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

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Abstract. Rat mast cells, pretreated with metabolic inhibitors and permeabilized by streptolysin-O, secrete histamine when provided with Ca\(^{2+}\) (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\(^{2+}\) plus ITP inhibit secretion in the order 2'-deoxyGDP > GDP > o-GDP > ADP ≈ 2'deoxyADP ≈ IDP.

To learn about the train of postreceptor processes which control exocytosis in secretory cells, or indeed, almost any other form of cellular activation, it is advantageous to gain access, by permeabilization of the plasma membrane, to the intracellular environment. A number of procedures for membrane permeabilization have been devised that allow secretory cells to respond to extracellular ligands and intracellular second messengers (such as Ca\(^{2+}\), GTP, etc.) applied alone and in combination (20, 32). From such studies, two separate pictures have emerged. In platelets (23, 31), the Ca\(^{2+}\) requirement for exocytosis is shifted to lower concentrations when GTP analogues are present. Since phorbol esters and thrombin (24) modulate Ca\(^{2+}\)-induced secretion in a similar manner, it has been concluded that the effect of internalized GTP (analogues), which activate polyphosphoinositide phosphodiesterase (13), is exerted through diacylglycerol, and hence phosphorylation by protein kinase C. In other systems such as rabbit neutrophils (5), it appears that Ca\(^{2+}\) and GTP analogues are both independently capable of activating exocytotic secretion by a mechanism not involving activation of protein kinase C.

We recently reported that mast cells permeabilized by treatment with streptolysin-O (a streptococcal cytotoxin) undergo an exocytotic secretory reaction, releasing histamine and \(\beta\)-N-acetylg glucosaminidase in response to Ca\(^{2+}\) buffered at concentrations in the micromolar range (25). The lesions generated by streptolysin-O in liposomal membranes have a diameter in excess of 12 nm and are sufficient to permit fluxes of large proteins such as catalase, urease, and ferritin (12). Mast cells permeabilized with streptolysin-O lose >65% of lactate dehydrogenase within 5 min yet remain responsive to step changes of Ca\(^{2+}\), from pCa8 to pCa5 up to 10 min after permeabilization (25). In the present paper we pay special attention to the role of nucleoside triphosphates in the secretory reaction of streptolysin-O-permeabilized mast cells. We show that Ca\(^{2+}\)-dependent exocytosis does not require the presence of phosphorylating nucleotides but that a ligand able to bind at GTP regulatory proteins is essential.

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 resuspended at \(\sim \! 0.2 \times 10^6\) ml\(^{-1}\) in a solution (pH 6.8) comprising 137 mM NaCl, 2.7 mM KCl, 20 mM Pipes, 5.6 mM glucose, and BSA (0.1 mg ml\(^{-1}\)). In all experiments except those illustrated in Fig. 3, the mast cells were finally resuspended in a glucose-free buffer and treated with metabolic inhibitors (5 \(\mu\)M antimycin A plus 6 mM 2-deoxyglucose) 2 min before (Fig. 1) or at the time of permeabilization (Figs. 2, 4, and 6) with streptolysin-O (0.4 IU ml\(^{-1}\)). 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 \(\mu\)l) was added to 3 or 4 vol of a solution containing streptolysin-O, nucleoside triphosphate (as the Mg\(^{2+}\) 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-
mine release was measured. Data are means ± SEM of three ex-

permeabilized with streptolysin O (0.4 IU ml⁻¹) in the presence of

Ca²⁺ was buffered at concentrations between 10⁻⁷ M and 10⁻³ M (pCa7
to pCa5), and Mg²⁺ was set at 2 mM by the use of appropriate EGTA

buffers which were prepared as previously described (4, 25). The maximum

temperature error due to varying the concentration of ATP in the range of 0–5 mM was

<0.02 pCa.

Streptolysin-O was obtained from Wellcome Diagnostics (Dartford,

Kent, UK) as a partially purified culture filtrate and used without further

purification. Compound 48/80 was purchased from Sigma Chemical Co.

Corporation Ltd., Lewes, Sussex, UK.

All nucleotides were purchased from Boehringer

Ingelheim (Mannheim, Germany) as a partially purified culture filtrate and used without further

purification. Compound 48/80 was purchased from Sigma Chemical Co.

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 experi-

ment, Ca²⁺ was buffered at 10 μM. The cells were rou-

tinely resuspended in a glucose-free buffer in the presence of

metabolic inhibitors (5 μM antimycin plus 6 mM 2-deoxy-

glucose) at the same time as or 2 min before permeabilization

in order to suppress the secretion (29 ± 3%) which other-

wise 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 sup-

port to the secretory reaction; ATP is without effect when

supplied to the permeabilized cells at concentrations <1

mM. GTP and XTP (xanthosine triphosphates) 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

β-adrenergic receptor-linked G₂-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-methylenediphosphate

(GTP-γ-S, GppNHp, and GppCH₂p, respectively) to support

Ca²⁺-induced secretion. In these experiments phosphorylating nucleotides were not provided and metabolic inhibi-

tors were added at the time of permeabilization. The results are shown in Fig. 2. All three nonphosphorylating an-

galogues of GTP are able to support Ca²⁺-dependent secre-

tion with the rank order GTP-γ-S > GppNHp > GppCH₂p.

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 Ca²⁺-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 5 mM ITP) by ~50%. Inosine diphosphate (IDP), xantho-
sine diphosphate (XDP), ADP, and 2'-deoxyADP caused

only marginal inhibition (<20%). The concentration dependence of inhibition by 2'-deoxyGDP on Ca²⁺-induced secre-
tion is illustrated in Fig. 3 (bottom). Inhibition by nucleoside
diphosphates is characterized by a reduction in the apparent

affinity for Ca²⁺ at the exocytotic site. The inhibition due to

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1. Abbreviations used in this paper: GppCH₂p, guanyl (β, γ-methylene) -
diphosphate; 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 μM Ca<sup>2+</sup> plus 20 μM GTP-γ-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 Ca<sup>2+</sup> is required to induce equivalent levels of secretion. Elevation of Ca<sup>2+</sup> above 10 μM (i.e., to 100 μM [pCa<sub>4</sub>] does not increase the extent of secretion which occurs at pCa<sub>5</sub> either in the absence, or at any concentration of guanine nucleotide (data not shown). These experiments indicate that the combined presence of Ca<sup>2+</sup> 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 Ca<sup>2+</sup> (pCa<sub>7</sub>) and GTP-γ-S (20 μM). After 5 min of preincubation with the combined metabolic inhibitors, ligand-induced secretion was totally suppressed; in contrast, secretion due to introduction of Ca<sup>2+</sup> plus GTP-γ-S was reduced <10%. After 30 min of pretreatment with inhibitors it was still possible to elicit ~40% secretion from the permeabilized cells; full inhibition requires ~1 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-γ-S at 20 μM) 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-γ-S is capable of eliciting the full expression of exocytic 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

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

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

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-γ-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 ≈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 (≈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 ATPase [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 adenylyl cyclase-activating systems it has been shown that GTP may be replaced by ITP with little loss of activity (15, 40, 46). Taken together, these observations suggest that the role of nucleoside triphosphates in the exocytotic reaction might be that of a ligand capable of binding and activating a GTP regulatory protein, rather than that of a phosphorylating agent. The demonstration of synergistic activation of histamine secretion by provision of Ca2+ together with nonphosphorylating derivatives of GTP to metabolically inhibited cells now provides supporting evidence for the role of a G protein in exocytosis. Again, the rank order among nonphosphorylating analogues of GTP (GTP·γ·S > GppNHp > GppCHp) is the same as that for other systems such as the activation of cGMP phosphodiesterase by transducin (47) and the activation of adenylate cyclase by G, in pigeon red blood cells (38).

Others have discussed the effects of metabolic inhibition and ATP depletion on the stimulus-secretion sequence of intact mast cells (26, 28, 34) and other secretory systems (e.g., platelets [2, 43, 44]) in terms of energy requirements. In contrast, it was recently reported (45) that trychocyst discharge in Paramaecium cells can occur in the absence of ATP and that in this system ATP is actually inhibitory. On treatment with the combined inhibitors, antimycin A plus 2-deoxyglucose, the ATP content of mast cells declines to 10% of its normal level within 2 min [26]. In our present experiments, intact cells treated with metabolic inhibitors are unable to respond to stimulation by compound 48/80 after 5 min. By working with a permeabilized cell system we are in the position to identify with somewhat better precision the actual sites of action of ATP in this overall process. We find that such cells, refractory to stimulation by compound 48/80, remain fully responsive to introduction of Ca2+ plus GTP·γ·S.

It would appear that ATP is not required for the exocytotic reaction itself but may be important in the communication of signals by cell surface receptors to the cell interior. Seen in this context, one vital role of ATP in intact cells must be in operation of the inositol 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 ATP. 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 (analouges) 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 Ca\(^{2+}\) 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 G\(_{\beta}\). We have called this second site G\(_{\beta}\) (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 Ca\(^{2+}\) and GTP whereas lysosomal enzyme secretion from neutrophils can occur under conditions in which Ca\(^{2+}\) is suppressed below \(<10^{-10} \text{M}\) (5). Similar to the neutrophils, guanine nucleotides also induce Ca\(^{2+}\)-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 Ca\(^{2+}\)-induced secretion is inhibited (30) so it is possible that guanine nucleotides exert both stimulatory and inhibitory effects on Ca\(^{2+}\)-induced secretion. We would stress that all evidence for the existence of G\(_{\beta}\) 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 G\(_{\alpha}\), G\(_{i}\), and G\(_{\beta}\), 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 cytolysin (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 ATP\(^{4-}\) (exclusion of inulin) (8) and Staphylococcus-permeabilized rabbit neutrophils (44) the expression of \(\alpha\)-adrenergic adenylate cyclase activity in membranes from turkey erythrocytes, J. Biol. Chem. 249:157-161.

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