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Cyclic AMP Inhibits Secretion From Electroporated Human Neutrophils

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It has long been known that intracellular cAMP inhibits and cGMP enhances intact neutrophil function. However, these effects are modest and require relatively high concentrations of the cyclic nucleotides. We decided to re-examine the effects of cyclic nucleotides on Ca^{2+}-induced secretion by electroporated cells. This system allowed us to bypass normal cell surface receptor-ligand interactions as well as to directly expose the intracellular space to native cyclic nucleotides. We found that concentrations of cAMP as low as 3 μM inhibited Ca^{2+}-induced secretion; 30–300 μM cAMP was maximally inhibitory. cAMP was actually slightly more potent than dibutyryl cAMP, a membrane-permeant derivative. In contrast, cGMP was only slightly stimulatory at 3 μM and modestly inhibitory at 300 μM; dibutyryl cGMP was ineffective. A more detailed investigation of the effects of cAMP showed that inhibition was only obtained in the presence of Mg^{2+}. Half-maximal inhibition by cAMP occurred at 10–30 μM. Inhibition by cAMP was achieved by shifting the Ca^{2+} dose-response curve for secretion to the right; this was observed for the release of both specific granules (vitamin B_{12} binding protein) and azurophil granules (B-glucuronidase). We previously showed that ATP could enhance Ca^{2+}-induced secretion in the presence of Mg^{2+}, apparently by interacting with a cell surface purine receptor. However, increasing concentrations of ATP could not overcome inhibition by cAMP; this suggested that cAMP acted at some site other than the purine receptor. Inhibition by cAMP was also less apparent in the presence of the protein kinase C agonist phorbol myristate acetate (PMA), suggesting that the cyclic nucleotide did not produce systemic desensitization of the neutrophils. In summary, these results demonstrate that low, physiologically relevant concentrations of cAMP can modulate neutrophil responsiveness.

Key words: degranulation, cyclic nucleotides, granulocytes

INTRODUCTION

We and others have used a variety of means to render neutrophil plasma membranes permeable for the purposes of studying intracellular signal transduction. Neutrophils permeabilized with saponin and digitonin can be induced to degranulate by exposure to Ca^{2+} alone [34,42,43]. Secretion from both specific and azurophil granule can be elicited by Ca^{2+}, with specific granules being the more sensitive. A different method of permeabilizing neutrophils was pioneered by Grinstein and Furuya [10], who used high voltage electric fields to render human neutrophils permeable to molecules of $M_r < 700$ Da. These investigators were thereby able to introduce Ca-EGTA buffers and NADPH into the cytosol and to stimulate O_2 consumption by these cells following exposure to surface stimuli; all experiments were done in the presence of Mg-ATP. More recently, Nasmith et al. [31] reported that a non-hydrolyzable guanine nucleotide, GTP-γ-S, induces both O_2 consumption and tyrosine phosphorylation on its own. Activation of the respiratory burst requires the presence of Mg^{2+} and ATP. Our own work showed that electroporated neutrophils secrete granule contents in response to increases in Ca^{2+} alone [41]. This degranulation is enhanced by Mg^{2+} and a wide variety of hydrolyzable and stable nucleotide phosphates in the presence of Mg^{2+}. The nucleotides appear to be interacting with a non-specific purine receptor which is coupled to protein kinase C.

Permeabilization also permits direct access of cyclic nucleotides to the cytoplasmic space. These compounds play a second messenger role in some cell types. Indeed, exposure of neutrophils to phagocytizable particles results in a rapid (maximal within 15 sec) and brief (returning to basal levels in 1–2 min) doubling of cyclic adenosine 3′,5′-monophosphate (cAMP) levels [12]. Stimulated increments in cAMP levels were later re-

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ported in rabbit [21] and human [11,13,28,30,36,40] neutrophils. The observations that changes in cAMP levels correlate closely with chemotaxis and degranulation [30], display specific desensitization after repeated exposures to the same stimulus, and that this desensitization is accompanied by a parallel decrease in superoxide (O_2^-) generation [35] suggest that changes in cyclic nucleotide levels may be of mechanistic significance. However, these observations are only correlative and a number of lines of evidence suggest that changes in cAMP are not important in stimulus-response coupling in neutrophils. First, cAMP increases can be induced by low concentrations of FMLP, without accompanying degranulation or O_2^- generation [40], suggesting that the former events are not *sufficient* for the latter. Second, increments in cAMP do not appear to be *necessary* for discharge of specific granules or O_2^- generation [30,44]. Finally, an inhibitor of adenylate cyclase, which blocks the stimulated increase in cAMP, does not impair neutrophil responses [37]. These findings seem to rule out cAMP in a second messenger role.

In spite of our previous report that cyclic nucleotides have no effect upon secretion from saponin- or digitonin-permeabilized neutrophils [42,43] and the evidence cited above militating against a role for these compounds in stimulus-response coupling, a number of factors persuaded us to re-examine the issue. The first was the development of an electroporation system for permeabilizing neutrophils, which proved to have substantially different properties from those obtained using cholesterol-complexing agents [41]; this is likely related to the sizes of the pores induced by the different techniques. The second factor was a renewed interest in this area of investigation: increments in cAMP have been linked to calmodulin-dependent processes [20], the effects of cyclic nucleotides have been shown to be dependent upon the stimulus employed [29,32,52], and cAMP has been shown to affect the functions of other permeabilized cell types [4,22,47–49]. In the studies reported here, we found that micromolar concentrations of cAMP inhibited Ca^{2+}-induced secretion from electropermeabilized human neutrophils. This inhibition required Mg^{2+} and resulted from a shift in the Ca^{2+} dose-response curve to the right.

MATERIALS AND METHODS

Reagents

cAMP, cGMP, dibutyryl cAMP, dibutyryl cGMP, ATP (adenosine 5′-triphosphate), and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Company (St. Louis, MO). [γ-^{32}P]-ATP was obtained from Amersham (Arlington Heights, IL). K-252a was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). All chemicals for SDS-PAGE were electrophoresis grade, and all other materials were reagent grade.

Preparation of Cell Suspensions

Heparinized (10 units/ml) venous blood was obtained from healthy adult donors. Purified preparations of neutrophils were isolated from this blood by means of Hypaque/Ficoll gradients [6] followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes [53]. This allowed studies of cell suspensions containing 98 ± 2% neutrophils with few contaminating platelets or erythrocytes. The cells were washed and finally suspended in a buffer consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4. For permeabilization studies, the cells were instead washed once and resuspended in KCl-HEPES buffer (100 mM KCl, 20 mM NaCl, 1 mM EGTA, and 30 mM HEPES, pH 7.0).

Neutrophil Permeabilization

Electropermeabilization was conducted essentially as described by Grinstein and Furuya [10] with some variations [41]. In essence, neutrophils were washed and then resuspended in ice-cold KCl-HEPES buffer; Mg-ATP and Ca^{2+} were not included at this time. The cells, at a concentration of 5×10^7 per ml, were transferred to a chilled Biorad Pulsar cuvette and subjected to three discharges of 5 kV/cm from a 25 μF capacitor (Biorad Gene Pulsar) with gentle stirring between each pulse. The capacitor discharged with a time constant of 0.5–0.7 ms under these conditions. All permeabilization operations were conducted at 4°C. Viability, as assessed by trypan blue exclusion, was consistently greater than 85%, in accord with Grinstein and Furuya [10].

Lysosomal Enzyme Release

Following permeabilization, neutrophils were used with or without washing, as indicated. Aliquots of the ice-cold permeabilized cell suspensions (100 μl, containing 5×10^6 cells) were added to 900 μl of pre-warmed KCl-HEPES buffer containing the indicated additions along with the desired concentration of free Ca^{2+} (determined for the buffer using a Ca^{2+} electrode [5]). The cells were then incubated at 37°C for 7 min; cells incubated without Ca^{2+} and intact neutrophils (in KCl-HEPES buffer) were employed as routine controls. The cell suspensions were then centrifuged at 750*g* for 10 min. Aliquots of the supernatants were taken for standard determinations of β-glucuronidase [7] (an enzyme found exclusively in azurophil granules), lysozyme [1] (an enzyme found in both specific and azurophil granules), and vitamin B_{12} binding protein [38] (a component of specific granules alone).

Electrophoresis and Autoradiography

Trichloroacetic acid precipitates from incubates with [γ-^{32}P]-ATP and appropriate cofactors [45] were extracted in acetone, dried, and resuspended in sample buffer (12.5% glycerol, 5% β-mercaptoethanol, 2% SDS, 62 mM Tris, pH 6.8, and 0.005% bromophenol blue). The samples were sonicated and heated at 100°C for 5 min before loading onto gels.

Electrophoresis was conducted using 10 or 12% mini-slab gels (0.5 × 55 × 85 mm) or 10, 12, or 15% slab gels (0.75 mm × 16 cm × 18 cm) and the discontinuous buffer system of Laemmli [27]. Samples were loaded on the mini-slab gels at approximately 5×10^5 cell equivalents per lane and were electrophoresed at a constant voltage of 180 V for 1 h at 4°C. Samples were loaded at a concentration of 2×10^6 cell equivalents per lane on 16 × 18 cm gels and electrophoresis was conducted at a constant current of 15 mA for 3 h at room temperature. All gels were stained with Coomassie Brilliant Blue. Mini-gels were air dried between cellophane membrane backing (BioRad), while 16 × 18 cm gels were dried between cellophane membranes under vacuum at 80°C on a Biorad gel dryer. For autoradiographic analysis, gels were exposed to X-Omat XAR-5 film at −70°C for 1–10 days, with or without enlightening plus intensifying screens.

RESULTS

We have previously shown that electroporated neutrophils can be induced to secrete lysosomal constituents in response to micromolar levels of free Ca^{2+} [41]. This secretion is enhanced by Mg^{2+} and several nucleotides (in the presence of Mg^{2+}). In the course of these studies, we found that micromolar concentrations of cAMP could inhibit degranulation in this system. Figure 1 demonstrates both the properties of Ca^{2+}-induced secretion and inhibition by cAMP. In the presence of Mg^{2+} alone (control), low concentrations of Ca^{2+} evoked secretion from specific granules, as indicated by the release of vitamin B_{12} binding protein (upper left panel, open circles). When 30 μM cAMP was also present, degranulation was inhibited over the entire range of Ca^{2+} (squares; solid symbols indicate statistical significance from corresponding control points). In the additional presence of ATP, control release of vitamin B_{12} binding

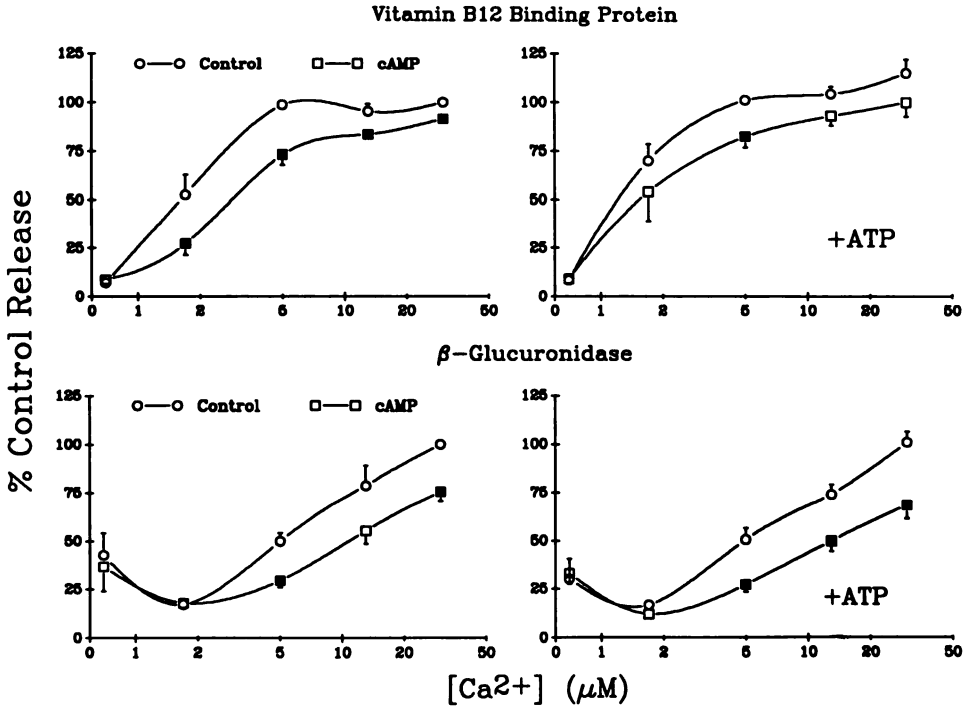

Fig. 1. Effects of ATP and cAMP on Ca^{2+}-induced secretion from electroporated human neutrophils. Human neutrophils were permeabilized as detailed in "Materials and Methods" and stimulated with the indicated concentrations of free Ca^{2+} in the presence of Mg^{2+} (1 mM). Where relevant, the incubations also included cAMP (30 μM; square symbols) and ATP (300 μM). The reaction mixtures were incubated for 7 minutes at 37°C and then centrifuged. Release of vitamin B_{12} binding protein (top) and B-glucuronidase (bottom) were measured in supernatant fluids. The results are given as a percentage of control release, namely that elicited by 30 μM Ca^{2+} in the absence of cAMP; these control release values were 49.9 ± 17.2% of total cellular vitamin B_{12} binding protein and 20.0 ± 4.0% B-glucuronidase. The results shown are the means ± s.e.m. for four experiments. Solid symbols denote those data points which were significantly different ($P < .05$, paired Student t-test) from the absence of added cAMP.

protein was slightly increased; inhibition by cAMP was still observed but was only statistically significant at 5 μM Ca^{2+}. A similar pattern was seen with secretion from azurophil granules, as measured by B-glucuronidase (lower panels), except that substantially more Ca^{2+} was required [41]. cAMP inhibited secretion from this granule type over most of the Ca^{2+} dose range. The clearest overall pattern was for vitamin B_{12} binding protein (upper left panel), which encompassed a full dose-response curve. In this case, the most pronounced effect of cAMP was in the middle ranges, as the nucleotide appeared to shift the Ca^{2+} dose-response curve to the right.

We wished to see whether inhibition by cAMP was unique to this nucleotide and whether permeant analogues of the cyclic nucleotides had any greater effect. For these experiments, we selected optimal conditions for detection of inhibition of vitamin B_{12} binding protein release by cAMP: i.e., a mid-range Ca^{2+} concentration (5 μM) in the presence of the full secretory system (Mg^{2+} plus ATP). As shown in Figure 2, cAMP inhibited secretion at concentrations as low as 3 μM (left set of bars), with greater effect at 30 and 300 μM. Dibutyryl cAMP (dbcAMP), a membrane-permeant derivative, was slightly less potent than cAMP itself; this is not unexpected since native cAMP should readily penetrate permeabilized cells and the larger dibutyryl derivative might traverse the pores even more slowly. cGMP activated degranulation only slightly at 3 μM; at high concentrations, this nucleotide was actually inhibitory. The dibutyryl derivative of cGMP had essentially no effect, with marginal but statistically significant inhibition at 30 μM only.

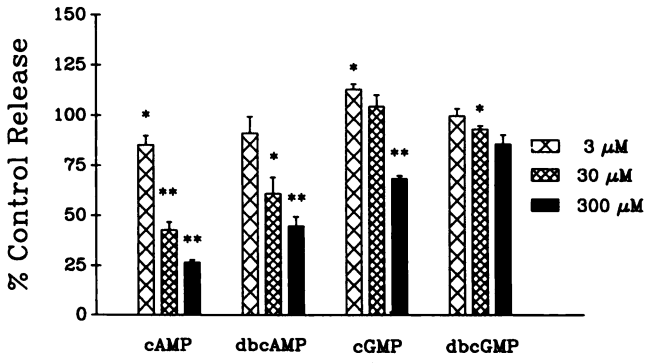

Fig. 2. Effects of various cyclic nucleotides on secretion of vitamin B_{12} binding protein. Permeabilized neutrophils were incubated with Ca^{2+} (5 μM), Mg^{2+} (1 mM), and ATP (300 μM) in the presence of 0, 3, 30, or 300 μM cAMP, dibutyryl cAMP (dbcAMP), cyclic GMP (cGMP), or dibutyryl cGMP (dbcGMP), as indicated. Vitamin B_{12} binding protein was assayed and release is expressed as a percentage of that obtained with control cells (absence of any nucleotide); this control value represented 21.2 ± 7.1% of the total cellular content (three experiments). Asterisks indicate the level of significant differences from control values (*$P < .05$; **$P < .01$, paired Student t-test).

In an effort to get a more detailed description of inhibition induced by cAMP, we varied the concentration of the cyclic nucleotide in the presence and absence of Mg^{2+} and ATP, at a fixed (5 μM) Ca^{2+} concentration. Figure 3 (upper panel) shows that in the absence of Mg^{2+} or ATP (circles), cAMP had no effect. In the presence of ATP alone (triangles), cAMP was only slightly inhibitory. The lower panel shows that Mg^{2+} (squares) was required for inhibition by cAMP at 30 and 100 μM. When ATP was present along with Mg^{2+} (diamonds), significant inhibition by cAMP was seen from 10 to 100 μM.

One possible model for our findings was that cAMP, in the presence of Mg^{2+}, was inhibiting the potentiation by Mg^{2+} of Ca^{2+}-induced secretion. We therefore wished to see if cAMP simply reversed the Mg^{2+}-enhancement of secretion and returned it to the level obtained with Ca^{2+} alone. In experiments not shown, we found that degranulation in the presence of cAMP and Mg^{2+} could be reduced below that obtained with Ca^{2+} alone. Thus, the data were not in accord with the

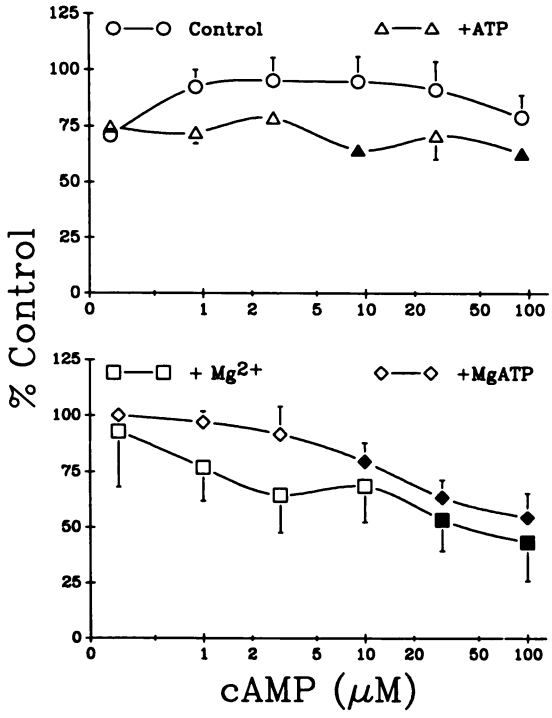

Fig. 3. Effects of various cAMP concentrations on secretion in the presence and absence of Mg^{2+} and ATP. Permeabilized neutrophils were incubated with Ca^{2+} (5 μM) and the indicated concentration of cAMP, with or without Mg^{2+} (1 mM) or ATP (300 μM). Vitamin B_{12} binding protein was assayed and release is expressed as a percentage of that obtained with control cells (absence of cAMP, presence of Mg-ATP); this control value represented 29.2 ± 12.2% of the total cellular content (four experiments). Solid symbols denote those data points which were significantly different ($P < .05$, paired Student t-test) from the absence of added cAMP.

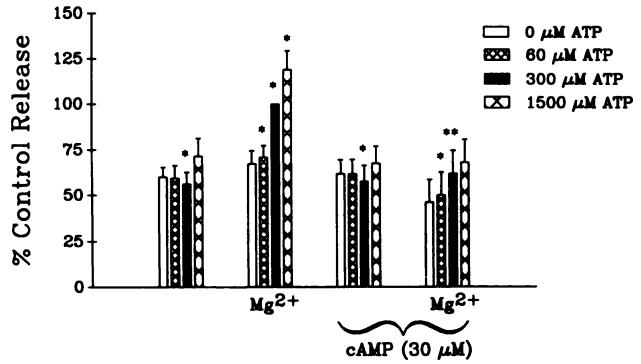

Fig. 4. Effect of ATP on inhibition by cAMP. Permeabilized neutrophils were incubated with Ca^{2+} (5 μM) and 30 μM cAMP, with or without Mg^{2+} (1 mM) or the indicated concentrations of ATP. Vitamin B_{12} binding protein was assayed and release is expressed as a percentage of that obtained with control cells (absence of cAMP, presence of 300 μM Mg-ATP); this control value represented 40.9 ± 7.0% of the total cellular content (three experiments). Asterisks indicate the level of significant differences from equivalent samples without ATP (*$P < .05$; **$P < .01$, paired Student t-test).

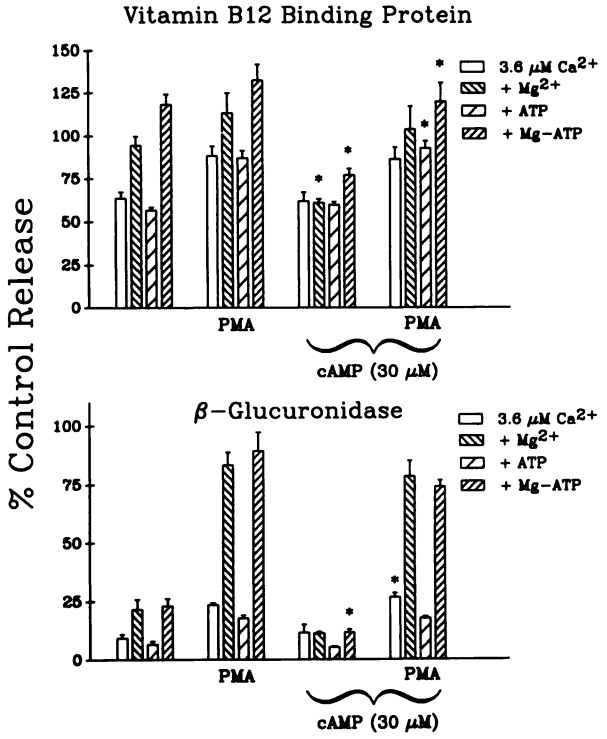

Fig. 5. Effect of PMA on inhibition by cAMP. Permeabilized neutrophils were incubated with Ca^{2+} (3.6 μM) and with or without cAMP (30 μM), Mg^{2+} (1 mM), ATP (300 μM), or PMA (10 ng/ml), as indicated. Vitamin B_{12} binding protein and B-glucuronidase were assayed and release is expressed as a percentage of that obtained with control cells (absence of cAMP, presence of PMA and 60 μM Ca^{2+}); this control value represented 34.1 ± 1.4% of the total cellular content of vitamin B_{12} binding protein and 16.6 ± 1.7% of the total B-glucuronidase (three experiments). Asterisks indicate samples which were significantly different in the presence of cAMP ($P < .05$, paired Student t-test).

model cited above and cAMP thus was not simply an antagonist for the action of Mg^{2+}. In other control experiments, we found that neither the phosphodiesterase inhibitor theophylline nor the adenylate cyclase activator PGE_1 potentiated the action of cAMP (not shown). This would be expected since in permeabilized, but not intact, cells the cAMP level would be "clamped" by the concentration present in the medium.

Because ATP appears to stimulate Ca^{2+}-induced secretion by interacting with a non-specific purine nucleotide binding site [41], we wished to determine whether cAMP also interacted with the same receptor. Unfortunately, no specific inhibitor of this receptor has been reported. Consequently, the most direct experiments we could perform were to see if ATP itself could compete with cAMP and overcome its inhibition. As shown in Figure 4, increasing concentrations of ATP from 0 to 1,500 M produced little change in Ca^{2+}-induced secretion in the absence of Mg^{2+} (left set of bars). When Mg^{2+} was present (second set of bars), increasing concentrations of ATP produced greater degranulation. As shown before, cAMP had no effect in the absence of Mg^{2+} (third set); however, in the presence of this divalent cation (fourth set of bars), cAMP did inhibit ATP-enhanced secretion, even in the presence of 1,500 μM nucleotide. The fact that high concentrations of ATP did not counteract inhibition by cAMP indicated that the cyclic nucleotide was interacting at a site other than that utilized by ATP (presumably the purine receptor).

In order to see whether cAMP produced a systemic desensitization of neutrophil secretion, we examined the effects of this nucleotide on cells in which protein kinase C (PKC) was stimulated by phorbol myristate acetate (PMA). We have previously shown that PMA synergistically enhances Ca^{2+}-induced secretion from electroporated neutrophils in the presence of Mg^{2+} and Mg^{2+}-ATP [41]; in this report, we showed that PMA reduces the Ca^{2+} requirement for half-maximal specific granule secretion from 3 to 0.5 μM. The upper panel of Figure 5 shows Ca^{2+}-induced release of vitamin B_{12} binding protein; the left set of bars presents control data. It can be seen that Mg^{2+} and Mg^{2+}-ATP (but not ATP alone) increased degranulation stimulated by Ca^{2+}, as expected. This pattern was enhanced in the presence of PMA (second set of bars). When cAMP (and Mg^{2+}) was present, release of vitamin B_{12} binding protein was strongly inhibited (compare the first and third sets of bars). However, when PMA was also present, inhibition by cAMP was much muted (compare second and fourth set of bars). A similar pattern was also seen for the release of B-glucuronidase (lower panel).

The most likely means by which cAMP inhibited degranulation was by stimulating a cAMP-dependent protein kinase (PKA). We were unable to overcome cAMP inhibition using K-252a, an inhibitor of this kinase (data not shown). This is probably because K-252a could also inhibit protein kinase C, which is required for ATP-enhanced secretion [41]. We also attempted to determine the molecular target of the putative PKA. Neutrophils were electroporated in the presence of [γ-^{32}P]-ATP, either carrier-free or with 30 μM unlabeled ATP, and then exposed to Ca^{2+}, Mg^{2+}, and cAMP under conditions which inhibited secretion. Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. In essence, we were unable to find any proteins which were specifically phosphorylated in the presence of cAMP, with or without the divalent cations (not shown).

DISCUSSION

It has long been known that agents which increase intracellular cAMP levels reduce neutrophil responsiveness while those agents which increase cGMP concentrations enhance cellular functions [54]. The potential importance of these second messengers is amplified by the prompt production of cAMP following neutrophil stimulation [12,36,40] and correlation of these increments with azurophil granule secretion [44]. While stimulus-elicited increases in cGMP have been reported [17–19], they have not been observed at early times by others [36,40].

One of the difficulties in cyclic nucleotide research is that the effects of these agents on neutrophil function are quite modest. High concentrations of dibutyryl cAMP (>150 μM) are often needed to obtain 20% inhibition of degranulation and the putative stimulation obtained with dibutyryl cGMP is often not evident [39]. Compounding this problem is the fact that the effects of cyclic nucleotides are dependent upon both the stimulus [20,29, 32,51,52] and the response [9,26] being examined. An additional difficulty with most studies is that intracellular cyclic nucleotide levels must be modulated either directly by adding membrane-permeant derivatives or indirectly by use of prostaglandins or beta-adrenergic agents. However, with permeabilized cells, it is possible to specifically introduce native cyclic nucleotides into the cytoplasmic space in order to test their functions. This model system bypasses cell surface receptors and the individual signal transduction pathways initiated by specific stimuli. It was therefore gratifying to find that low concentrations of cAMP (down to 3 μM) could inhibit Ca^{2+}-induced degranulation. Maximal inhibition required 30–100 μM cAMP and could reach 50%, substantially more than seen with intact cells. The data in Figures 2–4 show that the amount of inhibition obtained ranged from 25 to 60%, which is most attributable to the variability normally seen in the responses of permeabilized neutrophils [41]. cGMP did not stimulate degranulation in this system; in fact, high concentrations of this cyclic nucleotide inhibited responsiveness. Dibutyryl cAMP was less effective than the native compound. This is likely to be due to the larger size of the derivatized agent which makes it comparable to the pore dimensions of electropermeabilized neutrophils [10,41].

We also found that inhibition by cAMP required Mg^{2+} and was maximal when ATP was present. cAMP shifted the Ca^{2+} dose-response curve to the right and appeared to antagonize the potentiation by Mg-ATP. The requirement for Mg^{2+} suggests two main possibilities for the mechanism of action of cAMP. The first possibility is that cAMP is somehow interacting with a recently described cell surface purine nucleotide receptor [3,25]. This receptor binds a number of nucleotides, in addition to ATP, in the presence of Mg^{2+} and can lead to enhancement of cellular function in intact [8] and permeabilized [41] cells. In permeabilized neutrophils, the purine nucleotide apparently activates protein kinase C, promoting degranulation [41]. In view of the broad nucleotide specificity of this receptor, it was possible that cAMP was binding to it as an inhibitor. However, increasing concentrations of ATP did not antagonize the effects of cAMP and so these two nucleotides were likely to be operating at different sites.

A second possible mechanism for the action of cAMP, which is consistent with the requirement for Mg^{2+}, is the activation of a cAMP-dependent protein kinase (PKA). This enzyme has been isolated and characterized in neutrophils [15,23,50] and a number of its acceptor proteins determined [14]. More specifically, PKA can phosphorylate a component of the NADPH oxidase [24] and regulate a component in the platelet-activating factor biosynthetic pathway [33]. However, in intact cells, endogenous protein phosphorylation is not apparently altered by dibutyryl cAMP [2], suggesting that cAMP-dependent phosphorylation is only a fraction of the total kinase activity. Furthermore, we have shown that phosphorylation of endogenous proteins in neutrophil homogenates is primarily dependent upon Mg^{2+}, without a requirement for other cofactors [45,46]. In view of this high background from competing kinases, it is not surprising that we were unable to detect any obvious acceptors for PKA. It is possible that this enzyme is phosphorylating some relatively rare component, such as another enzyme, which mediates inhibition by cAMP.

While it is clear that increments in cAMP do not play a positive effector role in signal transduction in neutrophils, it is also clear that they can play a modulatory role. The studies reported here demonstrate that low, physio-

logically relevant concentrations of cAMP can inhibit secretion in a permeabilized model system in which normal receptor-ligand interactions are bypassed. The mechanism by which cAMP exerts its effects is unknown. Our own data indicate that PKC activation can overcome inhibition by cAMP (Fig. 5), suggesting that the nucleotide acts proximal to PKC. Other potential mechanisms were apparently excluded by Mueller and Sklar [29], who published evidence that inhibition of O_2^- generation by beta-adrenergic agonists (and, presumably, cAMP) does not involve intracellular Ca^{2+}, the activation of protein kinase C, or the activation of G-proteins. Other reports indicate that inhibition by cAMP appears to require Ca^{2+} or calmodulin [20,32] but does not involve perturbation of intracellular Ca^{2+} levels per se. Stimulated increases in endogenous cAMP also appear to require Ca^{2+} [51] and may be due to activation of adenylate cyclase by endogenously produced adenosine [16]. This close tie between cAMP and Ca^{2+} can be readily exploited by the permeabilized cell model and future experiments will attempt to more precisely localize the mode of action of this cyclic nucleotide.

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