Essential Synergy between Ca\textsuperscript{2+} and Guanine Nucleotides in Exocytotic Secretion from Permeabilized Rat Mast Cells

Tim W. Howell, Shamshad Cockcroft, and Bastien D. Gomperts

Department of Experimental Pathology, University College London, University Street, London WC1E 6JJ, United Kingdom

**Abstract.** Rat mast cells, pretreated with metabolic inhibitors and permeabilized by streptolysin-O, secrete histamine when provided with Ca\textsuperscript{2+} (buffered in the micromolar range) and nucleoside triphosphates. We have surveyed the ability of various exogenous nucleotides to support or inhibit secretion. The preferred rank order in support of secretion is ITP > XTP > GTP >> ATP. Pyrimidine nucleotides (UTP and CTP) are without effect. Nucleoside diphosphates included alongside Ca\textsuperscript{2+} plus ITP inhibit secretion in the order 2'-deoxyGDP > GDP > o-GDP > ADP ≡ 2'deoxyADP ≡ IDP.

**Materials and Methods**

All the methods used in this work have been described in earlier publications (21, 25). Mast cells were isolated from rat peritoneal washings by centrifugation (300 g, 15 min) through a cushion of BSA (35% wt/wt). They were washed by centrifugation and suspended in a glucose-free buffer and treated with metabolic inhibitors (5 lttM antimycin A plus 6 mM 2-deoxyglucose) 2 rain before (Fig. 1) or at the time of permeabilization (Figs. 2, 4, and 6) with streptolysin-O (0.4 IU ml\textsuperscript{-1}). In such experiments reagents used were also made up in glucose-free buffer. In practice we have found that these treatments (i.e., 2-min preincubation with metabolic inhibitors, or addition of inhibitors at the time of permeabilization) are both equally capable of reducing the background release of histamine (at pCa5) to low levels (25).

In a typical secretion experiment, 1 vol of cells (30 μl) was added to 3 or 4 vol of a solution containing streptolysin-O, nucleoside triphosphate (as the Mg\textsuperscript{2+} salt) and calcium buffer (3 mM EGTA final). After incubation for 10 min at 37°C, the cellular reactions were quenched by addition of ice cold 0.15 M NaCl, buffered at pH 7 with 10 mM K phosphate and sedimented by centrifugation at 2,000 g for 5 min at 4°C (the secretion terminates in 3 min [25]) so the use of 10 min incubations ensures that com-
Figure 1. Dependence of Ca\textsuperscript{2+}-induced histamine release from streptolysin-O-permeabilized mast cells on the concentration of exogenous nucleoside triphosphates. Mast cells were incubated at 37°C for 2 min in the presence of antimycin A (5 \mu M) plus 2-deoxyglucose (6 mM) in a glucose-free medium. Cells were then permeabilized with streptolysin O (0.4 IU ml\textsuperscript{-1}) in the presence of 3 mM Ca/EGTA (to maintain pCa5) and Mg/NTP as indicated. After a further 10-min incubation reactions were quenched and histamine release was measured. Data are means ± SEM of three experiments. O, ATP; Δ, XTP; A, GTP; ⋄, ATP; ▼, UTP; ◇, CTP. The curves describing the dependence of secretion on ATP and ITP previously appeared as part of a figure in reference 25.

Figure 2. Dependence on GTP analogues of Ca\textsuperscript{2+}-induced histamine secretion from metabolically inhibited mast cells permeabilized by streptolysin-O. Mast cells were permeabilized with streptolysin-O (0.4 IU ml\textsuperscript{-1}) in a glucose-free medium in the presence of metabolic inhibitors, 3 mM Ca/EGTA (to maintain pCa5), and either GTP-γ-S (●), GppNHp (■), or GppCH\textsubscript{2}p (▲) at the concentrations indicated. In the absence of guanine nucleotide there is 7% histamine release.

Results

Fig. 1 illustrates the dependence of histamine release from streptolysin-O permeabilized mast cells on the concentration of various exogenous nucleoside triphosphates. In this experiment, Ca\textsuperscript{2+} was buffered at 10 \mu M. The cells were routinely resuspended in a glucose-free buffer in the presence of metabolic inhibitors (5 \mu M antimycin plus 6 mM 2-deoxyglucose) at the same time as or 2 min before permeabilization in order to suppress the secretion (29 ± 3%) which otherwise occurs in the absence of applied nucleoside triphosphate (25). This procedure renders the requirement for exogenous nucleoside triphosphates absolute. Under these conditions inosine triphosphate (ITP) provides the most effective support to the secretory reaction; ATP is without effect when supplied to the permeabilized cells at concentrations <1 mM. GTP and XTP (xanthosine triphosphate) support secretion at intermediate levels. The pyrimidine nucleotides CTP and UTP do not support secretion.

In the knowledge that the supportive nucleotides (i.e., ITP, XTP, and GTP) are also those that permit the activation of \(\beta\)-adrenergic receptor-linked \(G\)-adenylate cyclase system of turkey red blood cells (10), we tested the ability of the stable analogues, guanosine 5'-O-(3-thiotriphosphate), guanyl imidodiphosphate, and guanyl β, γ-methylenediphosphosphate (GTP-γ-S, GppNHp, and GppCH\textsubscript{2}p, respectively) to support Ca\textsuperscript{2+}-induced secretion. In these experiments phosphorylating nucleotides were not provided and metabolic inhibitors were added at the time of permeabilization. The results are shown in Fig. 2. All three nonphosphorylating analogues of GTP are able to support Ca\textsuperscript{2+}-dependent secretion with the rank order GTP-γ-S > GppNHp > GppCH\textsubscript{2}p. If ITP and other nucleotides which support secretion do so through interaction with a guanine nucleotide–binding protein, then we should anticipate that GDP and appropriate analogues might be inhibitory. 2'-deoxyGDP (1 mM) caused total inhibition of Ca\textsuperscript{2+}-induced secretion (Fig. 3, top). GDP and periodate-oxidized GDP (o-GDP), both at 1 mM, reduced the extent of secretion due to pCa5 (in the presence of 3 mM ITP) by ~50%. Inosine diphosphate (IDP), xanthosine diphosphate (XDP), ADP, and 2'-deoxyADP caused only marginal inhibition (<20%). The concentration dependence of inhibition by 2'-deoxyGDP on Ca\textsuperscript{2+}-induced secretion is illustrated in Fig. 3 (bottom). Inhibition by nucleoside diphosphates is characterized by a reduction in the apparent affinity for Ca\textsuperscript{2+} at the exocytotic site. The inhibition due to

1. Abbreviations used in this paper: GppCH\textsubscript{2}p, guanyl (β, γ-methylenediphosphate; GppNHp, guanyl imidodiphosphate; GDP-β-S, guanosine 5'-O-(2-thiodiphosphate; GTP-γ-S, guanosine 5'-O-(3-thiotriphosphate); IDP, inosine 5'-diphosphate; ITP, inosine 5'-triphosphate; XDP, xanthosine 5'-diphosphate; XTP, xanthosine 5'-triphosphate.
it is necessary to apply a combination of 10 \mu M \text{Ca}^{2+} plus 20 \mu M \text{GTP-} \gamma \text{-S. Similar results were obtained with GppNHp though higher concentrations are required (data not shown, but see Fig. 2). At suboptimal concentrations of guanine nucleotide, a higher concentration of \text{Ca}^{2+} is required to induce equivalent levels of secretion. Elevation of \text{Ca}^{2+} above 10 \mu M (i.e., to 100 \mu M [pCa4]) does not increase the extent of secretion which occurs at pCa5 either in the absence, or at any concentration of guanine nucleotide (data not shown). These experiments indicate that the combined presence of \text{Ca}^{2+} together with a nonphosphorylating analogue of GTP is sufficient to cause exocytotic secretion from permeabilized mast cells.

It is well known that histamine secretion from intact cells due to stimulating ligands (e.g., antigen, compound 48/80, calcium ionophores, etc.) is prevented by treatments that cause depletion of intracellular ATP. In view of the apparent nonrequirement for ATP for secretion from the permeabilized cells, we compared the effects of metabolic inhibitors on the secretory response from intact and from permeabilized cells. Fig. 5 illustrates the progressive onset of inhibition due to application of metabolic inhibitors before stimulation of secretion from intact cells by compound 48/80, and from streptolysin-O-permeabilized cells by the combination of \text{Ca}^{2+} (pCa5) and \text{GTP-} \gamma \text{-S (20 \mu M). After 5 min of preincubation with the combined metabolic inhibitors, ligand-induced secretion was totally suppressed; in contrast, secretion due to introduction of \text{Ca}^{2+} plus \text{GTP-} \gamma \text{-S was reduced <10\%. After 30 min of pretreatment with inhibitors it was still possible to elicit } \sim 40\% \text{ secretion from the permeabilized cells; full inhibition requires } \sim 1 \text{ h of pretreatment with metabolic inhibitors.}

While these data suggest that phosphorylation does not comprise a necessary step in the exocytotic event, we have asked whether the pattern of secretion could be modulated...
by the presence of ATP. Fig. 6 compares the dependence on Ca\(^{2+}\) (GTP-\(\gamma\)-S at 20 \(\mu\)M) and the dependence on GTP analogues (at pCa5) from streptolysin-O-permeabilized cells in the absence and presence of added ATP (1 mM). It is striking that the combination of Ca\(^{2+}\) plus GTP-\(\gamma\)-S is capable of eliciting the full expression of exocytotic activity from the metabolically depleted cells, and that ATP serves only to enhance their effective affinities. The nonphosphorylating analogue AppNHp (1 mM) was without effect (data not shown).

Analysis of the data relating secretion to Ca\(^{2+}\) concentration (Fig. 6, top) using a computer program (3) to fit the logistic expression (37)

\[
\% \text{ secretion} = 100 \times \frac{[\text{Ca}^{2+}]^P}{([\text{Ca}^{2+}]^P + K^P)}
\]
gives

\[
\log K = 5.92 (\pm 0.02) \text{ and } P = 2.08 (\pm 0.18) \text{ (GTP-\(\gamma\)-S, no ATP)},
\]
and

\[
\log K = 6.34 (\pm 0.02) \text{ and } P = 2.415 (\pm 0.19) \text{ (GTP-\(\gamma\)-S + ATP)}.
\]

For comparison when using ITP, we have previously shown that \(\log K = 6.06 (\pm 0.05)\) and \(P = 1.66 (\pm 0.15)\) (25).

The effective affinity for Ca\(^{2+}\) in the exocytic process (stimulated by Ca\(^{2+}\) plus GTP-\(\gamma\)-S in the absence of ATP) is similar to that recorded earlier in experiments using Ca\(^{2+}\) plus ITP (25). In the presence of ATP the effective affinity for Ca\(^{2+}\) is enhanced by \(\sim 0.4\) pCa units (this is a threefold increase in effective affinity). The values of the exponent, \(P\), greater than unity, indicate that the concentration effect relationships are steeper than would be predicted by a simple Langmuir expression for Ca\(^{2+}\) binding.

The affinity for guanine nucleotides is increased to an even greater extent (\(\sim 10\)-fold) by ATP. We have not subjected these data to a formal analysis because there is a much greater variation between individual experiments (possibly due to the presence of impurities [13]) and also because the concentration of free guanine nucleotide at low (submicro-)molar concentrations must be subject to perturbation in the absence of a suitable buffering system.
Discussion

The use of streptolysin-O to permeabilize mast cells provides a valid system to investigate intracellular events related to the exocytotic process (25). Similar to the use of patch pipettes (20), streptolysin-O permits the rapid efflux of soluble proteins (measured here as lactate dehydrogenase). In contrast, other methods of permeabilization (i.e., high voltage discharge [29], use of ATP+ [8], Sendai virus [4, 21], and Staphylococcal α-toxin [1]) generate small lesions so that cytosol proteins are retained. Thus when using streptolysin-O as a permeabilizing agent, processes related to requirements for low molecular weight metabolites and the presence of high molecular weight proteins are fully and rapidly accessible to experimental manipulation. In this paper we have concentrated on the requirement of nucleotides for secretion.

Previously we have shown that mast cells permeabilized in the presence of Ca2+ (in the range pCa 7-5), but in the absence of added nucleotide, will release ~30% histamine; this can be enhanced to a maximum of ~80% by 5 mM ITP (25). Unexpectedly we found that ATP is actually inhibitory when supplied in the range 0.05–1 mM. With cells treated with metabolic inhibitors, no secretion occurs in the absence of added nucleoside triphosphate.

It is apparent that different secretory cells display varying preferences for nucleoside triphosphates in support of the exocytotic reaction. Thus, Ca2+-induced secretion of amines and lysosomal enzymes from permeabilized platelets can be supported by both ATP and CTP [33]: lysosomal enzyme secretion from permeabilized neutrophils shows a preference for ATP but can also be supported by GTP > UTP > XTP > ITP > CTP while specific granule secretion (measured as lysozyme release) has an absolute requirement for ATP (4). Now, in the mast cells, we find that ATP provides the least support and for this reason alone it appears unlikely that the role of nucleoside triphosphates in secretion from these cells involves a conventional phosphorylation reaction.

Of six nucleoside triphosphates tested, ITP provides the best support to histamine secretion and at concentrations above 1 mM ITP > XTP > GTP >> ATP. The pyrimidine nucleoside triphosphates are without significant effect. At concentrations below 0.5 mM, ITP remains the most effective, but GTP > XTP. GDP and GDP analogues can inhibit Ca2+-induced secretion from cells supplemented with ITP (reversible by GTP-γ-S). Of the nucleoside diphosphates that we tested the most potent inhibitor is 2'-deoxyGDP which at concentrations less than 1 mM causes total blockade of secretion supported by 5 mM ITP: by comparison, the inhibitory effect of IDP is no greater than that due to ADP and XDP. The rank orders for activating nucleotides are similar to those described for the β-adrenergic system of turkey red blood cells (10): here, the sole difference lies in the absence of an inversion to give GTP > ITP at low nucleotide concentrations. In many other adenylate cyclase-activating systems in operation of the inositide kinases for the maintenance of phosphatidylinositol-4,5-bisphosphate (16, 36), depletion of which would lead to failure to generate the second messengers inositol-1,4,5-trisphosphate and diacylglycerol. However, in view of the susceptibility of Ca2+ ionophore-induced secretion to metabolic inhibition (7, 18, 27), an alternative role for ATP in intact cells unrelated to the mechanism of receptor activation could be to sustain the level of Ca2+. This, as discussed below, may be involved both at the level of receptor activation and in the control of membrane fusion leading to exocytosis.

Our experiments also point to other roles for ATP since the sensitivities to Ca2+ and GTP (analogues) are shifted by approximately threefold and tenfold when it is supplied. Thus it is at least plausible that those proteins which bind and transduce the exocytotic response to Ca2+ and GTP are themselves phosphoproteins.

We have previously presented evidence for two sites of action of GTP in the stimulus-secretion coupling sequence of neutrophils (5, 19, 22). One site of action, Gα, transduces receptor signals to activation of polyphosphoinositide phosphodiesterase (13) and this results in the generation of two products, inositol 1,4,5-trisphosphate (which mobilizes Ca2+ from intracellular storage sites [9]) and 1,2-diacylglycerol (the activator of protein kinase C [35]). If it is Gα which is probed in the current experiments then effects of inositol 1,4,5-trisphosphate in liberating Ca2+ can safely be neglected in the permeabilized cells in which the concentration of Ca2+ is buffered by the presence of 3 mM EGTA. The other product, diacylglycerol, is likely to be retained in the plasma membrane, as it is in stimulated neutrophils (6). However,
since secretion can occur from metabolically inhibited cells and in the absence of added phosphorylating substrates, any effect of diacylglycerol is likely to be due to processes other than the activation of a protein kinase.

We consider that GTP also acts at a second site together with Ca²⁺ directly to cause secretion. In Sendai virus-permeabilized neutrophils we have shown that GTP (analogues) can stimulate exocytosis independent of polyphosphoinositide phosphodiesterase activation and involvement of Gₛ. We have called this second site Gₑ (5, 19, 22). The characteristics of the control of secretion by guanine nucleotides in the streptolysin-O-permeabilized rat mast cells and the Sendai virus-permeabilized rabbit neutrophils (5) differ in the sense that the mast cells exhibit a dual requirement for both Ca²⁺ and GTP whereas lysosomal enzyme secretion from neutrophils can occur under conditions in which Ca²⁺ is suppressed below <10⁻¹⁰ M (5). Similar to the neutrophils, guanine nucleotides also induce Ca²⁺-independent secretion of insulin from RINm5F cells permeabilized by high voltage discharge (42) and of catecholamines from digitonin-permeabilized adrenal medullary cells in primary culture at pCa7 (11). A contrary effect of GTP and GTP analogues has been reported in freshly disaggregated bovine adrenal cells (permeabilized by high voltage discharge) in which Ca²⁺-induced secretion is inhibited (30) so it is possible that guanine nucleotides exert both stimulatory and inhibitory effects on Ca²⁺-induced secretion. We would stress that all evidence for the existence of Gₑ is based on the measurement of secretion, and at this stage we can offer no information about its location, nor about possible structural relationships or homologies with the other G proteins exemplified by Gₛ, Gᵢ and Gₛ all of which are likely also to be present in these secretory cells.

A particular feature of this work has been the use of streptolysin-O to permeabilize cells in order to introduce otherwise impermeant aqueous solutes into the cytosol. Streptolysin-O, a cholesterol-directed bacterial cytolsin (41) has been shown to permit efflux of proteins of molecular weight in excess of 400,000 in model systems (12) and in the mast cells we find that >65% lactate dehydrogenase leaks from the cells within 5 min of applying 0.4 IU ml⁻¹ of the toxin (25). By comparison with other methods of cell permeabilization such as high voltage electric discharge (29, 32, 39), use of ATP₄⁻ (exclusion of insulin) (8) and Staphylococcal alpha toxin (1), none of which permit efflux of cytosol proteins, the lesions generated by streptolysin-O are very large indeed. An alternative method of gaining access to and controlling the composition of the cytosol is by the use of patch pipettes in the whole cell mode. Patch pipettes communicate with the cytosol through a single orifice having micrometer dimensions, larger by far than those generated by streptolysin-O. In the mast cells, patch pipettes have also been used as sensing electrodes to monitor the course of the exocytotic reaction (17). Mast cells attached to patch pipettes rapidly become refractory to the introduction of Ca²⁺ buffers (and Ca²⁺-mobilizing ligands). They nonetheless degranulate in response to nonhydrolyzable analogues of GTP introduced through the pipette together with EGTA (to suppress cytosol Ca²⁺). The failure of Ca²⁺ to induce exocytosis was rationalized on the basis of the dilution of cytosol protein into the effective infinite volume of the pipette. In contrast, when using streptolysin-O which generates lesions having intermediate dimensions, a dependence on Ca²⁺ for secretion remains an absolute requirement.

In addition to the requirement for Ca²⁺, the data presented in this paper are suggestive of involvement of Gₑ in mast cell secretion. We have already remarked on the differential effect of ATP depletion on receptor-stimulated secretion from intact cells versus Ca²⁺ plus GTPγS-stimulated secretion from permeabilized cells. This suggests that Gₑ activation of polyphosphoinositide phosphodiesterase is necessary for transmembrane signaling but is not intrinsic to the exocytotic mechanism. Confirmation of a role for Gₑ comes from our observation that neomycin can selectively inhibit activation of polyphosphoinositide phosphodiesterase whilst leaving secretion intact (Cockcroft, S., T. W. Howell, and B. D. Gomperts, manuscript in preparation).

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