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DIFFERENTIAL MODULATION BY N^4,2'-O-DIBUTYRYL CYTIDINE 3':5'-CYCLIC MONOPHOSPHATE OF NEUTROPHIL ACTIVATION

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The cyclic pyrimidine nucleotide, cCMP, is an endogenous substance in mammalian cells but little is known on its functional role. We studied the effects of cCMP, its cell-permeant analogue, N^4,2'-O-dibutyryl cytidine 3':5'-cyclic monophosphate (Bt_2cCMP), and of butyrate on superoxide (O_2^-) formation and cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in human neutrophils. Bt_2cCMP inhibited O_2^- formation and the rise in $[Ca^{2+}]_i$ induced by a chemotactic peptide at submaximally effective concentrations. O_2^- formation induced by platelet-activating factor was potentiated by Bt_2cCMP, whereas the cyclic nucleotide had no effect on the rise in $[Ca^{2+}]_i$ induced by this agonist. Bt_2cCMP enhanced O_2^- formation induced by τ-hexachlorocyclohexane at a submaximally effective concentration. O_2^- formation stimulated by complement C5a, concanavalin A, NaF, A 23187, phorbol myristate acetate and arachidonic acid was not affected by Bt_2cCMP. cCMP was less effective than Bt_2cCMP to inhibit fMet-Leu-Phe-induced O_2^- formation, and butyrate was without effect on any of the functional parameters studied. Our data show that a cell-permeant analogue of cCMP differentially inhibits and potentiates activation of human neutrophils.

Human neutrophils possess an O_2^--forming NADPH oxidase which is activated by the chemoattractants, fMet-Leu-Phe, complement C5a and PAF [1-3]. The signal transduction pathways by which chemoattractants induce O_2^- formation are assumed to involve activation of phospholipase C and protein kinase C, rises in $[Ca^{2+}]_i$ and direct activation of NADPH oxidase through guanine nucleotide-binding proteins [1-3]. Various substances not acting through chemoattractant receptors activate O_2^- formation as well, among them being concanavalin A, which binds to membrane glycoproteins, NaF (AlF_4^-) as a general activator of guanine nucleotide-binding proteins and HCCH which may activate phospholipase C [1,2]. Phorbol myristate acetate activates NADPH oxidase *via* protein kinase C, and activation of O_2^- formation by A 23187 involves a rise in $[Ca^{2+}]_i$ [1,2]. Stimulation of O_2^-

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ABBREVIATIONS

Bt_2cAMP, N^6,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate; Bt_2cCMP, N^4,2'-O-dibutyryl cytidine 3':5'-cyclic monophosphate; Bt_2cGMP, N^2,2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; fMet-Leu-Phe, *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; HCCH, τ-hexachlorocyclohexane; O_2^-, superoxide anion; PAF, platelet-activating factor.

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formation by arachidonic acid may involve alterations in the lipid environment of NADPH oxidase [1,2].

The cyclic purine nucleotides, cAMP and cGMP, differentially modulate chemoattractant-induced O_2^- formation in human neutrophils [4]. After a debate on whether the cyclic pyrimidine nucleotide, cCMP, is an endogenous substance in mammalian cells [for review see Ref. 5], recent data suggest that cCMP, in fact, is present in these cells and that it is synthesized by a cytidylyl cyclase [6-8]. However, very little is known on the functional role of cCMP in general and in neutrophils in particular [5-8]. We studied a possible role of cCMP in neutrophil activation by using its cell-permeant analogue, Bt_2cCMP. We report that Bt_2cCMP differentially modulates O_2^- formation and rises in $[Ca^{2+}]_i$ in human neutrophils.

MATERIALS AND METHODS

Bt_2cCMP (sodium salt, purity approximately 95%) and cCMP (free acid, purity approximately 98%) were obtained from Sigma Chemie (Taufkirchen, Germany). Sources of other materials have been described elsewhere [4,9-11]. Bt_2cCMP and cCMP were used without further purification. Experiments with eight different lots of Bt_2cCMP during a period of fifteen months gave consistent results.

Neutrophils were isolated from fresh blood of healthy volunteers [9]. Cell preparations consisted of more than 95% viable neutrophils as revealed by trypan blue dye exclusion.

O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, FRG) [9]. Reaction mixtures (0.5 ml) contained 100 µmol/l ferricytochrome C and a buffer consisting of (mmol/l) 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose and 20 Hepes/NaOH, pH 7.4. Neutrophils (2.0 x 10^6 cells/cuvette) were suspended in the solution described above and were incubated for 3 min in the absence or presence of various compounds at 37°C prior to the addition of stimuli.

$[Ca^{2+}]_i$ was determined as described [11] with modifications. Briefly, neutrophils were suspended at 5 x 10^6 cells/ml in a buffer consisting of (mmol/l) 138 NaCl, 6 KCl, 1 $MgSO_4$, 1.1 $CaCl_2$, 0.1 EGTA, 1 Na_2HPO_4, 5 $NaHCO_3$, 5.5 glucose, and 20 Hepes/NaOH, pH 7.4, supplemented with 0.1% (w/v) bovine serum albumin. Cells were incubated for 1 h at 37°C in the presence of Fura-2 acetoxymethylester (4 µmol/l). Subsequently, cells were diluted with the above buffer to a concentration of 0.5 x 10^6 cells/ml and were centrifuged at 250 x g for 10 min at 20°C. Cells were suspended at 2.5 x 10^6 cells/ml in the above buffer and were kept at 20°C until measurement of $[Ca^{2+}]_i$. Fluorescence of neutrophils (1.25 x 10^6 cells/ml) was determined at 37°C using a Ratio II™ spectrofluorometer (Aminco, Silver Spring, Maryland, USA). Cells were incubated for 3 min in the absence or presence of various compounds prior to the addition of stimuli. Excitation and emission wavelengths were 340 nm and 500 nm, respectively.

RESULTS

The effects of Bt_2cCMP on chemoattractant-induced O_2^- formation were studied. fMet-Leu-Phe activated O_2^- formation with an EC_{50} of 40 nmol/l and a maximum at 300 nmol/l (Fig. 1). Bt_2cCMP (1 mmol/l) *per se* did not stimulate O_2^- formation and increased the EC_{50} of fMet-Leu-Phe to 80 nmol/l ($p < 0.01$) without altering the maximal effectiveness of the agonist. The effects of Bt_2cCMP on

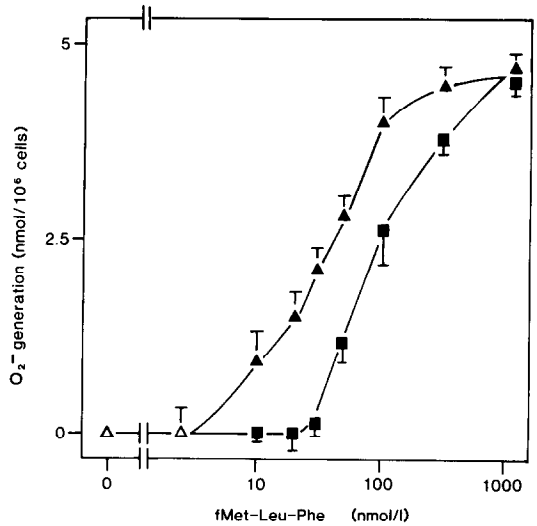

Fig. 1. Effect of Bt_2cCMP on concentration-response function to fMet-Leu-Phe-induced O_2^- formation in human neutrophils. Bt_2cCMP (1 mmol/l) or solvent (control) was added to reaction mixtures 3 min prior to fMet-Leu-Phe at various concentrations. (▲), control; (■), Bt_2cCMP. Open triangles indicate that fMet-Leu-Phe did not activate O_2^- formation. Data shown are the means ± S.D. of eight independent experiments.

fMet-Leu-Phe-induced O_2^- formation were concentration-dependent. In the presence of fMet-Leu-Phe at a submaximally effective concentration (50 nmol/l), Bt_2cCMP at 0.1 mmol/l reduced O_2^- formation by 15% (Table 1). Bt_2cCMP at 1 mmol/l inhibited the effect of fMet-Leu-Phe by 55%. cCMP was considerably less effective than Bt_2cCMP to diminish O_2^- formation, and butyrate was without effect. The inhibitory effect of Bt_2cCMP on O_2^- formation induced by fMet-Leu-Phe at 50 nmol/l was also evident when the cyclic nucleotide was added to cells simultaneously with the chemoattractant. Preincubation of neutrophils with Bt_2cCMP for periods longer than 3 min did not result in greater inhibition of formyl peptide-induced O_2^- formation (data not shown).

Table 1. Effects of Bt_2cCMP, cCMP and butyrate on fMet-Leu-Phe-induced O_2^- formation in human neutrophils

Concentration (mmol/l)	O_2^- generation (nmol/10^6 cells)		
	Bt_2cCMP	cCMP	butyrate
0.01	2.7 ± 0.3[a]	2.8 ± 0.1[a]	2.7 ± 0.3[a]
0.03	2.6 ± 0.3[a]	2.7 ± 0.2[a]	2.8 ± 0.4[a]
0.1	2.3 ± 0.2[b]	2.8 ± 0.1[a]	2.7 ± 0.2[a]
0.3	1.6 ± 0.1[c]	2.3 ± 0.2[b]	2.9 ± 0.3[a]
1.0	1.2 ± 0.1[c]	2.1 ± 0.4[b]	2.6 ± 0.2[a]

Bt_2cCMP, cCMP or butyrate at the indicated concentrations was added to reaction mixtures 3 min prior to fMet-Leu-Phe (50 nmol/l). In the presence of solvent (control) neutrophils generated 2.7 ± 0.2 nmoles of O_2^- per 10^6 cells. Data shown are the means ± S.D. of eight independent experiments. The significance of the effects of compounds *versus* control was assessed using the Wilcoxon test. [a], not significant; [b], $p < 0.05$; [c], $p < 0.01$.

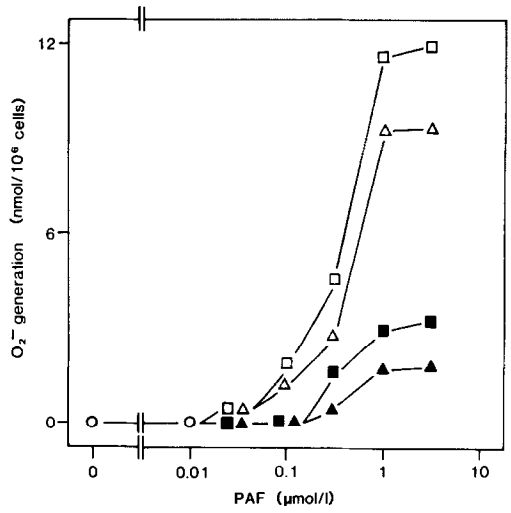

Fig. 2. Effect of Bt_2cCMP on concentration-response function to PAF-induced O_2^- formation in human neutrophils. Bt_2cCMP (1 mmol/l) or solvent (control) was added to reaction mixtures 3 min prior to PAF at various concentrations. Experiments were conducted in the presence of cytochalasin B (1 μg/ml, open symbols) or in its absence (closed symbols). (△ , ▲), control; (□ , ■), Bt_2cCMP. Open circles indicate that PAF did not activate O_2^- formation. Data shown are the means of five independent experiments; the S.D. of the data generally amounted to less than 10% of the mean values.

PAF activated O_2^- formation with an EC_{50} of 0.3 μmol/l and a maximum at 1 μmol/l (Fig. 2). Bt_2cCMP (1 mmol/l) enhanced the effect of PAF by up to 150% without altering its EC_{50} and without priming neutrophils to respond to PAF at non-stimulatory concentrations. The relative but not the absolute magnitude of the stimulatory effect of Bt_2cCMP was greatest with PAF at submaximally effective concentrations. PAF did not prime neutrophils to respond to Bt_2cCMP after the PAF-induced O_2^- formation had ceased (data not shown). Cytochalasin B, which prevents actin polymerization and internalization of plasma membrane receptors [12], increased the maximal effectiveness of PAF by about five-fold. In the presence of cytochalasin B, Bt_2cCMP at 1 mmol/l enhanced the effects of PAF by up to 65%. Bt_2cCMP at 0.3 mmol/l enhanced the effects of PAF in the presence of cytochalasin B by up to 30%, and butyrate (1 mmol/l) did not affect PAF-induced O_2^- formation (data not shown).

O_2^- formation was activated by C5a at concentrations ranging from 1 nmol/l to 1 μmol/l [4]. Bt_2cCMP (1 mmol/l) did not inhibit or stimulate C5a-induced O_2^- formation (data not shown).

Modulation of $[Ca^{2+}]_i$ by Bt_2cCMP was investigated. Bt_2cCMP reduced the increase in $[Ca^{2+}]_i$ induced by fMet-Leu-Phe at a submaximally effective concentration (1 nmol/l) from 290 nmol/l to 120 nmol/l and considerably reduced the duration of the response (Fig. 3). By contrast, Bt_2cCMP did not affect the rise in $[Ca^{2+}]_i$ induced by fMet-Leu-Phe at a maximally effective concentration (10 nmol/l) (see Fig. 3) and by PAF at a submaximally and a maximally effective con-

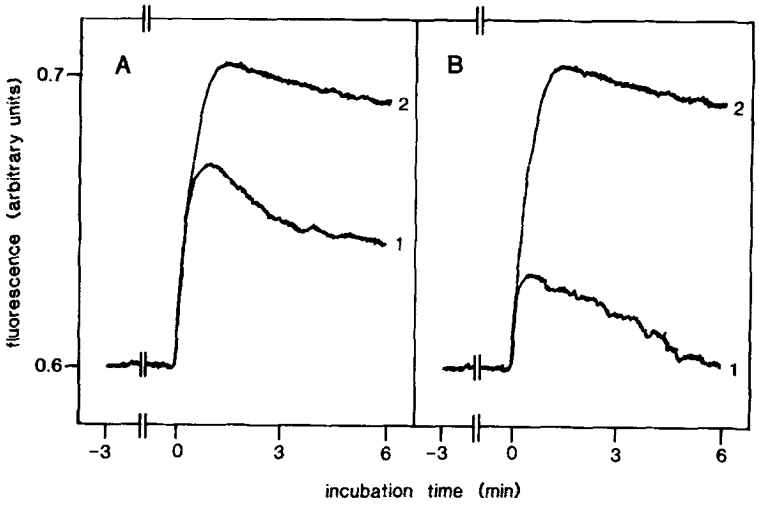

Fig. 3. Effect of Bt_2cCMP on fMet-Leu-Phe-induced increases in $[Ca^{2+}]_i$ in human neutrophils. Bt_2cCMP (1 mmol/l) or solvent (control) was added to reaction mixtures 3 min prior to fMet-Leu-Phe (t = 0 min). Panel **A**: Experiments with solvent (control). Trace 1, fMet-Leu-Phe (1 nmol/l). Trace 2, fMet-Leu-Phe (10 nmol/l). Panel **B**: Experiments with Bt_2cCMP. Trace 1, fMet-Leu-Phe (1 nmol/l). Trace 2, fMet-Leu-Phe (10 nmol/l). Superimposed original registrations from one experiment are shown. Similar results were obtained in five independent experiments.

centration (10 and 100 nmol/l) (Fig. 4). In addition, the rise in $[Ca^{2+}]_i$ induced by C5a at submaximally and maximally effective concentrations (0.1 and 1.0 nmol/l) was not affected by Bt_2cCMP, and butyrate at 1 mmol/l was without effect

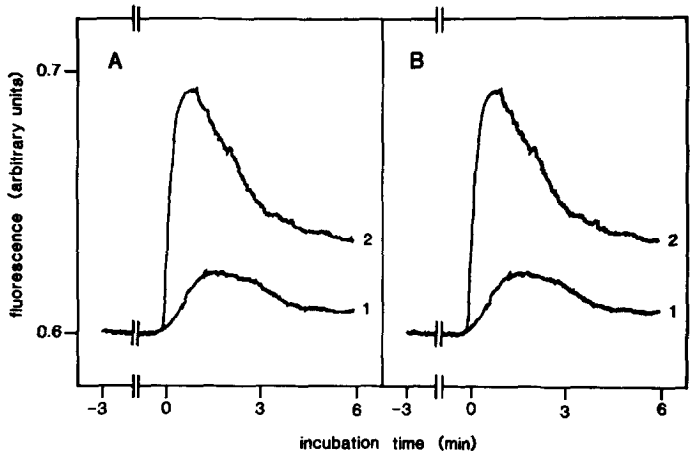

Fig. 4. Effect of Bt_2cCMP on PAF-induced increases in $[Ca^{2+}]_i$ in human neutrophils. Bt_2cCMP (1 mmol/l) or solvent (control) was added to reaction mixtures 3 min prior to PAF (t = 0 min). Panel **A**: Experiments with solvent (control). Trace 1, PAF (10 nmol/l). Trace 2, PAF (100 nmol/l). Panel **B**: Experiments with Bt_2cCMP. Trace 1, PAF (10 nmol/l). Trace 2, PAF (100 nmol/l). Superimposed original registrations from one experiment are shown. Similar results were obtained in five independent experiments.

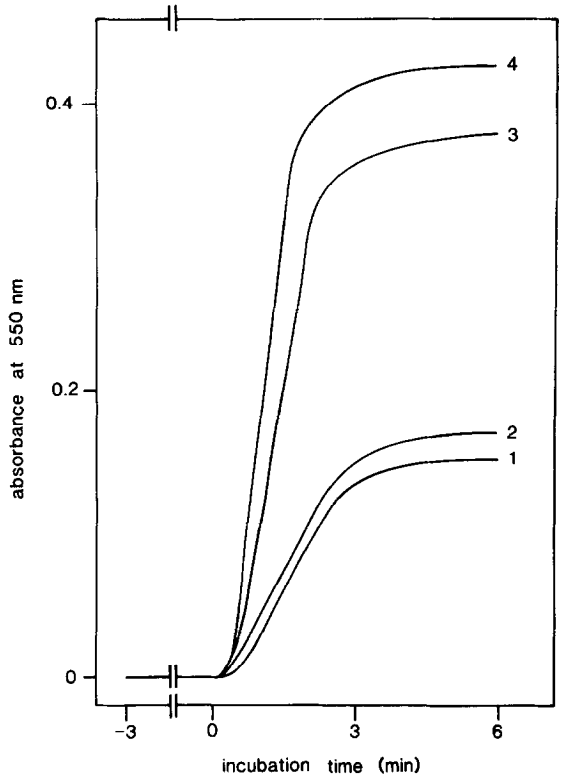

Fig. 5. Effects of Bt_2cCMP on HCCH-induced O_2^- formation in human neutrophils. Bt_2cCMP at the indicated concentrations or solvent (control) was added to reaction mixtures 3 min prior to HCCH (125 µmol/l) (t = 0 min). Trace 1, Bt_2cCMP (0.1 mmol/l) or solvent; trace 2, Bt_2cCMP (0.2 mmol/l); trace 3, Bt_2cCMP (0.5 mmol/l); trace 4, Bt_2cCMP (1 mmol/l) Superimposed original registrations from one experiment are shown. Similar results were obtained in five independent experiments.

on increases in $[Ca^{2+}]_i$ induced by any of the chemoattractants studied (data not shown).

The effects of Bt_2cCMP on O_2^- formation induced by stimuli which circumvent receptor stimulation were studied. HCCH (125 µmol/l) activated O_2^- formation with a lag-phase of about 20 s, and O_2^- formation ceased after about 4 min (Fig. 5). Bt_2CMP enhanced HCCH-induced O_2^- generation with a very steep concentration-response function and reduced the lag-phase to < 10 s, but the cyclic nucleotide did not prolong the duration of O_2^- formation. Butyrate at 1 mmol/l did not affect HCCH-induced O_2^- formation (data not shown). With HCCH at higher concentrations (250 and 500 µmol/l), no stimulatory effect of Bt_2cCMP on O_2^- formation was evident (data not shown). Finally, O_2^- formation was activated by concanavalin A (25, 50 and 100 µg/ml), NaF (10 and 20 mmol/l), phorbol myristate acetate (10, 30 and 100 ng/ml), A 23187 (2.5 µmol/l) and by arachidonic acid (25, 50, 100 and 150 µmol/l). Bt_2cCMP (1 mmol/l) showed no effect on O_2^- formation induced by these stimuli (data not shown).

DISCUSSION

The cell-permeant analogue of cCMP, Bt_2cCMP, effectively modulates O_2^- formation induced by fMet-Leu-Phe, PAF and HCCH and the formyl peptide-induced rise in $[Ca^{2+}]_i$ in human neutrophils. Bt_2cCMP is more effective than cCMP to inhibit fMet-Leu-Phe-induced O_2^- formation, and butyrate is without effect on the functional parameters studied. These data indicate that the effects of Bt_2cCMP depend on the cyclic nucleotide moiety and that the presence of butyrate is not sufficient to account for the effects of the intact molecule. O_2^- formation and the rises in $[Ca^{2+}]_i$ are differentially regulated by Bt_2cCMP, both the type of stimulus and its concentration determining its effects. The opposite effects of Bt_2cCMP on O_2^- formations induced by fMet-Leu-Phe on one hand and PAF and HCCH on the other hand and its lack of effect on C5a-, Con A-, NaF-, phorbol myristate acetate-, A 23187- and arachidonic acid-induced O_2^- formations suggest that Bt_2cCMP does not interfere with activation of NADPH oxidase in an unspecific manner.

The effects of Bt_2cCMP on neutrophil activation are rapid in onset and do not require long preincubation times. The concentrations of Bt_2cCMP necessary to modulate neutrophil activation range from 0.1-1 mmol/l and are comparable to those of Bt_2cAMP and Bt_2cGMP to alter functions of various types of blood cells in short term- and long-term experiments [4,13-16]. Previously, cCMP as low as 100 nmol/l was claimed to rapidly stimulate the growth of leukemia cells *in vitro* [17]. With respect to O_2^- formation, an effect of cCMP is evident only at concentrations which are at least 1000-fold higher than those reported by Bloch et al. [17]. However, these authors did not compare the effects of cCMP with those of Bt_2cCMP. Chan et al. [18] reported that in long-term experiments, Bt_2cCMP as low as 10 µmol/l promoted early embryonic development but data on the effects of Bt_2cCMP at lower and higher concentrations were not shown. Unfortunately, Chan et al. [18] did not study the effects of butyrate in their test system. These experiments would have been important as butyrate is known to mimick certain effects of butyrylated analogues of cyclic nucleotides [19,20]. Thus, future studies dealing with the effects of Bt_2cCMP on cell functions should include control experiments with cCMP and butyrate.

The biochemical basis for a possible functional role of cCMP is very poorly defined [for review see Ref. 5], and several possibilities have to be considered to explain the effects of Bt_2cCMP on neutrophil activation. Apparently, the effects of Bt_2cCMP on NADPH oxidase are not mediated through plasma membrane pyrimidinoceptors as their activation results in potentiation of both fMet-Leu-Phe- and PAF-induced O_2^- formations [10], but Bt_2cCMP shows opposite effects on O_2^- formations induced by these agonists. The fact that Bt_2cCMP stimulates PAF-induced O_2^- formation in the presence and absence of cytochalasin B suggests that the cyclic nucleotide does not act at the level of actin poly-

merization. With regard to fMet-Leu-Phe but not with respect to PAF, the effects of Bt_2cCMP on O_2^- formation and $[Ca^{2+}]_i$ are in parallel, and C5a-induced O_2^- formation and rises in $[Ca^{2+}]_i$ are resistant to modulation by Bt_2cCMP. These divergent effects of Bt_2cCMP on neutrophil activation induced by fMet-Leu-Phe, PAF and C5a suggest that the cyclic pyrimidine nucleotide differentially interacts with proximal signal transduction components, e.g. chemoattractant receptors, and with more distal components being involved in the activation of effector systems, e.g. components of NADPH oxidase. By analogy, cAMP-increasing agents also differentially inhibit neutrophil activation, depending on the type of stimulus and functional parameter studied [21-23]. The failure of Bt_2cCMP to modulate O_2^- formations induced by various substances which act at more distal steps of the signal transduction cascade supports the view that cCMP may interfere with chemoattractant receptors. On first glance, our finding that HCCH-induced O_2^- formation is potentiated by Bt_2cCMP, is puzzling as HCCH is assumed not to act through specific receptors [3,24,25]. However, recent studies indicate that HCCH mimicks some aspects of chemoattractant-induced phagocyte activation [24,25] as is the case for other agents, i.e. lipopeptides and substance P [26,27].

Intriguingly, the effects of Bt_2cAMP, Bt_2cGMP and Bt_2cCMP on O_2^- formation differ in several regards. Specifically, Bt_2cAMP and Bt_2cGMP inhibit PAF-induced O_2^- formation [4] but Bt_2cCMP potentiates O_2^- formation induced by this agonist. Additionally, Bt_2cCMP does not affect C5a-induced O_2^- formation, whereas Bt_2cAMP inhibits and Bt_2cGMP potentiates O_2^- formation induced by this chemoattractant [4]. Furthermore, Bt_2cCMP enhances HCCH-induced O_2^- formation, but Bt_2cAMP and Bt_2cGMP do not modulate activation of NADPH oxidase by this substance [4]. These data show that Bt_2cCMP does not mimick the effects of Bt_2cAMP and Bt_2cGMP and suggest that this cyclic pyrimidine nucleotide apparently does not act through activation of cAMP- and/or cGMP-dependent protein kinases or through inhibition of cAMP- and/or cGMP-degrading phosphodiesterases [28,29]. Whether or not stimulation by cCMP of certain forms of phosphodiesterases occurs, remains to be clarified [30]. We are also not aware of published data on the existence of a cCMP-dependent protein kinase in mammalian cells [31]. Finally, it cannot be ruled out that cCMP, in analogy to cGMP, directly regulates the activity of effector proteins [32]. In case of ion channels in the renal collecting duct, cCMP at 100 µmol/l, unlike cGMP, is without effect on channel open probability [33]. In this system, cGMP at 0.1 and 1 mmol/l is similarly effective, but the effect of cCMP at 1 mmol/l was not reported by Light et al. [33].

It is not yet possible to assess the physiological relevance of our findings until a number of problems is solved. Bt_2cCMP above 30 µmol/l inhibits fMet-Leu-Phe-induced O_2^- formation, but the intracellular concentration of cCMP achieved subsequently to the exogenous application of its butyrylated analogue is unknown. It has to be determined whether deacylation of Bt_2cCMP is required

for modulation of neutrophil activation or whether the cyclic nucleotide analogue acts *per se*. In analogy to cGMP, brominated analogues of cCMP would be expected to be more lipophilic and resistant to degradation than cCMP itself and to be useful experimental tools to study the functional role of this nucleotide [34]. Another question is whether human neutrophils possess a cytidylyl cyclase and what the physiological concentration of cCMP in these cells may be. Most importantly, it has to be clarified whether the intracellular concentration of cCMP is subject to regulation by intercellular signal molecules. Previous results suggested the presence of cCMP and a cCMP-degrading phosphodiesterase in leukemia cells [35-37], and more recent results indicate that cytidylyl cyclase is widely distributed in mammalian tissues [7,8]. Suprisingly, there are substantial differences between cytidylyl cyclases from mouse and rat tissues concerning specific activity, tissue distribution and regulation by divalent cations [7,8]. Finally, it will be important to investigate the effects of butyrylated and brominated analogues of cUMP on neutrophil functions, as cUMP was previously reported to be a substrate and an inhibitor of cCMP-degrading phosphodiesterases [29,38].

In conclusion, we have shown that Bt_2cCMP differentially modulates neutrophil activation. Functionally, Bt_2cAMP, Bt_2cGMP and Bt_2cCMP are not equivalent. Regardless of the mechanism involved in the biological activities of Bt_2cCMP, its differential effects on neutrophil activation may render this compound a useful experimental tool to dissect signal transduction pathways.

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