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# $Ca^{2+}$-induced secretion by electropermeabilized human neutrophils. The roles of $Ca^{2+}$, nucleotides and protein kinase C

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**Studies of stimulus-response coupling have benefitted from the availability of permeabilization techniques, whereby putative second messengers and intracellular modulators can be introduced into the cell interior. Electropermeabilization, which uses high-intensity electric fields to breach the plasma membrane, creates small pores, permitting access of solutes with molecular masses below 700 KDa. Neutrophils permeabilized by this technique, but not intact cells, discharged lysosomal constituents when exposed to micromolar levels of $Ca^{2+}$. Secretion by electroporated neutrophils was significantly enhanced by the presence of Mg-ATP (0.3–1.0 mM). Contrary to expectations, it was determined that ATP was not the only nucleotide which enhanced $Ca^{2+}$-induced secretion in the presence of $Mg^{2+}$. Not only could GTP, XTP, ITP, UTP or ADP partially or completely replace ATP, but even non-hydrolyzable nucleotides such as ADPβS ATPγS, and App[NH]p were effective. GTPγS and GDPβS were inhibitory, while Gpp[NH]p was inactive. None of these nucleotides induced secretion on its own. In contrast, neutrophils which were permeabilized and then washed, were only slightly activated by Mg-ATP and other nucleotides; even the response to $Ca^{2+}$ alone was less. This hyporesponsiveness of washed cells proved to be due to a time-dependent deactivation of the permeabilized neutrophils taking place at 4 °C. In an effort to assess the role for protein kinase C (PKC) in secretion in this system, we examined the effects of phorbol myristate acetate (PMA), a PKC agonist. PMA enhanced degranulation induced by $Ca^{2+}$ by lowering the requirement for this divalent cation; enhancement by PMA was not dependent upon exogenous ATP. Three inhibitors of PKC with varying specificity, namely H-7, K-252a, and staurosporine, all abrogated PMA-enhanced secretion. These agents also inhibited secretion stimulated by $Ca^{2+}$ plus ATP in parallel with that induced by $Ca^{2+}$ plus PMA, strongly suggesting a role for PKC in modulation of degranulation by ATP. Our results show that electropermeabilized neutrophils provide a convenient, useful model for stimulus-secretion coupling. These data also suggest that the 'requirement' for Mg-ATP, which has been observed in other permeabilized cell systems, is not simply for metabolic energy or as a substrate for kinases. It is possible that these nucleotides all interact with a recently described neutrophil receptor for adenine nucleotides or with a recently postulated exocytosis-linked G-protein.**

## Introduction

We and others have used a variety of means to render neutrophil plasma membranes permeable for the purposes of studying intracellular signal transduction. Using neutrophils permeabilized with saponin and digitonin, it has been shown that secretion can be induced by elevated intracellular $Ca^{2+}$ alone [1–3]. Both specific and azurophil granule secretion was elicited by $Ca^{2+}$, with specific granules being the more sensitive. Granule secretion from neutrophils permeabilized with Sendai virus [4–6] also displayed differential sensitivity to $Ca^{2+}$. In addition, a role for G-proteins in stimulus-response coupling in neutrophils permeabilized by Sendai virus or digitonin was indicated by the fact that stable guanine nucleotides could both induce secretion on their own, as well as enhance $Ca^{2+}$-induced degranulation [4,7]. These guanine nucleotides have been shown to regulate interconversion between states of the formyl peptide receptor [8], indicating that receptor-G protein interactions remain intact in this model system. Permeabilized cell systems have also been used to show that guanine nucleotides can regulate phosphatidyl inositol-specific phospholipase C [9] and that the resultant $IP_3$ interacts with a specific intracellular receptor [10] to stimulate

Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; XTP, xanthine 5′-triphosphate; ITP, inosine 5′-triphosphate; UTP, uridine 5′-triphosphate; App[NH]p, adenylyl imidodiphosphate; Gpp[NH]p, 5′-guanylyl imidophosphate; GDPβS, guanosine 5′-[2-thio]diphosphate; ADPβS, adenosine 5′-[2-thio]diphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PKC, protein kinase C; PMA, phorbol myristate acetate; IP, inositol triphosphate.

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the release of $Ca^{2+}$ [2,11]. Finally, we have exploited our ability to have direct access to the neutrophil cytoplasm to show that the fusion process between granule and plasma membranes is not governed by an osmotic mechanism [12].

Each of the permeabilized techniques used in the above-mentioned studies has its own advantages and disadvantages; each has limitations with respect to the size of pores created, the stability of the permeabilized state and the extent to which the 'normal' stimulus-response coupling pathway has been left intact. Electropermeabilization has been used for some time on cells other than neutrophils (primarily platelets and pancreatic acinar cells), apparently producing small pores but leaving signal transduction pathways relatively intact [13–26]. The physical basis for this technique is the imposition of an electric field of approx. 1.5 V (applied by a high-voltage field) across the cell membrane (reviewed by Knight and Scrutton in Ref. 17). The conditions of voltage, charge and solution conductance must all be determined semi-empirically [17,27]. The use of electropermeabilization with neutrophils was pioneered by Grinstein and Furuya [28]. High-voltage electric fields were shown to make human neutrophils permeable to molecules of $M_r < 700$. By this technique, these investigators were 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. [29] reported that a non-hydrolyzable guanine nucleotide, GTPγS, induced both $O_2$ consumption and tyrosine phosphorylation on its own. Activation of the respiratory burst required the presence of $Mg^{2+}$ and ATP.

Our own work has focused on secretion of granule constituents, rather than oxidase activation, in response to surface or intracellular (second messenger) stimulation. Our primary model has been the neutrophil permeabilized with cholesterol-complexing agents such as saponin or digitonin. In order to take advantage of the benefits of electroporation and to compare its properties with permeabilization by digitonin, we extended our secretion studies to neutrophils rendered permeable by this novel technique. In essence, we found that electroporated neutrophils secreted granule constituents following exposure to $Ca^{2+}$. This process was facilitated by ATP and other nucleotides in the presence of $Mg^{2+}$. However, rapid desensitization of the electroporated neutrophils does take place and can be a problem for some studies, such as those requiring gentle washing of the cells. Our data also indicate that protein kinase C (PKC) could be activated in these cells and that this enzyme might be involved in modulation of secretion by ATP and other nucleotides. These results show that neutrophils permeabilized by high voltage discharge provide a good model system for the study of secretion.

## Materials and Methods

*Reagents*

GTP, Gpp[NH]p, GDP, GDPβS, ATP, ADP, App[NH]p, ADPβS, XTP, ITP and UTP were purchased from Sigma (St. Louis, MO). GTPγS was obtained from Boehringer-Mannheim (Indianapolis, IN). K-252a and staurosporine were purchased from Kamiya (Thousand Oaks, CA) and H-7 was from Seikagaku America. All other materials were reagent grade.

*Preparation of cell suspension*

Heparinized (10 U/ml) venous blood was obtained from healthy adult donors. Purified preparations of neutrophils were isolated from this blood by means of Hypaque/Ficoll gradients [30] followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes [31]. This allowed studies of cell suspensions containing $98 \pm 2\%$ 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 20 mM Hepes (pH 7.0)).

*Neutrophil permeabilization*

Electropermeabilization was conducted essentially as described by Grinstein and Furuya [28] with some variations. 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 (except when indicated). The cells, at a concentration of $5 \cdot 10^7$ per ml, were transferred to a chilled Bio-Rad Pulsar cuvette and subjected to three discharges of 5 kV/cm from a 25 μF capacitor (Bio-Rad 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 [28].

*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 $5 \cdot 10^6$ 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 [32]). 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 $750 \times g$

for 10 min. Aliquots of the supernatants were taken for standard determinations of β-glucuronidase [33] (an enzyme found exclusively in azurophil granules), lysozyme [34] (an enzyme found in both specific and azurophil granules), and vitamin B-12 binding protein [35] (a component of specific granules alone).

## Results

### *$Ca^{2+}$, $Mg^{2+}$ and ATP*

In view of our interest in secretion by permeabilized neutrophils, we wished to see if electroporation, already established for the study of oxidase activation [28,29], could be extended to degranulation. In the original work from Grinstein's laboratory, Mg-ATP was present during both permeabilized and respiratory burst activation [28] and then later shown to be necessary for the support of $O_2$ consumption [29]. Since we wished to examine nucleotide requirements separately, we permeabilized our cells in a KCl-Hepes buffer which we had employed extensively (for permeabilized studies with digitonin and saponin) [1,3] in the absence of $Ca^{2+}$, ATP or $Mg^{2+}$. The conductance of this solution resulted in a discharge time constant of 0.5–0.7 ms, somewhat longer than the 0.3–0.4 ms reported by Grinstein and Furuya [28]. We also used more concentrated cell suspensions, which seemed to have no impact on the electroporation process. Otherwise, permeabilization was performed exactly as they had described.

As shown in Fig. 1, electroporated neutrophils secreted lysosomal constituents when exposed to elevated $Ca^{2+}$ alone (open circles). Vitamin B-12 binding protein (top), a constituent of specific granules, was released at $Ca^{2+}$ concentrations as low as 2 μM, while β-glucuronidase (bottom), a constituent of azurophil granules, required as much as 10 μM; lysozyme (middle), contained in both granule types, was released with intermediate sensitivity. This is typical of the differential degranulation which is observed with both intact and digitonin-permeabilized cells [3,6]. When the electroporated neutrophils were also incubated with 1 mM $Mg^{2+}$ and 300 μM ATP, a substantial enhancement of secretion was observed for all three granule constituents. This was most noticeable as a shift in the $Ca^{2+}$ dose/response curve to the left.

We next wished to see what concentrations of ATP were required to promote $Ca^{2+}$-induced secretion. For these experiments, we incubated the permeabilized cells with 5 μM $Ca^{2+}$, a mid-range concentration deemed optimal for detecting nucleotide-enhanced release of vitamin B-12 binding protein (although not necessarily the other granule constituents), along with 1 mM $Mg^{2+}$. Control (100%) release was then induced by 'saturating' concentrations of $Ca^{2+}$, in this case 30 μM. As shown in Fig. 2, increasing concentrations of ATP resulted in enhanced release of vitamin B-12 binding protein, with

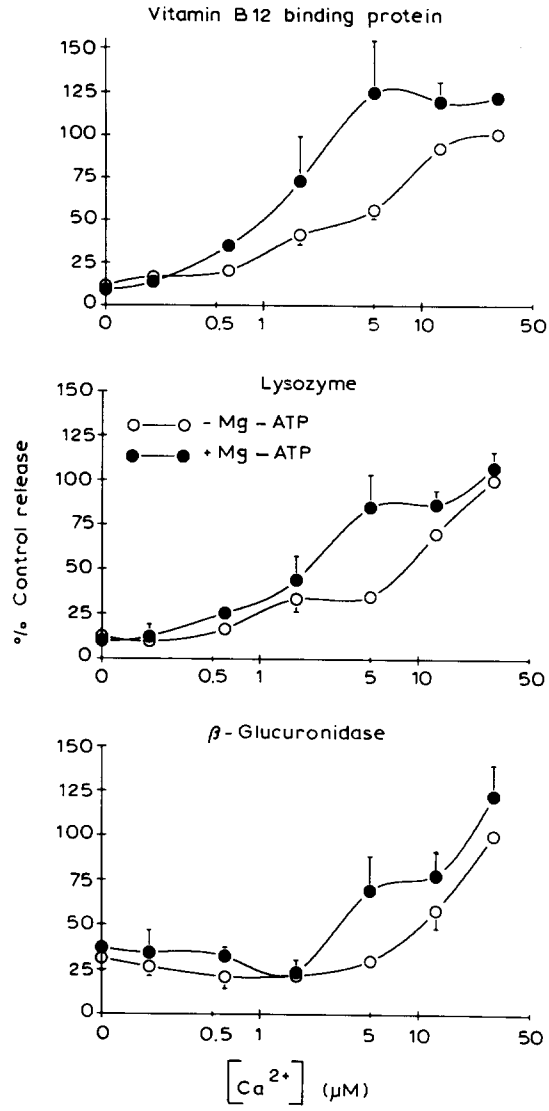


Fig. 1. Release of granule constituents by electropermeabilized neutrophils in response to $Ca^{2+}$. Human neutrophils were isolated and permeabilized as described in Materials and Methods. Aliquots of the cell suspension were added to prewarmed tubes containing KCl-Hepes buffer and the indicated free $Ca^{2+}$ concentrations and with (closed symbols) or without (open symbols) $Mg^{2+}$ (1 mM) plus ATP (0.3 mM). The reaction mixtures were incubated for 7 min at 37°C and then centrifuged. Release of vitamin B-12 binding protein (top panel), lysozyme (middle panel) and β-glucuronidase (bottom panel) 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 Mg-ATP; these control release values were 42.3±3.0% of total cellular vitamin B-12 binding protein, 16.7±7.1% lysozyme, and 9.2±5.1% β-glucuronidase. The results shown are the means±S.E. for three experiments.

an optimal response at 300–1000 μM. The experimental conditions were not optimized for the release of lysozyme and β-glucuronidase; secretion of lysozyme was slightly, but not significantly enhanced by ATP while the release of β-glucuronidase was not changed. In separate experiments, it was shown that optimal secretion of all granule constituents also required 1–3 mM

$Mg^{2+}$ (not shown); without this divalent cation, ATP did not enhance $Ca^{2+}$-induced release.

*Other nucleotides*

Mg-ATP is often added to permeabilized cell preparations under the assumption that it is required for protein kinases or for metabolic energy. To check the strictness of this requirement for ATP, we tested a number of hydrolyzable and non-hydrolyzable nucleotides as replacements. We again chose (as our screen) conditions under which Mg-ATP maximally enhanced secretion of vitamin B-12 binding protein by mid-range levels of $Ca^{2+}$. As shown in Table I, we first looked at the effects of adenine nucleotides on secretion induced by 3.6 $\mu$M $Ca^{2+}$ in the presence of 1 mM $Mg^{2+}$. All of these compounds enhanced secretion significantly, with ATP, ADP$\beta$S, and ADP being the most potent. None of these nucleotides stimulated degranulation on its own (i.e., in the absence of added $Ca^{2+}$).

Among the guanine nucleotides, only GTP was consistently stimulatory, being almost as effective as ATP (Table I). GDP and Gpp[NH]p were inactive, while GDP$\beta$S was inhibitory. The most potent antagonist was GTP$\gamma$S, which was effective only at the moderate $Ca^{2+}$ concentrations shown in Table I. Other preliminary data showed that 10 $\mu$M GTP$\gamma$S inhibited secretion induced by 3.6 $\mu$M $Ca^{2+}$ more than 50%, while 100 $\mu$M GTP$\gamma$S had no effect when saturating $Ca^{2+}$ (60 $\mu$M) was used (not shown).

Other investigators have reported that XTP, ITP and UTP can substitute for ATP in secretion [36]. We therefore tested these nucleotides for potency in electroporated neutrophils. As shown in Table I, all three of these nucleotides enhanced $Ca^{2+}$-induced secretion as effectively as ATP.

Thus, a very broad range of nucleotides, both hydrolyzable and stable, could substitute for ATP in enhancing secretion. In virtually all of these cases, $Mg^{2+}$ was required for this to occur. The right side of Table I shows the results obtained using the various nucleotides in the absence of $Mg^{2+}$. Only GTP still enhanced secretion significantly under these conditions and the extent of this enhancement was slight. GDP$\beta$S was inhibitory in both the absence and presence of $Mg^{2+}$.

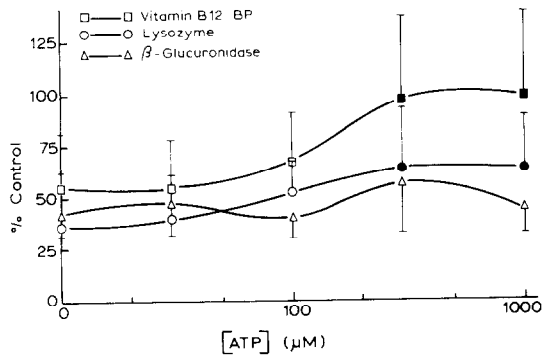


Fig. 2. Enhancement by Mg-ATP of $Ca^{2+}$-induced secretion by electroporated neutrophils. Aliquots of the cell suspension were added to prewarmed tubes containing KCl-Hepes buffer, 5 $\mu$M free $Ca^{2+}$, 1 mM $Mg^{2+}$ and the indicated concentrations of ATP. See Materials and Methods and the Legend to Fig. 1 for more details. The results are given as a percentage of control release, namely that elicited by 30 $\mu$M $Ca^{2+}$ in the absence of Mg-ATP; these control release values were 34.1 ± 9.6% of total cellular vitamin $B_{12}$ binding protein, 16.3 ± 5.5% lysozyme, and 10.2 ± 4.1% $\beta$-glucuronidase. Solid symbols denote those data points which were significantly different from the absence of exogenous ATP.

TABLE I

*Enhancement of $Ca^{2+}$-induced degranulation of various nucleotides*

Electroporated neutrophils were incubated with $Ca^{2+}$ (3.6 $\mu$M), with or without $Mg^{2+}$ (1 mM), and the individual nucleotides, as indicated. The nucleotides were present at 300 $\mu$M concentrations, except for ATP$\gamma$S and GTP$\gamma$S (both 30 $\mu$M). 100% values were provided by release, observed with 60 $\mu$M $Ca^{2+}$ with ATP (29.5 ± 1.3%, $n = 18$ of total cellular vitamin B-12 binding protein). The release values for vitamin B-12 binding protein are given as means ± S.E. with number of experiments in parentheses. Statistical significance was determined by paired Students' *t*-tests and are given as: * $P < 0.05$; ** $P < 0.01$ compared to no addition.

| Nucleotide | Vitamin B-12 binding protein (% control release) | | | |
|---|---|---|---|---|
| | $+Mg^{2+}$ | | $-Mg^{2+}$ | |
| None | 54.3 ± 2.8 | (22) | 35.5 ± 1.6 | (18) |
| ATP | 91.0 ± 3.0 ** | (22) | 35.8 ± 1.7 | (18) |
| ADP$\beta$S | 99.8 ± 20.9 * | (5) | 34.5 ± 3.1 | (3) |
| ADP | 87.1 ± 23.9 * | (4) | 29.5 ± 1.2 | (3) |
| ATP$\gamma$S | 61.0 ± 11.8 * | (6) | 48.5 ± 10.0 | (6) |
| App[NH]p | 58.0 ± 10.9 * | (6) | 42.6 ± 9.3 | (6) |
| GTP | 82.8 ± 8.2 ** | (7) | 38.6 ± 4.3 * | (7) |
| GDP | 65.0 ± 27.7 | (3) | 25.3 ± 3.1 | (3) |
| GppNHp | 62.3 ± 14.8 | (6) | 42.2 ± 8.6 | (5) |
| GDP$\beta$S | 40.5 ± 23.2 * | (3) | 25.5 ± 4.7 ** | (3) |
| GTP$\gamma$S | 18.8 ± 7.0 * | (3) | 21.0 ± 2.0 | (3) |
| XTP | 86.8 ± 5.1 ** | (3) | 21.6 ± 7.7 | (3) |
| ITP | 86.5 ± 6.2 ** | (3) | 20.9 ± 6.9 | (3) |
| UTP | 81.0 ± 10.9 * | (3) | 17.7 ± 7.5 | (3) |

*Desensitization*

In view of the variety of effects of the various nucleotides, it was possible that endogenous nucleotides released from the permeabilized neutrophils might complicate our interpretations. This concern was substantiated by the finding that the permeabilized cell suspensions contained approx. 15 $\mu$M ATP, 7 $\mu$M ADP and 4 $\mu$M GTP (Balazovich, K., personal communication). The nucleotides would be diluted to 1.5 $\mu$M ATP, 0.7 $\mu$M ADP and 0.4 $\mu$M GTP by the experimental protocol; these concentrations were low but potentially significant levels. Consequently, we performed a number of experiments in which the permeabilized cells were

washed, to remove this endogenous component, before incubation with the nucleotides and stimulus.

We found that washed cells responded to nucleotides in a much different manner than unwashed neutrophils. Stimulation by ATP (in the presence of $Mg^{2+}$) was much less and only marginally significant statistically (not shown). Many of the effects of the nucleotides observed with unwashed cells (Table I) were reflected in the washed neutrophils; however, the magnitudes of the responses were much lower.

In order to see if hyporesponsiveness to $Ca^{2+}$ or Mg-ATP were taking place in the washed cells, $Ca^{2+}$ dose–response characteristics were measured. As can be seen in Fig. 3, washed neutrophils secreted lysosomal

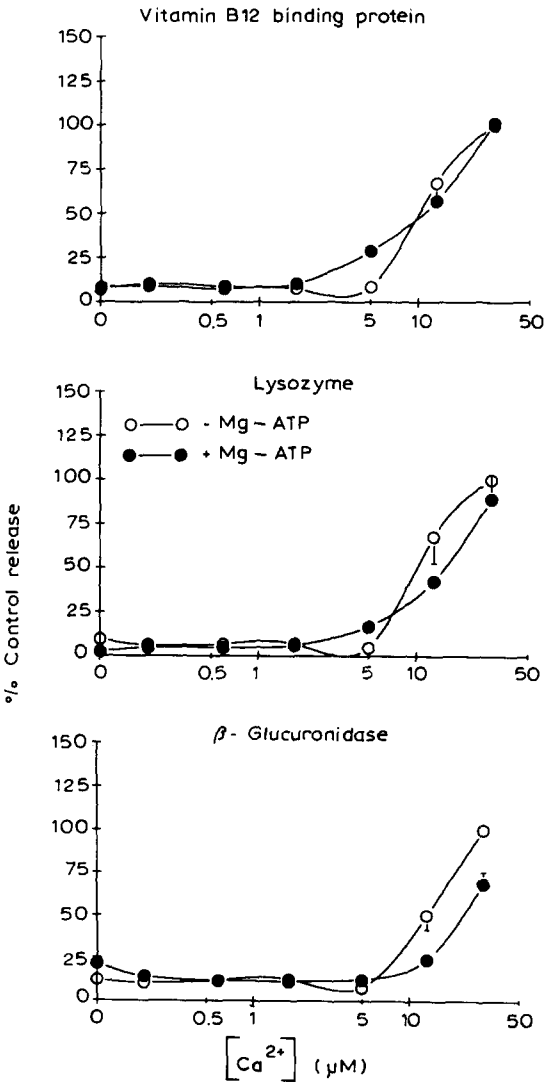


Fig. 3. Release of granule constituents by washed electropermeabilized neutrophils in response to $Ca^{2+}$. Human neutrophils were isolated, permeabilized and then washed as described in the Legend to Fig. 2. Control release values were 31.7±3.5% of total cellular vitamin B-12 binding protein, 21.3±4.9% lysozyme, and 20.3±5.1% β-glucuronidase. The results shown are the means±S.E. for three experiments.

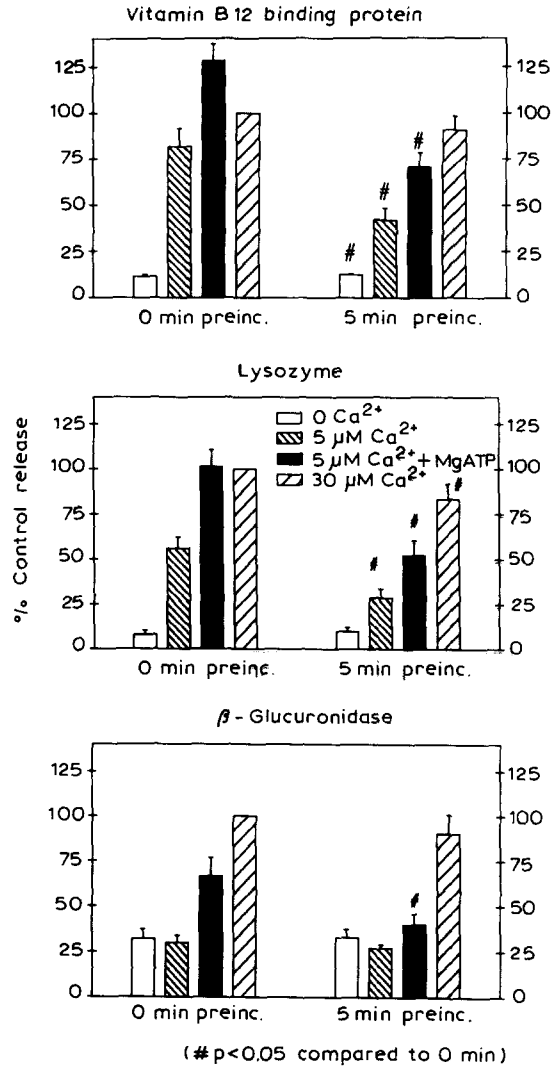


Fig. 4. Time-dependent desensitization of $Ca^{2+}$-induced and Mg-ATP-enhanced secretion by electroporated neutrophils. Electroporated neutrophils were preincubated for either 0 (left set of bars) or 5 min (right set of bars) at 4°C. These cells were then incubated with $Ca^{2+}$ (0, 5 or 30 μM) and with or without Mg-ATP (1 mM $Mg^{2+}$, 0.3 mM ATP) for 7 min at 37°C, as indicated. Secretion of vitamin B-12 binding protein (top), lysozyme (middle), or β-glucuronidase (bottom) were measured and are expressed as a percentage of control release (induced by 30 μM $Ca^{2+}$ plus Mg-ATP). Conditions under which 5 min preincubation resulted in secretion which was significantly different from 0 min controls are denoted by #. The results are given as the means±S.E. from seven experiments. Control release values were 36.7±7.5% of total cellular vitamin B-12 binding protein, 21.9±5.1% lysozyme and 11.2±4.0% β-glucuronidase.

constituents in response to $Ca^{2+}$, but the curves were shifted to the right in comparison with Fig. 1. To an even greater extent, enhancement of secretion by Mg-ATP was virtually eliminated. The decreased responsiveness to Mg-ATP could not be overcome by increasing the ATP concentration (not shown).

One possible cause of the hyporesponsiveness to Mg-ATP was that some critical endogenous component

was leaking out of the permeabilized cells during the washing step. We therefore conducted experiments, in which supernatants from permeabilized neutrophils were harvested (and concentrated in some cases) and then added back to cell suspensions in the hope of restoring responsiveness to Mg-ATP; no such restoration occurred (not shown). However, the controls we conducted during these experiments revealed that the permeabilized cells desensitized over time, without washing, at 4°C. 50% of the neutrophil responsiveness to $Ca^{2+}$ and to Mg-ATP plus $Ca^{2+}$ was lost after 5 min on ice (Fig. 4); this progressive loss continued over at least 20 min (not shown).

In an effort to forestall this time-dependent desensitization to Mg-ATP, we tried a number of strategies. The continued presence of GTP, ADPβS, phorbol myristate acetate, phosphatase or proteinase inhibitors, or low concentrations of $Mg^{2+}$, $Ca^{2+}$ or ATP were all without effect (not shown). The permeabilized cells were probably not resealing as electroporating them a second time had no effect and, in any case, high concentrations of $Ca^{2+}$ evoked normal levels of secretion. We were thus unable to achieve any amelioration of the time-dependent desensitization.

*Protein kinase C*

Although the lack of nucleotide specificity (Table I) strongly suggested that ATP was serving as a ligand rather than a substrate, it was still desirable to see if protein kinase C (PKC) had any role in secretion by this system. We found that, unlike neutrophils permeabilized with digitonin [37], secretion from electroporated cells could be synergistically enhanced by the PKC agonist phorbol myristate acetate (PMA). Fig. 5 shows $Ca^{2+}$ dose-response curves for secretion of the three granule constituents, all in the presence of $Mg^{2+}$ (1 mM); PMA (10 ng/ml) and ATP (300 μM) were used as modulators. PMA only slightly stimulated background secretion (in the absence of $Ca^{2+}$) of vitamin B-12 binding protein and lysozyme; the most prominent effect of PMA was to shift the $Ca^{2+}$ dose–response curves for secretion of all three granule constituents to the left. PMA was more effective than ATP in diminishing the $Ca^{2+}$ requirements, but the combination of the two agents produced the most degranulation. Preliminary studies showed that GTP and UTP promoted the effects of PMA as much as did ATP. In control experiments (not shown), PMA induced an increase in background secretion from intact cells; however, no enhancement with $Ca^{2+}$ was seen and virtually no release was observed with β-glucuronidase. Furthermore, 4α-phorbol, which does not activate PKC, did not promote secretion (not shown). Thus, the results shown in Fig. 5 could not be attributed to intact cells and indicated that PKC was activatable in electroporated neutrophils.

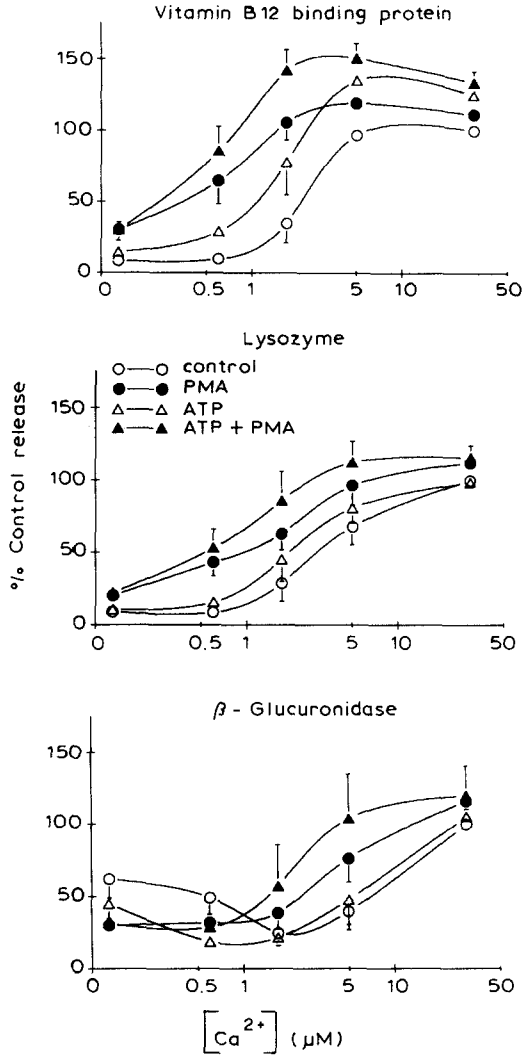


Fig. 5. Modulation of $Ca^{2+}$-induced secretion by ATP and PMA. Electroporated neutrophils were incubated with $Mg^{2+}$ (1 mM) and the indicated free $Ca^{2+}$ concentrations and with 10 ng/ml PMA (closed symbols) and/or 300 μM ATP (triangles). Release of vitamin B-12 binding protein (top panel), lysozyme (middle panel) and β-glucuronidase (bottom panel) 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 ATP; these control release values were 28.2±2.1% of total cellular vitamin B-12 binding protein, 21.3±2.1% lysozyme and 13.2±3.7% β-glucuronidase. The results shown are the means±S.E. for three experiments.

The ability of PMA to enhance $Ca^{2+}$-induced secretion was used as a tool to examine the role of PKC in nucleotide-enhanced responses. Our rationale was that if PKC were involved in these processes, then they should have similar sensitivity to PKC inhibitors as PMA-enhanced degranulation. We first looked at the effects of staurosporine, a relatively potent (although not entirely specific) inhibitor of PKC [38], on $Ca^{2+}$-induced secretion modulated by ATP and PMA. In these experiments, low (1.7 μM) concentrations of $Ca^{2+}$ were used to amplify the enhancement of secretion induced by PMA and ATP. As shown in Fig. 6, increasing

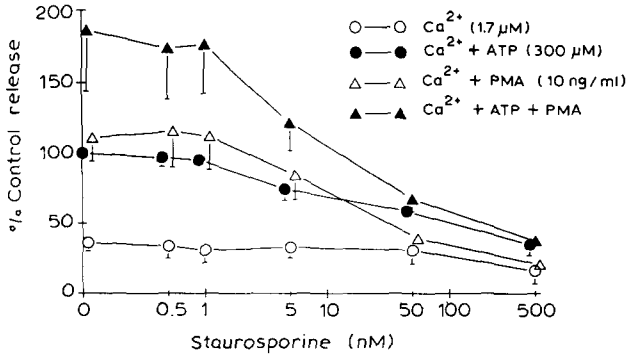


Fig. 6. Effect of staurosporine on $Ca^{2+}$-induced secretion modulated by ATP and PMA. Electroporated neutrophils were incubated with $Mg^{2+}$ (1 mM), 1.7 $\mu$M free $Ca^{2+}$ and the indicated concentrations of staurosporine and with 10 ng/ml PMA (triangles) and/or 300 $\mu$M ATP (closed symbols). Release of vitamin B-12 binding protein was measured in supernatant fluids. The results are given as a percentage of control release, namely that elicited by 1.7 $\mu$M $Ca^{2+}$ in the presence of ATP alone: 27.2 ± 4.4% of total cellular vitamin B-12 binding protein. The results shown are the means ± S.E. for three experiments.

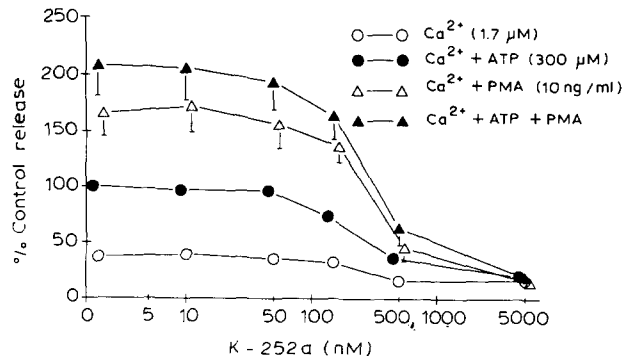


Fig. 8. Effect of K-252a on $Ca^{2+}$-induced secretion modulated by ATP and PMA. Electroporated neutrophils were incubated with $Mg^{2+}$ (1 mM), 1.7 $\mu$M free $Ca^{2+}$ and the indicated concentrations of K-252a and with 10 ng/ml PMA (triangles) and/or 300 $\mu$M ATP (closed symbols). Release of vitamin B-12 binding protein was measured in supernatant fluids. The results are given as a percentage of control release, namely that elicited by 1.7 $\mu$M $Ca^{2+}$ in the presence of ATP alone: 24.3 ± 4.0% of total cellular vitamin B-12 binding protein. The results shown are the means ± S.E. for three experiments.

concentrations of staurosporine inhibited release of vitamin B-12 binding protein enhanced by PMA, either in the presence or absence of ATP. Furthermore, staurosporine was equipotent against ATP- and PMA-enhanced degranulation while that induced by $Ca^{2+}$ alone was virtually unaffected. That ATP hydrolysis was not required was demonstrated by obtaining identical results when ATP was replaced by ADP$\beta$S (not shown).

H-7, a somewhat less specific inhibitor of PKC, showed similar results (Fig. 7). At concentrations of 100 and 300 $\mu$M, H-7 inhibited degranulation stimulated by PMA (with or without ATP) as well as ATP-enhanced secretion. However, lower concentrations of H-7 actually stimulated $Ca^{2+}$-induced and ATP-enhanced responses. This effect of H-7 complicated interpretations of the results and might be related to the relative nonspecificity of the inhibitor. K-252a, an inhibitor with equal potency against PKC, cAMP-dependent protein kinase, and cGMP-dependent protein kinase, also inhibited PMA-enhanced degranulation (Fig. 8). Secretion by $Ca^{2+}$ alone was relatively unaffected, but the responses enhanced by ATP and/or PMA were decreased in parallel.

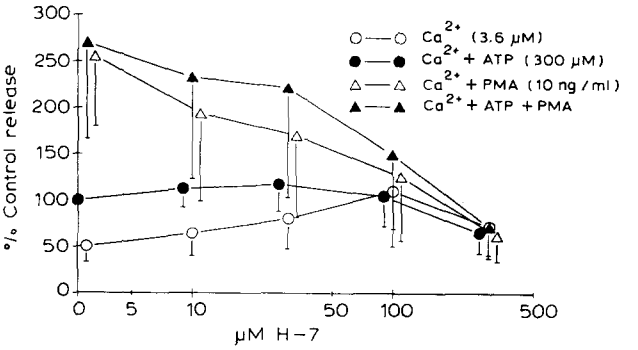


Fig. 7. Effect of H-7 on $Ca^{2+}$-induced secretion modulated by ATP and PMA. Electroporated neutrophils were incubated with $Mg^{2+}$ (1 mM), 1.7 $\mu$M free $Ca^{2+}$ and the indicated concentrations of H-7 and with 10 ng/ml PMA (triangles) and/or 300 $\mu$M ATP (closed symbols). Release of vitamin B-12 binding protein was measured in supernatant fluids. The results are given as a percentage of control release, namely that elicited by 1.7 $\mu$M $Ca^{2+}$ in the presence of ATP alone: 22.9 ± 8.5% of total cellular vitamin B-12 binding protein. The results shown are the means ± S.E. for three experiments.

## Discussion

Our main finding was that electropermeabilized neutrophils can secrete granule constituents in response to elevated $Ca^{2+}$. Similar findings have been made with platelets [13–15,18,22], pancreatic acinar cells [16], adrenal chromaffin cells [19], RINm5F cells [20,23], and parathyroid cells [21]. The amount of $Ca^{2+}$ required to induce secretion by electroporated neutrophils was comparable to that observed with other permeabilization methods [1–6]. However, it was with some interest that we observed that Mg-ATP shifted the dose–response curve for $Ca^{2+}$ to the left (Fig. 1). Depending upon the cell type and permeabilization method employed, the role of ATP can range from none [1,3,36,37,39–43] to enhancing [44–46] to required [6,19,47,48]. ATP has been found to be inhibitory under some conditions [40]. For human neutrophils permeabilized by saponin [1] or digitonin [3,37], no enhancement of secretion was found with $Mg^{2+}$ and/or ATP. In contrast, HL60 cells permeabilized with streptolysin O [43] and rabbit neutrophils permeabilized with Sendai virus [6] were almost completely dependent upon ATP to support secretion. The differences in these reports can be ascribed to both the species and the permeabilized system employed. In the work reported here, however, we found an effect of

Mg-ATP which was intermediate between those obtained with detergents [1,3,37] and the other permeabilization techniques [6,43], namely a strong enhancement of $Ca^{2+}$-induced secretion without an absolute requirement. In view of the maintained requirement for Mg-ATP, it is possible that electroporation preserves some mechanisms of stimulus-response coupling which are otherwise destroyed by treatment with digitonin or saponin. Alternatively, Mg-ATP utilizing components which could leak out of cells treated with digitonin or saponin might be retained in the cytosol following electroporation. This is a distinct possibility, since the detergents permit movement of large molecules ($M_r \leq 160\,000$).

One of our most interesting findings is that the requirement for ATP could be met by a number of other nucleotides, both hydrolyzable and stable (Table I). This contrasts with the usual assumption, either implicit or explicit, that ATP is required for metabolic energy or as a substrate for protein kinases. This assumption has basis, since permeabilized cells do phosphorylate endogenous proteins from exogenous [$\gamma$-$^{32}$P]ATP [26], are responsive to protein kinase C inhibitors [49,50] and agonists [50,51], and since depletion of ATP, either before or after permeabilization, does inhibit degranulation [42,47].

However, it is clear that the situation is not so simple in some other systems. Howell et al. [36] report that in mast cells, ATP was not even the nucleotide of choice for secretion; ITP, XTP, and GTP were all more effective. Using Sendai virus-permeabilized neutrophils, researchers from the same laboratory reported that GTP substituted well for ATP [6]; even UTP had some effect. These investigators interpreted these findings as indicating that the nucleotides were acting at some exocytosis-linked GTP-binding site ($G_e$) other than the conventional G-proteins. Our findings are consonant with theirs. We have also found an array of nucleotides which could substitute for ATP; GTP was among the most potent. In Table I, we showed that ITP, XTP, and UTP were all comparable to ATP in supporting secretion. We also found that some stable analogues were effective, an observation which, while inexplicable using the metabolic energy or protein kinase models, is compatable with the $G_e$ model.

An additional possibility is that the several guanine and adenine nucleotides were interacting with a recently described ATP receptor on the neutrophil surface [52,53]. Binding of several stable and hydrolyzable nucleotides to this receptor, which has also been found on a variety of other cells, primes the neutrophils for enhanced responses to conventional stimuli. A recent report indicates that ATP$\gamma$S, UTP, ITP and App[NH]p can all substitute for ATP in stimulating intact neutrophils and HL60 cells [54]. Such an interaction in permeabilized neutrophils could also conceivably lead to enhanced secretory responses and indeed, the above-mentioned nucleotide specificity is very close to the one we report there (Table I). However, such a model is difficult to test without specific antagonists, since the nucleotides have access to both the intracellular and extracellular spaces in permeabilized cells.

*Guanine nucleotides*

Permeabilized cells have been used extensively to show that guanine nucleotides interact synergistically with $Ca^{2+}$ to enhance secretion [4–7,18,20,22,23,36,43,55,56]. In a number of cell types (including neutrophils [4,7] and HL60 cells [43]), guanine nucleotides have been shown to stimulate degranulation on their own. In spite of the fact that permeabilized neutrophils have functional surface receptor-linked G-proteins [8,57], it has been postulated (on the basis of indirect data) that guanine nucleotides also interact with an additional site, such as an exocytosis-linked GTP-binding protein [4,5,7,36,43].

Our finding that degranulation by electroporated neutrophils was enhanced by GTP is compatable with this interpretation. However, our unconventional findings were that none of the guanine nucleotides stimulated substantial secretion on its own and that GTP$\gamma$S and GDP$\beta$S were inhibitory. However, even these data are in accord with the two hypotheses mentioned above. One possibility is that the hypothetical exocytosis-linked G-protein, with which the stimulatory nucleotides interact, was inhibited by GTP$\gamma$S and GDP$\beta$S. Another possibility is that these latter two nucleotides are antagonists for a putative cell surface receptor. ATP$\gamma$S, App[NH]p, UTP, and ITP (all of which also replaced ATP) are agonists for this receptor [52,54] as is GTP (Axtell, R.A., Ward, P.A., Sandborg, R.R., Smolen, J.E. and Boxer, L.A., unpublished data). While less likely, it is also possible that GTP$\gamma$S and GDP$\beta$S inhibited $Ca^{2+}$-induced secretion by interacting with conventional receptor-linked G-proteins, but such interactions should be agonistic rather than antagonistic.

*Desensitization*

The process of time-dependent desensitization of permeabilized cell responsiveness has been reported for adrenal chromaffin cells [58] and mast cells [40,59]. We found a half-time for loss of responsiveness of approx. 5 min at 0 °C; this is comparable to the rate of desensitization to $Ca^{2+}$ reported for mast cells at 37 °C [40]. In mast cells, this process can be obviated by the presence of ATP [60], but the addition of ATP to our system was not effective (not shown).

Stimulus-elicited, as opposed to $Ca^{2+}$-induced, secretion by mast cells desensitizes in a matter of seconds, which can be overcome by GTP [59]. This process is different from the one we are measuring; furthermore, we found that guanine nucleotides did not prevent

desensitization (not shown). For digitonin-permeabilized adrenal chromaffin cells, the desensitization process is much slower, involves the leakage of cytosolic proteins and can be reversed by re-addition of concentrated cytosol [58]. This is almost certainly not taking place in our system, since electroporated neutrophils have much smaller holes and desensitization was not reduced by the re-addition of concentrated released materials (not shown).

We have also tried to block the desensitization process with low concentrations of $Ca^{2+}$, $Mg^{2+}$, PMA and phosphatase inhibitors, all without success. While we unfortunately do not yet know the cause of this phenomenon, our results indicate that electroporated neutrophils are useful for biochemical studies of the secretion process. Furthermore, it should be noted that secretion still occurred at high $Ca^{2+}$ concentrations in desensitized cells, perhaps by a mechanism distinct from the one operating in sensitized neutrophils.

*Protein kinase C*

Electroporated neutrophils appear to have the potential to be regulated by PKC. Grinstein and Furuya reported that oxygen consumption by these cells could be stimulated by the PKC agonists TPA and DiC8 and inhibited by H-7 [28]. Oxygen consumption stimulated by GTPγS and DiC8 was also inhibited by staurosporine [29]. Furthermore, we showed here that PMA could enhance $Ca^{2+}$-induced secretion, presumably by activating PKC and that this enhancement could be blocked by three PKC inhibitors. This is in distinct contrast to the situation found in digitonin-permeabilized neutrophils. Secretion by these detergent-treated cells is not affected by Mg-ATP, PMA, H-7, or mezerein (a PKC antagonist) [37]. There are two likely explanations for this difference. The first is that the larger pores inflicted in the membranes by digitonin would permit PKC and/or its essential substrates to leak away. However, it is more likely that the substrates would be depleted, since most of the total PKC activity remains associated with digitonin-permeabilized neutrophils [37]. The second possibility is that digitonin disrupts the signal transduction mechanisms leading to or away from PKC, while electroporation leaves them intact. This explanation would be in accord with the perceived 'gentleness' of the latter technique.

Thus, it appears that PKC can be activated in electroporated neutrophils and that this activation can enhance $Ca^{2+}$-induced secretion. Even more intriguing is the observation that the three PKC antagonists employed also inhibited ATP-enhanced degranulation. Since the concentrations of the antagonists required for this inhibition were the same as those needed to block PMA-enhanced responses, it is likely that these agents were operating by a common mechanism, namely PKC. However, it is also clear that in this situation ATP is not simply serving as a substrate for PKC, but rather as a ligand for either a nucleotide receptor or a postulated $G_e$-like protein (see previous discussion). This is emphasized by the ability of ADPβS to substitute for ATP in these experiments. It is pertinent that all three PKC inhibitors block the ATP binding site of the enzyme. It is therefore possible that the inhibitors could also block ATP-receptor interactions. This model strains credibility as it requires identical $K_i$ values for these inhibitors at two sites (PKC and nucleotide receptor) with similarly broad nucleotide specificities. Most likely, the nucleotides are activating PKC subsequent to their interactions with receptors and G-proteins and this activation serves to enhance $Ca^{2+}$-induced degranulation.

Thus, degranulation from electroporated neutrophils seems to have both $Ca^{2+}$- and PKC-dependent components. $Ca^{2+}$ alone can cause secretion, which is probably in its most unambiguous form in washed, electroporated cells or digitonin-permeabilized neutrophils. PKC can also be activated, either by PMA or nucleotides acting as ligands, leading to the enhancement of $Ca^{2+}$-induced secretion. ATP for PKC activity is most likely provided from residual endogenous intracellular stores (approx. 50% of the original complement, as determined by direct analysis). Separation of the intracellular and extracellular pools of nucleotides in electroporated neutrophils is supported by data indicating that they are only slowly exchanged (not shown). This kinetic isolation, wherein the two pools of poorly permeant molecules do not freely exchange during the crucial first 2 min of incubation, suggests that the exogenous nucleotides enhance secretion by interacting at the cell membrane rather than in the cytoplasm. Experiments designed to clarify these sites of interaction are currently ongoing.

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