Exocytosis in electroporabilized neutrophils

Responsiveness to calcium and guanosine 5'-[γ-thio]triphosphate

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Electroporabilized neutrophils were used to study the exocytotic response in rabbit neutrophils. Enzyme release from electroporabilized neutrophils could be induced by elevating the Ca^{2+} concentration. Ca^{2+} -induced secretion was significantly enhanced by guanosine 5'-[γ-thio]triphosphate (GTP[S]) in a concentration-dependent manner. The effect of GTP[S] could be blocked by guanosine 5'-[β-thio]diphosphate (GDP[S]) and was not affected by pertussis toxin. GTP[S] did not induce enzyme release in the absence of Ca^{2+} . Induction of an exocytotic response did not require addition of ATP. However, neutrophils permeabilized in the absence of ATP became refractory to stimulation due to a reduction in their affinity for Ca^{2+} . Responsiveness to the effectors Ca^{2+} or Ca^{2+} + [GTP[S] could be prolonged or restored by ATP. ATP was not the only agent that prolonged responsiveness; other nucleotides and inorganic phosphates were also effective. The protein kinase C inhibitors staurosporine and 1-*O*-hexadecyl-2-methyl-*sn*-glycerol did not inhibit exocytosis and had only a small effect on the prolongation and restoration of responsiveness by ATP. A hypothesis is presented suggesting that the loss of responsiveness is caused by dephosphorylation and that the restoration or prolongation of responsiveness is not mediated by protein kinase C. It is possible that an as yet unidentified Ca^{2+} -binding protein is dephosphorylated, resulting in a decrease in Ca^{2+} affinity.

INTRODUCTION

Investigation of stimulus–secretion coupling is facilitated by the use of permeabilized cells, whereby intracellular modulators can be introduced into the cell interior. For example, exocytotic release has been investigated in bovine adrenal chromaffin cells permeabilized by Staphylococcal α-toxin [1,2], in platelets permeabilized with high voltage discharge [3,4], and in rat mast cells permeabilized with streptolysin-O (SLO) [5].

In neutrophils, exocytosis has been investigated using several different permeabilization techniques [6–11], and it has been shown that Ca^{2+} and guanine nucleotides play important roles. In digitonin-permeabilized neutrophils, exocytosis was induced by elevating the intracellular Ca^{2+} level. In the presence of GTP analogues the requirement for Ca^{2+} was shifted to lower concentrations [6,7]. In Sendai-virus- and SLO-permeabilized neutrophils, Ca^{2+} and GTP analogues were both independently capable of activating exocytotic secretion [11,12]. In electroporabilized human neutrophils it was found that GTP analogues could inhibit Ca^{2+} -induced exocytosis [13].

The role of ATP in exocytosis has also been investigated in permeabilized cells, and it has been observed that there is not always an obligatory requirement for ATP in exocytosis [1,14–16]. However, ATP does modulate the exocytotic process in neutrophils and other cell types: it enhances the effective affinity for the effectors, Ca^{2+} and guanine nucleotides [17,18], it increases the absolute amount of secretion [18], and it plays a role in sustaining exocytosis [14,16].

SLO-permeabilized rat mast cells [14] and digitonin-permeabilized bovine adrenal chromaffin cells [16] become refractory after permeabilization, and their ability to respond to the different effectors is reduced. In the absence of ATP the decrease in this so-called responsiveness was faster than in the presence of ATP. When ATP was provided immediately after

permeabilization, the period during which the permeabilized cells remained susceptible to stimulation by the effectors was prolonged. In rat mast cells this decline in responsiveness is characterized as a time-dependent decrease in the affinity for Ca^{2+} [14,19].

We report here that, in rabbit neutrophils, responsiveness to the effectors Ca^{2+} and the stable GTP analogue guanosine 5'-[γ-thio]triphosphate (GTP[S]) decreases after electroporabilization. It is shown that responsiveness can be prolonged and restored by different nucleotides and inorganic phosphates. Prolongation of responsiveness is not controlled by protein kinase C. The data presented here suggest that the decrease in responsiveness is caused by dephosphorylation.

MATERIALS AND METHODS

Materials

ATP, adenosine 5'-[βγ-methylene]triphosphate (App[CH]p), adenosine 5'-[αβ-methylene]triphosphate (Ap[CH]pp), ADP, AMP, cyclic AMP, XTP, pyrophosphate, pertussis toxin, 2-deoxyglucose and *Micrococcus lysodeikticus* were obtained from Sigma Chemical Co. GTP, GTP[S], adenosine 5'-[βγ-imido]triphosphate (App[NH]p), adenosine 5'-[γ-thio]triphosphate (ATP[S]), guanosine 5'-[β-thio]diphosphate (GDP[S]), CTP, UTP, *p*-nitrophenyl phosphate and staurosporine were purchased from Boehringer–Mannheim Biochemicals, Mannheim, Germany; 1-*O*-hexadecyl-2-methyl-*sn*-glycerol (AMG- C_{16}) was from Bachem Feinchemikalien AG, Bubendorf, Switzerland, and SLO was from Wellcome Diagnostics.

Methods

Neutrophils were isolated from the peritoneal cavity of rabbits [20]. Rabbits were injected intraperitoneally with 150 ml of isotonic saline containing 1.5 mg of glycogen/ml and after 4 h

Abbreviations used: AMG- C_{16} , 1-*O*-hexadecyl-2-methyl-*sn*-glycerol; GTP[S], guanosine 5'-[γ-thio]triphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate; SLO, streptolysin-O; LDH, lactate dehydrogenase; App[NH]p, adenosine 5'-[βγ-imido]triphosphate; Ap[CH]pp, adenosine 5'-[αβ-methylene]triphosphate; App[CH]p, adenosine 5'-[βγ-methylene]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate.

the exudate was collected by flushing the peritoneal cavity with iso-osmotic saline containing citrate (0.4%, pH 7.4). The cells were centrifuged (400 g, 3 min) and washed with medium containing 140 mM-KCl, 20 mM-Pipes, 1 mM-MgCl₂ (pH 6.8) and, where indicated, 10 mM-glucose. The cell concentration used in all experiments was 3×10^6 cells/ml.

Electropermeabilization was performed according to Grinstein & Furuya [21] with some modifications. Cells were washed and resuspended in medium at a concentration of 5×10^7 cells/ml and kept on ice. For electropermeabilization, 800 μ l of the cell suspension (4×10^7 cells) was transferred to a pre-cooled Gene-Pulser cuvette (0.4 cm) and permeabilized by two discharges of 1.9 kV (4.75 kV/cm; capacitance 25 μ F) in a Bio-Rad Gene Pulser (time constant 0.4 ms). Between the two discharges the cells were gently stirred. After permeabilization the cells were transferred to tubes containing ice-cold medium (1×10^7 cells/ml) and used immediately. Lactate dehydrogenase (LDH) release, determined according to Elferink & Deierkauf [20], never exceeded the amount released during permeabilization, indicating that no more cells were damaged during the incubations.

Permeabilization with SLO was performed according to Cockcroft [11]. Cells were washed and resuspended in buffer (containing 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Pipes, pH 6.8) at a concentration of 1×10^7 cells/ml. Portions of 300 μ l were transferred to tubes containing 700 μ l of buffer supplemented with the permeabilization agent SLO (0.4 unit/ml final concn.) and different effectors as indicated.

Lysozyme was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* according to the method of Shugar [22]. Samples of 0.2 ml were mixed with 1.7 ml of phosphate buffer (0.1 M, pH 6.2). The reaction was started by addition of 0.2 ml of *Micrococcus lysodeikticus* suspension (3.5 mg of lyophilized cells/ml), and the decrease in absorbance was recorded at 450 nm. Lysozyme release was expressed as a percentage of the maximum release obtained by treating the cells with 0.05% (w/v) Triton X-100.

Calcium concentrations up to 30 μ M (pCa = 4.5) were obtained by the use of Ca-EGTA buffers. These were prepared by mixing end-point-titrated equimolar solutions of EGTA and Ca-EGTA in proportions calculated with a computer programme, as described by Tatham & Gomperts [23]. The calcium buffers were diluted in the medium so the final EGTA concentration was 3 mM. Free Ca²⁺ concentrations higher than pCa 4.5 were calculated using the same computer program and checked with a Ca²⁺ electrode, but were not buffered with EGTA. The Mg²⁺ concentration was set at 1 mM.

RESULTS

Electropermeabilized neutrophils

Electropermeabilized rabbit neutrophils showed secretion of granule constituents when exposed to increasing Ca²⁺ concentrations (Fig. 1). The release of β -glucuronidase, a constituent of azurophilic granules, followed the same pattern as that of lysozyme release (results not shown).

When electropermeabilized cells were incubated in the presence of Ca²⁺ + GTP[S], a substantial shift to the left of the Ca²⁺ dose/response curve was observed (Fig. 1). This enhancement by GTP[S] at low Ca²⁺ concentrations was concentration-dependent, and maximal with 50 μ M-GTP[S]. GTP[S] did not induce lysozyme release in the absence of Ca²⁺ in electropermeabilized neutrophils (Table 1). Both in the absence and in the presence of GTP[S], Ca²⁺-induced lysozyme release was unaffected by the addition of ATP (Table 1). Pertussis toxin did not inhibit lysozyme release induced by Ca²⁺ or Ca²⁺ + GTP[S]. Addition of

GDP[S] to a final concentration of 500 μ M resulted in a considerable inhibition of the lysozyme release induced by Ca²⁺ + GTP[S], but had no effect on lysozyme release induced by Ca²⁺ alone (results not shown).

SLO-permeabilized neutrophils

In neutrophils permeabilized with SLO, both GTP[S] alone and Ca²⁺ alone induced lysozyme release. In both cases lysozyme release required the presence of ATP. Lysozyme release induced by Ca²⁺ + GTP[S] was ATP-independent in both SLO-permeabilized cells and electropermeabilized cells. In the presence of ATP a difference in the sensitivity for Ca²⁺ was found between SLO-permeabilized and electropermeabilized cells. At low Ca²⁺ concentrations (10 μ M), SLO-permeabilized cells were more sensitive to Ca²⁺ than were electropermeabilized cells. At high Ca²⁺ concentrations (100 μ M) this difference in sensitivity decreased (Table 1).

Fig. 1. Dependence on Ca²⁺ and GTP[S] of secretion from electropermeabilized neutrophils stimulated by addition of Ca²⁺ or Ca²⁺ + GTP[S] immediately and at 5 min after electropermeabilization

Neutrophils were pretreated with 2-deoxyglucose (5 mM) for 10 min, centrifuged and resuspended in ice-cold medium (5×10^7 cells/ml). After electropermeabilization, cells were immediately transferred (●) to tubes containing different Ca²⁺ concentrations with (b) or without (c) 50 μ M-GTP[S]; or cells were incubated for 5 min at 37 °C (○) in the absence of effectors and then transferred to the tubes containing the effectors. The cells were incubated for a further 10 min, and lysozyme released into the supernatant was determined.

Table 1. Dependence on Ca²⁺, GTP[S] and ATP of lysozyme secretion from electroporabilized and SLO-permeabilized neutrophils

Neutrophils were electroporabilized and then transferred to tubes containing EGTA, 100 μM-Ca²⁺ or 3 mM-Ca. EGTA (10 μM-Ca²⁺) and, where indicated, GTP[S] (50 μM) and ATP (1 mM). Alternatively, intact neutrophils were directly transferred to tubes containing SLO (0.4 unit/ml), EGTA, Ca²⁺ and, where indicated, GTP[S] (50 μM) and ATP (1 mM). The cells were incubated for 10 min at 37 °C and lysozyme released into the supernatant was determined.

	Lysozyme release (%)			
	Electroporabilized		SLO-treated	
	-ATP	+ATP	-ATP	+ATP
200 μM-EGTA	7	6	1	2
200 μM-EGTA + GTP[S]	6	5	1	35
10 μM-Ca ²⁺	3	8	8	41
10 μM-Ca ²⁺ + GTP[S]	49	51	67	77
100 μM-Ca ²⁺	70	77	8	82
100 μM-Ca ²⁺ + GTP[S]	83	85	86	77

ATP and responsiveness

With increasing incubation time between permeabilization and addition of effectors ('permeabilization interval'), lysozyme release considerably decreased, and vanished completely with permeabilization intervals exceeding 7 min (Fig. 2). This loss of responsiveness was found in control cells and in cells that were incubated in the presence of glucose, but was most profound in cells that were preincubated with 2-deoxyglucose (90 % ATP depletion). For that reason all experiments concerning responsiveness were carried out with 2-deoxyglucose-treated cells.

The time during which the cells remained responsive to subsequent addition of the effector(s) was considerably prolonged

Table 2. Effect of protein kinase C inhibitors on Ca²⁺- and Ca²⁺ + GTP[S]-induced secretion from electroporabilized neutrophils

Neutrophils were preincubated with deoxyglucose for 10 min. After 5 min the protein kinase C inhibitors were added: AMG-C₁₆ at 100 μM or staurosporine (STAU) at 200 nM. Cells were centrifuged, resuspended and electroporabilized. Cells were immediately transferred (0 min) to tubes containing 100 μM-Ca²⁺, 3 mM-Ca. EGTA (10 μM-Ca²⁺), GTP[S] (50 μM), ATP (1 mM), AMG-C₁₆ (100 μM) or staurosporine (200 nM) as indicated. Alternatively, cells were incubated for 5 min at 37 °C in the presence or absence of 1 mM-ATP and in the presence of inhibitors (where indicated), and then transferred to tubes containing Ca²⁺ or Ca²⁺ + GTP[S]. After incubation for 10 min, lysozyme released into the supernatant was determined.

Time (min)...	Lysozyme release (%)					
	Control		AMG-C ₁₆		STAU	
	0	5	0	5	0	5
10 μM-Ca ²⁺ + GTP[S]	70±4	10±1	63±1	3±1	70±3	3±2
10 μM-Ca ²⁺ + GTP[S] + ATP	56±2	52±1	51±3	38±4	53±3	41±1
100 μM-Ca ²⁺	58±2	24±4	57±6	19±4	64±4	27±3
100 μM-Ca ²⁺ + ATP	64±2	57±5	60±4	43±2	59±2	49±1

when ATP was present during the permeabilization interval (Fig. 2). Addition of ATP together with the effector(s) after the permeabilization interval also restored responsiveness (results not shown). Prolongation of responsiveness by ATP was much stronger with Ca²⁺ + GTP[S] as effector than with Ca²⁺ alone (Fig. 2). Addition of ATP did not affect initial enzyme release (t = 0 min) induced by Ca²⁺ or Ca²⁺ + GTP[S] (Fig. 2).

Stimulation of cells with increasing Ca²⁺ concentrations after a 5 min permeabilization interval not only resulted in an increase

Fig. 2. Duration of responsiveness of electroporabilized neutrophils to the late addition of Ca²⁺ or Ca²⁺ + GTP[S] in the presence and absence of ATP

Neutrophils were pretreated with 2-deoxyglucose (5 mM) for 10 min, centrifuged and resuspended in ice-cold medium (5 × 10⁷ cells/ml). After electroporabilization, cells were incubated at 37 °C in the presence (○, □) or absence (●, ■) of 1 mM-ATP. After the indicated time (permeabilization interval), aliquots of cells were transferred to tubes containing 100 μM-Ca²⁺ (a) or 3 mM-Ca. EGTA (10 μM-Ca²⁺) + 50 μM-GTP[S] (b). After further incubation for 10 min, lysozyme released into the supernatant was determined. Data have been normalized for the purpose of comparison; control values (100 %): 54 ± 4 % (Ca²⁺), 62 ± 4 % (Ca²⁺ + ATP), 85 ± 2 % (Ca²⁺ + GTP[S]), 82 ± 3 % (Ca²⁺ + GTP[S] + ATP).

Table 3. Effect of different nucleotides on prolongation of the responsiveness of neutrophils to the addition of Ca^{2+} or $\text{Ca}^{2+} + \text{GTP[S]}$

Cells were pretreated with deoxyglucose. After electropor-meabilization, cells were incubated at 37 °C for 5 min in the presence of different nucleotides (1 mM) and then transferred to tubes containing 100 μM - Ca^{2+} , or 3 mM-Ca. EGTA (10 μM - Ca^{2+})+GTP[S] (50 μM). After a 10 min incubation, lysozyme released into the supernatant was determined. Data have been normalized for the purpose of comparison at control values (100%): $81 \pm 2\%$ (10 μM - Ca^{2+} + GTP[S]), $54 \pm 4\%$ (100 μM - Ca^{2+})

Nucleotide	Responsiveness (% of control)	
	100 μM - Ca^{2+}	10 μM - Ca^{2+} + GTP[S]
–	38	20
ATP	70	75
GTP	72	26
CTP	74	78
UTP	72	80
XTP	60	80

Table 4. Effect of different adenine nucleotides and inorganic phosphates on prolongation of responsiveness to addition of Ca^{2+} + GTP[S]

Cells were pretreated with deoxyglucose, electropor-meabilized and transferred to tubes containing 3 mM-Ca. EGTA (10 μM - Ca^{2+})+GTP[S] (50 μM) and different nucleotides (1 mM) or inorganic phosphates (1 mM). Alternatively, cells were incubated at 37 °C for 5 min in the presence of different nucleotides (1 mM) or inorganic phosphates (1 mM) and then transferred to tubes containing 3 mM-Ca. EGTA (10 μM - Ca^{2+})+GTP[S] (50 μM). After further incubation for 10 min, lysozyme released into the supernatant was determined.

	Lysozyme release (%)	
	0 min	5 min
–	82	19
ATP	78	51
ADP	78	58
AMP	80	50
Adenosine	82	14
App[NH]p	80	62
App[CH]p	84	59
Ap[CH]pp	80	46
ATP[S]	78	60
Cyclic AMP	77	19
Orthophosphate	82	69
Pyrophosphate	77	16
p-Nitrophenyl phosphate	81	60

in lysozyme release but also decreased the loss of responsiveness (Fig. 1).

Role of protein kinase C in responsiveness

To investigate the role of protein kinase C in the prolongation of responsiveness, the cells were preincubated with two different protein kinase C inhibitors. When the cells were preincubated with AMG- C_{16} or staurosporine, no inhibition of lysozyme release was found when the cells were activated immediately after permeabilization ($t = 0$) with Ca^{2+} or $\text{Ca}^{2+} + \text{GTP[S]}$ in the presence or absence of ATP. The ATP effect after a 5 min permeabilization interval was only slightly inhibited by AMG- C_{16} and staurosporine (Table 2).

Fig 3. Effect of inorganic phosphate on restoration and prolongation of responsiveness to addition of Ca^{2+} + GTP[S]

Cells were pretreated with 2-deoxyglucose and electropor-meabilized followed by: ●, immediate transfer to tubes containing the effectors + P_i (1 mM); ○, preincubation for 5 min in the presence of P_i (1 mM) and transfer to tubes containing the effectors + P_i (1 mM); ■, preincubation for 5 min in the absence of P_i and then transfer to tubes containing the effectors + P_i (1 mM); □, preincubation for 5 min in the absence of P_i and then transfer to tubes containing the effectors without P_i . After transfer to the effectors [3 mM-Ca. EGTA (10 μM - Ca^{2+})+GTP[S] (50 μM)] the cells were incubated for the indicated time and lysozyme released into the supernatant was determined.

Role of other nucleotides and of inorganic phosphates in responsiveness

Other purine and pyrimidine nucleotides were studied with regard to their ability to prolong cell responsiveness. All triphosphate nucleotides that were investigated were able to prolong responsiveness to Ca^{2+} , and all triphosphate nucleotides except GTP were able to prolong responsiveness to $\text{Ca}^{2+} + \text{GTP[S]}$ (Table 3). The nucleotides showed the same effects in the presence of equimolar Mg^{2+} concentrations, indicating that the effects are not caused by chelation of Mg^{2+} . Stable analogues of ATP, other adenine nucleotides and different inorganic phosphates were able to prolong responsiveness to $\text{Ca}^{2+} + \text{GTP[S]}$. Responsiveness could not be prolonged by adenosine or cyclic AMP (Table 4). Prolongation of responsiveness with these different compounds was only investigated with $\text{Ca}^{2+} + \text{GTP[S]}$ as effector because it was necessary to use buffered Ca^{2+} concentrations.

To investigate the effect of inorganic phosphate on the restoration and prolongation of responsiveness, the time course of lysozyme release was determined. When cells were stimulated immediately after permeabilization with $\text{Ca}^{2+} + \text{GTP[S]}$ in the presence of P_i , lysozyme release started immediately and was complete within 4 min. When cells were stimulated after a 5 min permeabilization interval during which P_i was present, lysozyme release also started immediately but the rate was slower. When P_i was added together with the effectors after a 5 min permeabilization interval, lysozyme release started after a lag time of 3 min and the rate was slower. In the absence of P_i hardly any lysozyme release was found (Fig. 3).

DISCUSSION

Ca^{2+} induced an exocytotic response in electropor-meabilized neutrophils which was enhanced by GTP[S]. This enhancement was inhibited by GDP[S] and unaffected by pertussis toxin,

suggesting the involvement of a pertussis toxin-insensitive GTP-binding protein. These findings are in accordance with studies in human neutrophils and other cell types permeabilized by different methods [2,3,20,24], although inhibition of Ca^{2+} -induced exocytosis by GTP[S] has also been observed in human neutrophils [25].

In electroporabilized neutrophils GTP[S] did not elicit a Ca^{2+} -independent exocytotic response, in contrast to studies by Cockcroft and co-workers, who found that GTP[S] alone induced exocytosis in SLO-permeabilized [11] and Sendai-virus-permeabilized [9] human neutrophils. A difference in sensitivity to Ca^{2+} and a difference in ATP requirement were observed between electroporabilized and SLO-permeabilized cells. The results indicate that electroporabilization is significantly different from permeabilization with SLO [11] or Sendai-virus [9], but strongly resembles permeabilization with digitonin, where no addition of ATP is required for exocytosis [21]. This indicates that the permeabilization method can have a strong influence on the sensitivity of the cells towards the different effectors and ATP. One can only speculate on the basis of these differences. It appears most likely, however, that, depending on the permeabilization method used, essential components for exocytosis leak out of the cell. Treatment of neutrophils with 2-deoxyglucose resulted in a decline of the ATP level by 90%, and neither electroporabilization nor treatment with SLO gave a further decrease in the ATP level (results not shown). The external ATP that is required for exocytosis in SLO-permeabilized cells is possibly used to compensate for the loss of essential components that leak from SLO-permeabilized cells, in contrast with electroporabilized cells.

Although addition of ATP is not required for the onset of exocytosis under the conditions of our experiments, it plays a modulatory role in exocytosis. In the absence of ATP, electroporabilized cells showed a time-dependent loss of responsiveness to the different effectors. This was also observed by other investigators with other cell types and different permeabilization methods [14,16]. This decline in responsiveness is due to a decrease in the affinity for Ca^{2+} of the Ca^{2+} -binding component, as increasing the Ca^{2+} concentration restored responsiveness. In the presence of ATP the affinity for Ca^{2+} is restored and the duration of responsiveness is prolonged. In rat mast cells the loss of responsiveness was also characterized as a decline in affinity for Ca^{2+} [14].

In the presence of ATP, neutrophils retain sensitivity to Ca^{2+} + GTP[S] for longer than to Ca^{2+} alone. This result, and the observation that GTP[S] enhances Ca^{2+} -induced exocytosis, suggest that GTP[S] plays an important role in increasing the affinity for Ca^{2+} .

A comparable decrease of responsiveness in the absence of ATP was found in SLO-permeabilized mast cells. The loss of responsiveness in mast cells, however, could be prevented completely by pretreatment with protein kinase C inhibitor AMG- C_{16} [14]. Because AMG- C_{16} and staurosporine, potent inhibitors of protein kinase C in neutrophils [26,27], had only a small effect on the loss of responsiveness of electroporabilized neutrophils, it seems evident that the decline of responsiveness in neutrophils is not controlled by protein kinase C in the same way as in mast cells.

The difference in loss of responsiveness between mast cells and neutrophils is further emphasized by the effects of other nucleotides, which were ineffective in mast cells but prevented loss of responsiveness in neutrophils. Our results with other nucleotides resemble to some degree those obtained by Smolen & Sandborg [13]. They found that ATP, purine and pyrimidine nucleotides, and non-hydrolysable analogues of ATP, enhanced Ca^{2+} -induced exocytosis in electroporabilized human

neutrophils. Under the conditions of our experiments we did not find an enhancement by the different nucleotides, but there was a prolongation of responsiveness.

Responsiveness to the different effectors could be restored and prolonged not only by nucleotides but also by inorganic phosphates. A possible explanation is that this loss of responsiveness is due to the involvement of a phosphatase. It has been observed that protein phosphatases show differences in substrate specificity and that some protein phosphatases can be inhibited by phosphate nucleotides and inorganic phosphates [28–30], while some phosphatases have been described that cannot be inhibited by pyrophosphate [31]. The putative phosphatase could cause a loss of responsiveness by dephosphorylating a crucial phosphoprotein involved in the exocytotic response. In this hypothesis the phosphoprotein plays a role in the binding of Ca^{2+} , and the affinity for Ca^{2+} of this phosphoprotein is also affected by GTP[S]. Incubating the neutrophils in the presence of nucleotides or P_i prevents dephosphorylation of this phosphoprotein and responsiveness is maintained. When P_i is added together with the effectors after the permeabilization interval, lysozyme release starts after a lag time which is probably needed for the recovery of the binding capacity for Ca^{2+} and the restoration of responsiveness. During this lag time the phosphatase is inhibited by P_i , while phosphorylation of the Ca^{2+} -binding protein is not inhibited. Therefore this will result in accumulation of the phosphoprotein, finally resulting in a restoration of responsiveness. However, what type of phosphatase is involved remains to be elucidated.

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