (±7%, range 67–56%, $n = 5$), respectively in Quin-2 loaded cells. Whilst the percentage inhibitory effect of Ca^{2+}-buffering on O_2^- secretion was greater after fMet-Leu-Phe stimulation than that observed after A23187 stimulation, it must be stressed that A23187 only stimulates about 20% of the O_2^- secretion stimulated by fMet-Leu-Phe: the rates of Ca^{2+}-buffered O_2^- secretion in both A23187 and fMet-Leu-Phe stimulated cells were approximately equal.

The rate of PMA stimulated O_2^- secretion (Figure 2b) was, however, greater in

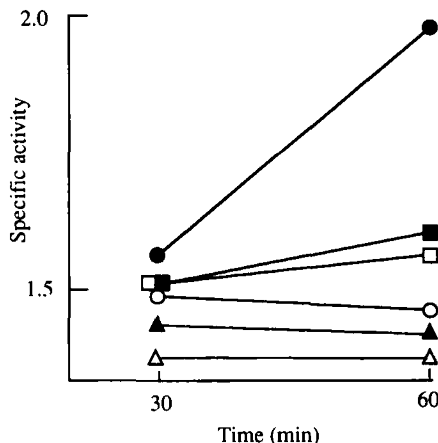

FIGURE 3. Effect of Ca^{2+}, EGTA and methylhydroxybenzoate on trypsin activity. Trypsin activity was assayed as described in Methods after 30 or 60 min incubation with the following: no extra additions (open circles); 2 mM $CaCl_2$ (closed circles); 2 mM $CaCl_2$ + 2 mM EGTA (closed squares); 2 mM $CaCl_2$ + 2 mM methylhydroxybenzoate (open squares); 2 mM EGTA (open triangles); 2 mM methylhydroxybenzoate (closed triangles). Specific activities were calculated using a molar absorption coefficient of p-nitroanilide of 9.96×10^3 and are expressed as μmole/min/mg protein. Similar experiments were obtained in 4 other experiments.

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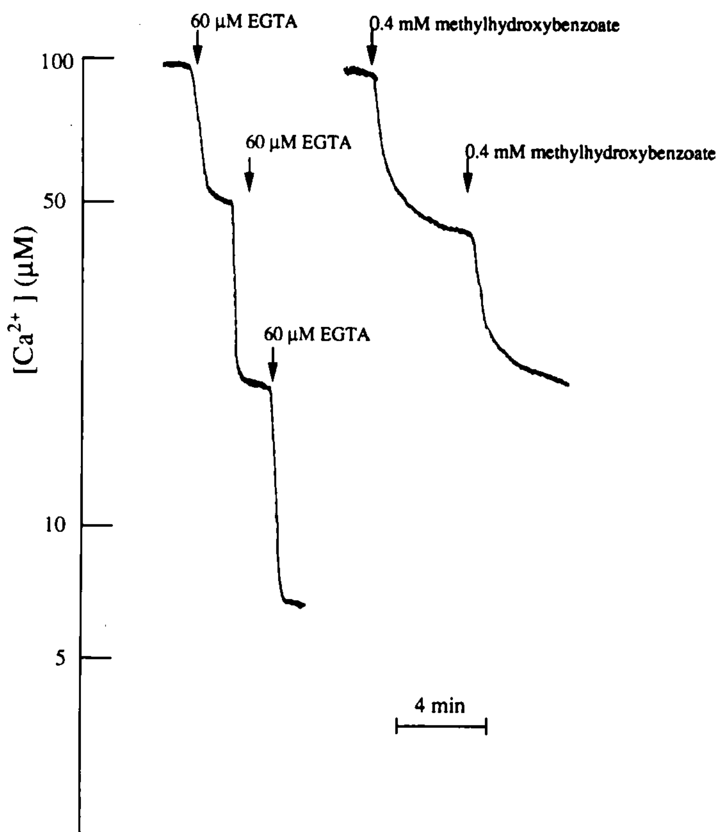

FIGURE 4. Chelation of Ca^{2+} by EGTA and methylhydroxybenzoate. The chelation of Ca^{2+} by EGTA and methylhydroxybenzoate was recorded as described in Methods in a buffer containing 5 mM Pipes (pH 6.5), 100 μM KCl and 100 μM $CaCO_3$. At the points shown by arrows, additions of either EGTA or methylhydroxybenzoate were made from concentrated stock solutions to give the final concentrations indicated.

Quin-2 loaded cells than in control cells (increased by 38% $\pm$ 5%, range 42–31%, $n = 5$). Thus, buffering intracellular free Ca^{2+} **enhanced** the rate of PMA-stimulated O_2^- secretion and reduced the lag time before activation occurred. When extracellar Ca^{2+} was buffered with EGTA, both the Quin-2 enhanced and control rates of O_2^- secretion were reduced, and the lag times before activation occurred were extended.

Effect of EGTA and methylhydroxybenzoate on trypsin activity

The above experiments suggested that methylhydroxybenzoate inhibited O_2^- secretion activated via cytoplasmic free Ca^{2+} increases. Thus, we then examined whether this compound could affect a cell free Ca^{2+}-dependent process. The activity of trypsin was therefore measured in the presence and absence of added Ca^{2+} and in the presence and absence of methylhydroxybenzoate and EGTA. Figure 3 shows that trypsin activity was constant when incubated in the absence of exogenously-added Ca^{2+}, whereas when incubated for 60 min in the presence of 2 mM Ca^{2+}, the activity increased by

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over 30%. The additions of 2 mM EGTA and 2 mM methylhydroxybenzoate largely prevented this Ca^{2+}-dependent enhancement. EGTA and methylhydroxybenzoate alone had little effect on enzyme activity other than would be predicted by chelating endogenous levels of Ca^{2+}. It was also found that supplementing Ca^{2+}/EGTA or Ca^{2+}/methylhydroxybenzoate incubations with extra Ca^{2+} (i.e. 4 mM Ca^{2+} and 2 mM chelator) resulted in a return of trypsin activity to that obtained after addition of 2 mM Ca^{2+} alone (data not shown).

Ca^{2+}-chelation by EGTA and methylhydroxybenzoate

The ability of EGTA and methylhydroxybenzoate to chelate Ca^{2+} was assessed using a Ca^{2+} selective electrode. Sequential additions of EGTA resulted in a rapid decrease in the free Ca^{2+} concentration as the cation was chelated (Figure 4a). Sequential additions of methylhydroxybenzoate similarly resulted in a similar decreased level of free Ca^{2+}, but the effects were different from those observed with EGTA in two ways. Firstly, whereas the decrease in free Ca^{2+} level observed after addition of EGTA was rapid, there was a more gradual decrease in free Ca^{2+} after addition of methylhydroxybenzoate. Secondly, whereas EGTA bound Ca^{2+} on an approximately equimolar basis, 400 μM methylhydroxybenzoate bound approx 60 μM Ca^{2+}: hence, an approximate molar ratio of methylhydroxybenzoate: Ca^{2+} binding was 7:1.

DISCUSSION

The data presented here show that the preservative methylhydroxybenzoate inhibits O_2^- secretion from human neutrophils and that the extent of inhibition of this process is dependent upon the nature of the stimulus used to elicit the response. The stimuli used are known to activate neutrophils via distinct intracellular signalling mechanisms and hence these observations provide insights into both the mechanisms by which methylhydroxybenzoate may exert its pharmacological effects (see Introduction) and also into the intracellular signalling processes required for oxidant secretion by activated neutrophils.

In vivo the respiratory burst of neutrophils is activated following binding of physiological agonists to specific receptors on the plasma membrane: these receptors are coupled to G-proteins which activate phospholipase C to release diacylglycerol (which remains in the membrane to form a substrate for protein kinase C) and inositol-1,4,5-triphosphate (which is released into the cytoplasm where it releases Ca^{2+} from intracellular stores). Hence, receptor-mediated (such as fMet-Leu-Phe) activation results in an elevation of cytosolic free Ca^{2+} and this rise precedes oxidase activation.[20,21] However, experimental manipulation of this system has shown that oxidase activation can occur in the absence of detectable intracellular Ca^{2+} rises if the cells are stimulated by phorbol esters which activate protein kinase C directly. Furthermore, under some experimental conditions, fMet-Leu-Phe stimulated activation can be uncoupled from intracellular Ca^{2+} rises e.g. if cells are first stimulated with low concentrations of phorbol esters[24] or if intracellular Ca^{2+} levels are previously elevated by ionophores.[25] These experiments have led to the proposal that a third intracellular mechanism for oxidase activation can occur under certain circumstances which is independent of both intracellular Ca^{2+} and protein kinase C activity, and which may involve phospholipases A_2 and D.[26,27]

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However, two points must be borne in mind. Firstly, when intracellular Ca^{2+} levels are carefully buffered, oxidant production in response to fMet-Leu-Phe is inhibited[21,25] (Figure 2a). Secondly, neutrophils can generate reactive oxidants both intra- and extra-cellularly[28] and we have recently shown that the intracellular signalling processes required for either oxidant secretion or intracellular oxidase activity are different (Watson, F.M., Robinson, J. and Edwards, S.W. in preparation). In the present study we have used superoxide dismutase-inhibitable cytochrome c reduction to specifically measure O_2^- secretion, as opposed to measurements of O_2 consumption[24] or luminol chemiluminescence[24] which monitor both intra- and extra-cellular oxidase activity.

When neutrophils were loaded with the Ca^{2+} indicator Quin-2 at concentrations sufficient to buffer elevated free Ca^{2+} rises, the ability of these cells to secrete O_2^- in response to fMet-Leu-Phe was reduced by over 90% (Figure 2a), confirming earlier reports of the role of Ca^{2+} in this process.[21] Whilst A23187, which leads to increases in cytosolic Ca^{2+} via a process by-passing intracellular signalling mechanisms, also activated O_2^- secretion, this was only 20% of that activated by fMet-Leu-Phe (Figure 2c), confirming previous observations:[22] thus, intracellular Ca^{2+} increases *per se* are insufficient to fully activate oxidant secretion. In Quin-2 loaded (Ca^{2+}-buffered) cells, O_2^- secretion activated by A23187 was reduced to levels that were just barely detectable and indistinguishable from the residual (Ca^{2+}-buffered) fMet-Leu-Phe response. Whether this was due to incomplete Ca^{2+} buffering after Quin-2 loading, Ca^{2+} release at sites inaccessible to the Ca^{2+} buffer or else due to Ca^{2+}-independent activation pathways, has not been established.

In neutrophils incubated with methylhydroxybenzoate, oxidant secretion in response to fMet-Leu-Phe, PMA and to A23187 was inhibited although the extent of inhibition was dependent upon the stimulus used. We propose that methylhydroxybenzoate inhibits O_2^- secretion via perturbation of Ca^{2+} dependent processes, for the following reasons.

1. O_2^- secretion activated via processes which are inhibited when intracellular Ca^{2+} is buffered are also inhibited by methylhydroxybenzoate.

2. Methylhydroxbenzoate could mimic the Ca^{2+} chelator, EGTA, in preventing the Ca^{2+} dependent enhancement of trypsin activity (Figure 3): this effect was restored by incubation with excess Ca^{2+}.

3. Methylhydroxybenzoate was shown to directly bind Ca^{2+} (using a Ca^{2+} electrode, Figure 4), although the binding was slower and the preservative had a lower affinity than EGTA.

4. Methylhydroxybenzoate has recently been shown to directly prevent the rapid Ca^{2+} spikes induced by phenylephrine in aequorin-loaded rat hepatocytes:[10] these Ca^{2+} transients were restored when the preservative was removed.

Our attempts to directly show that methylhydroxybenzoate prevented intracellular Ca^{2+} increases in neutrophils loaded with fluorescent indicators such as Quin-2 and Indo-1 were frustrated by fluorescence quenching due to spectral overlap between methylhydroxbenzoate and the Ca^{2+} indicators: binding with Ca^{2+} also caused spectral changes in methylhydroxybenzoate which further complicated this approach: we could neither monitor intracellular Ca^{2+} levels in aequorin-loaded neutrophils as these cells are too small to micro-inject. However, it has recently been shown that the rapid Ca^{2+} transients in individual rat hepatocytes induced by phenylephrine were prevented by methylhydroxygenzoate,[10] lending support to our conclusions as to its effects upon neutrophil function.

The kinetics of O_2^- secretion in methylhydroxybenzoate-treated and Quin-2 buffered neutrophils in response to PMA were not identical, however: in preservative-

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treated neutrophils the response was inhibited, whereas in Ca^{2+} buffered cells oxidant secretion was enhanced (Figure 1b and 2b). By virtue of its lipid solubility,[28] methylhydroxybenzoate can buffer Ca^{2+} both intra- and extra-cellularly, unlike EGTA which can only act extracellularly. EGTA reduced the elevated (intracellular Ca^{2+} buffered) PMA response, suggesting that methylhydroxybenzoate has the potential to act both as an intra-and extra-cellular Ca^{2+} buffer. It is interesting to note that PMA activates a Ca^{2+} efflux system in pig neutrophils,[30] and it is possible that intracellular Ca^{2+} may activate a process which normally regulates PMA-dependent activation: one such Ca^{2+}-activated regulator may be calpain.[31]

Further work is now necessary to establish whether a) any of the other observed effects of methylhydroxybenzoate (see Introduction) may also be explained by its ability to chelate Ca^{2+}, b) to establish if any of the clinical effects of Narcon® may be due to inhibition of neutrophil O_2^- secretion by the preservative and c) to establish if methylhydroxybenzoate has any anti-inflammatory activity by way of its ability to inhibit reactive oxidant secretion from activated neutrophils.

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