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Relationship between arachidonate generation and exocytosis in permeabilized mast cells

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Using rat mast cells permeabilized with streptolysin O we show that release of arachidonate generally occurs under similar but not identical conditions to those that cause exocytosis of β-*N*-acetylglucosaminidase (hexosaminidase). Thus, hexosaminidase secretion and arachidonate release both require provision of Ca^{2+} together with a guanine nucleotide but exocytosis occurs at lower concentrations of both effectors. The kinetics of both processes are similar, with a delay in onset only when ATP is present. Arachidonate release occurs largely from a pool of arachidonyl phosphatidylcholine which appears to represent less than 1% of the total phosphatidylcholine of the cells. Despite the general similarity of the conditions causing exocytosis and arachidonate release, our results show that under some circumstances it is possible to obtain exocytosis without measurable release of arachidonate and that therefore phospholipase A_2 activation is not an essential precursor of secretion.

INTRODUCTION

The stimulus to secretion in many cell types frequently results also in the activation of phospholipase A_2, releasing arachidonate, which can subsequently be converted into other biologically active metabolites through the reactions of the cyclo-oxygenase or lipoxygenase pathways. Although this can be understood as an entirely relevant function in those cells such as neutrophils [1], eosinophils [2], platelets [3], monocytes [4] and mast cells [5,6], which are involved in the secretion of inflammatory mediators, arachidonate release also occurs in non-inflammatory situations, such as the secretion of catecholamines from stimulated adrenal chromaffin cells [7] and that of insulin from pancreatic β-cells [8]. The question thus arises whether the activation of phospholipase A_2 is an essential step in the pathway leading to exocytosis, or whether it is an adjunct process not strictly necessary for secretion.

No clear resolution of this problem has been obtained previously, although a central role for phospholipase A_2 is suggested by the observation that mast cells [9] and eosinophils [10] undergo exocytotic secretion and morphological degranulation after the exogenous application of highly purified pancreatic phospholipase A_2. On the other hand, there are situations in which substantial uncoupling has been described. Thus, in ionophore- or antigen-stimulated bone-marrow-derived mast-cell cultures (which resemble mucosal cells, not the serosal connective-tissue-type cells used in the present investigation), O_2 deprivation selectively blocks arachidonate release, but has little effect on lysosomal enzyme release [11]. Activation of rodent bone-marrow-derived mast cells with thrombin occurs without detectable generation of the products of the 5-lipoxygenase system [12]. Stimulation of these same cells with IgE-directed ligands or with Ca^{2+} ionophore (A23187) is accompanied by substantial leukotriene C_4 production. In RBL-2H3 cells (a cell line related to rat mast cells) an almost total suppression (> 97%) of antigen-induced release of arachidonate can be achieved by 18 h pretreatment with dexamethasone (1 nM), which has only a marginal effect on histamine secretion [13].

Recent investigations on the mechanism of exocytosis have involved the use of permeabilized secretory cells, which allow the direct introduction of normally impermeant effectors and inhibitors into the cytosol [14,15]. In permeabilized mast cells we have shown that exocytosis [monitored as the release of histamine or β-*N*-acetylglucosaminidase (hexosaminidase)] requires the presence of both Ca^{2+} and GTP [16] (in practice it is normal to use a non-hydrolysable analogue such as GTP[S]); both are necessary, and together they are sufficient for secretion to occur. Exocytosis occurs independently of the activation of inositide-specific phospholipase C and the generation of diacylglycerol [17].

Some mammalian cells possess a phospholipase A_2 activity which releases arachidonate and which is activated by Ca^{2+} at concentrations in the micromolar range when GTP or its analogues are provided [18,19]. The possibility exists that this enzyme is responsible for the Ca^{2+}- and/or GTP-sensitivity of the secretory response in mast cells. In the present paper we demonstrate that, although the conditions controlling arachidonate production and exocytosis in permeabilized mast cells are very similar, their characteristics differ sufficiently to support the conclusion that arachidonate production (and hence phospholipase A_2 activation) is not essential for the secretory response.

METHODS

Radiochemicals were obtained from Amersham International. Streptolysin-O was obtained from Wellcome Diagnostics plc. GTP[S] was purchased as a 100 mM

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; hexosaminidase, β-*N*-acetylglucosaminidase; pCa, $-\log[Ca^{2+}]$.

solution from Boehringer Mannheim; other biochemicals were from the same company or from Sigma Chemical Co.

Mast cells were obtained by peritoneal lavage of male Sprague–Dawley rats. They were purified to near homogeneity as previously described by centrifugation through a 2 ml cushion of Percoll [20], washed twice by centrifugation and suspended at approx. 10^6 ml^{-1} in a buffered salt solution which comprised NaCl (137 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), Pipes (20 mM), glucose (5.6 mM) and bovine serum albumin (1 mg/ml). The pH was adjusted to 6.8 with NaOH. [^{3}H]Arachidonate (2 μCi/ml) was added and the cells were incubated at 37 °C for 1 h. Time-course experiments (Fig. 1) indicated that equilibration of the label with the main phospholipids is almost complete at this time. The cells were then sedimented and washed twice by centrifugation in a buffered solution (pH 6.8) which comprised NaCl (137 mM), KCl (2.7 mM), Pipes (20 mM) and defatted albumin (1 mg/ml). The cells were finally suspended at a concentration of approx. 0.3×10^6 ml^{-1}. Before use, they were incubated for 5 min with metabolic inhibitors [2-deoxyglucose (6 mM) plus antimycin A (5μM)], at which time intracellular ATP was decreased to less than 30 μM and the cells were no longer responsive to stimulation with receptor-directed ligands [16]. For some experiments the cells were pre-labelled with [^{32}P]P_i, [^{3}H]palmitate or [^{3}H]choline instead of [^{3}H]arachidonate, but otherwise treated identically.

Ca^{2+} was buffered at concentrations between 0.1 and 10 μM (pCa 7–5), and Mg^{2+} was set at 2 mM by the use of appropriate EGTA buffers, which were prepared as described in ref. [20]. The maximum error caused by varying the concentration of ATP in the range 0–5 mM was < 0.02 pCa.

Experiments were initiated by transferring 30 μl of cells to 90 μl of medium containing streptolysin O (0.4 i.u./ml final concn.), calcium buffer (3 mM final concn., to regulate pCa as indicated), MgATP and GTP[S] as indicated. Reactions were terminated after 10 min by addition of 0.7 ml of ice-cold NaCl (0.15 M, buffered at pH 7 with 10 mM-potassium phosphate), and the cells were sedimented by centrifugation. Samples of supernatant were removed for measurement of β-*N*-acetylglucosaminidase as previously described [21] and for counting of radioactivity. In some experiments, the sedimented cells were retained for isolation and measurement of individual phospholipids as described below.

For the kinetic experiments (Fig. 4) cells were initially labelled and prepared as described above. The metabolically inhibited cells were then permeabilized and loaded with GTP[S] (as indicated) in the presence of 0.2 mM calcium buffer to maintain pCa 7. After 2 min to allow equilibration of the guanine nucleotide, they were transferred to tubes containing 3 mM calcium buffer at pCa 5, and timed samples were removed and quenched as described above. For further details see ref. [22].

For separation of phospholipids, cell pellets were resuspended in 0.5 ml of 0.15 M-NaCl (buffered at pH 7 with 10 mM-potassium phosphate), to which was then added 1.9 ml of chloroform/methanol (1:2, v/v) plus a small quantity of carrier phospholipid (an unfractionated mixture of phospholipids isolated from neutrophils or red cells). After thorough mixing, the phases were separated by addition of 0.6 ml of 0.15 M-NaCl and 0.6 ml of chloroform [23]. The lower (chloroform) layer was removed and evaporated to dryness under vacuum. The dried lipids were redissolved in 50 μl of chloroform and applied to 20 cm × 20 cm silica t.l.c. plates. The lipids were separated with chloroform/methanol/acetic acid/water in the proportions 75:45:12:2 (by vol.) [24] (or 74:45:3:1 [25] to ensure separation of phosphatidylinositol from phosphatidylserine). The plates were exposed to I_2 vapour, and the stained spots were scraped and suspended in 0.5 ml of methanol/acetic acid/water (5:2:3, by vol.) Then 4 ml of Fluran HV scintillation fluid (BDH) was added, and radioactivity was determined by liquid-scintillation counting.

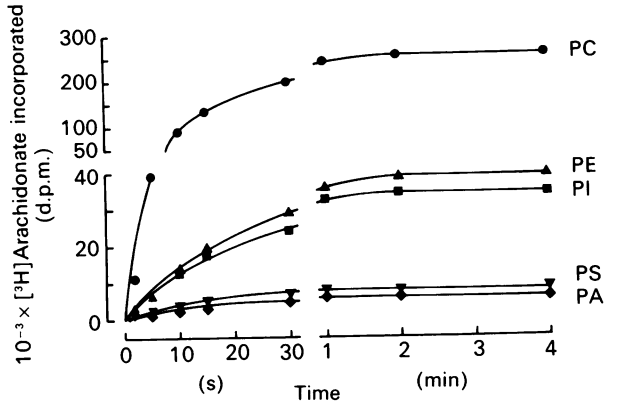

Fig. 1. Time course of [^{3}H]arachidonate uptake into mast-cell phospholipids

[^{3}H]Arachidonate (2 μCi) was added to mast cells (2×10^6 in 2 ml) and incubated at 37 °C. Samples were withdrawn and added directly into a mixture of chloroform and methanol at the times indicated. The lipid extracts were separated on t.l.c. Key: ●, phosphatidylcholine (PC); ▲, phosphatidylethanolamine (PE); ■, phosphatidylinositol (PI); ◆, phosphatidate (PA); ▼, phosphatidylserine (PS).

RESULTS

Addition of streptolysin-O to mast cells in a simple salts medium buffered at low Ca^{2+} was sufficient to cause almost complete loss of cytosolic components, including free arachidonate, but without release of the contents of secretory granules [21] or of radioactivity from labelled lipids (Fig. 2).

Exocytosis of histamine and lysosomal enzymes from permeabilized mast cells requires the presence of a dual effector system, comprising Ca^{2+} and a guanine nucleotide [16]. Fig. 2 illustrates the results of an experiment in which the release of a secretory product (hexosaminidase) and the release of arachidonate from permeabilized cells were compared. In this experiment the cells were treated with streptolysin-O in the presence of a range of concentrations of Ca^{2+} and GTP[S] so that the stimulus was applied as the cells became permeable. The data relating effector concentration to exocytosis are very similar to those previously reported [16,26], and show that the operative affinity for both effectors is enhanced when ATP is provided, although this has no effect on the maximal extent of release that can be elicited. Release of arachidonate also requires the presence of both effectors, but, unlike secretion, their effective

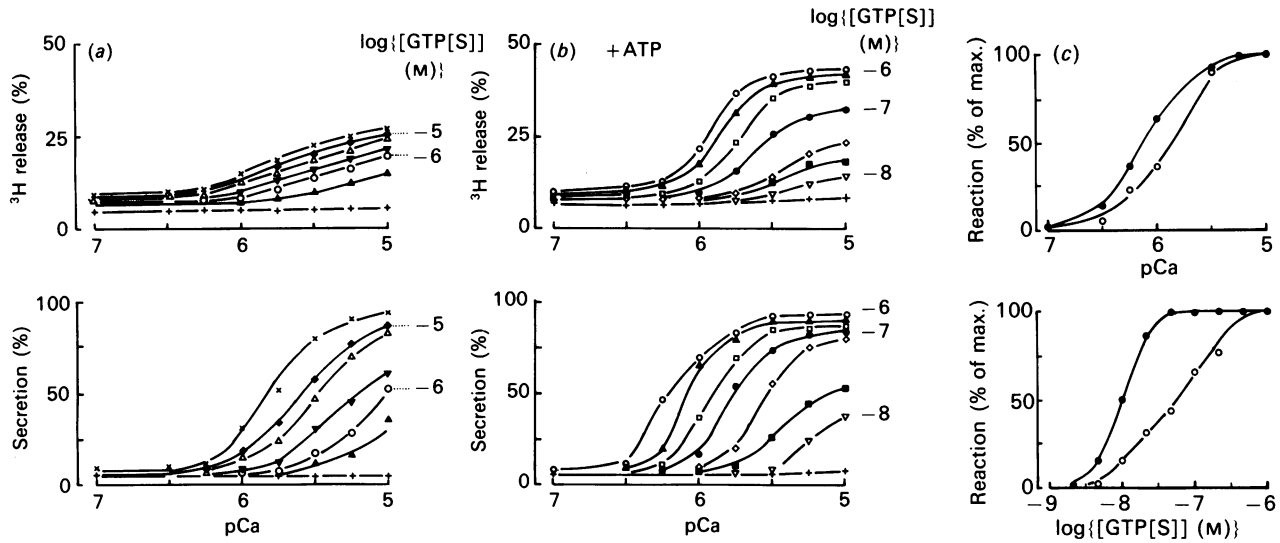

Fig. 2. Dependence on [Ca^{2+}] and GTP[S] for release of arachidonate and hexosaminidase from rat mast cells permeabilized by streptolysin-O in the presence and absence of ATP

(*a*) and (*b*) Mast cells, labelled with [^{3}H]arachidonate and treated with metabolic inhibitors to the point of non-responsiveness towards stimulation by exogenous secretagogues, were permeabilized in the presence of Ca^{2+} (buffered with 3 mM-EGTA) and GTP[S] as indicated, and in the absence (*a*) or presence (*b*) of ATP (1 mM). GTP[S]: ×, 21.6 μM; ◆, 10 μM; △, 4.64 μM; ▼, 2.16 μM; ○, 1.0 μM; ▲, 0.464 μM; □, 0.216 μM; ●, 0.1 μM; ◇, 0.0464 μM; ■, 0.0216 μM; ▽, 0.01 μM; +, zero. After 10 min incubation the reactions were quenched, the cells sedimented and the supernatants analysed for hexosaminidase and [^{3}H]arachidonate as described in the Methods section. (*c*) This represents the averaged normalized data from experiments in which mast cells were treated and permeabilized (in the presence of 1 mM-ATP) as above, and triggered either with 10 μM-GTP[S] and a range of Ca^{2+} concentrations (six experiments) or with 10 μM-Ca^{2+} and a range of GTP[S] concentrations (four experiments) as indicated. ●, Hexosaminidase secretion; ○, arachidonate release. The S.E.M. did not exceed 4.1 % for any point represented.

affinity is not substantially altered when ATP is provided, though the extent of release is considerably enhanced.

Fig. 3 and Table 1 present an analysis of the distribution of arachidonate radioactivity among phospholipids and the aqueous fraction after permeabilization in the presence of 10 μM-GTP[S], 1 mM-MgATP and a range of Ca^{2+} concentrations. Quantitatively the most significant change is the large loss (up to 60%) of label from phosphatidylcholine, which represents the main cellular pool of labelled arachidonate. This was paralleled by a corresponding increase in the amount of radioactivity in the extracellular medium, which chromatographed largely as fatty acid and was assumed to represent mainly free arachidonate. Of the other lipids, only phosphatidylinositol showed decreased radioactivity on stimulation of permeabilized cells, but slight increases in labelling of phosphatidate were observed. It was not possible from these experiments to determine whether the decrease in phosphatidylinositol labelling was due to attack by phospholipase A_2 or by phospholipase C, although the increase in phosphatidate radioactivity could be a consequence of breakdown of polyphosphoinositides by phospholipase C and phosphorylation of the resulting diacylglycerol. In this case, the loss of phosphatidylinositol could be due to its phosphorylation to refill the polyphosphoinositide pool. It is not clear from our results whether any of the released arachidonate radioactivity is derived from phospholipase A_2 attack on phosphatidylinositol, but certainly there was no indication of the production of any lysophosphatidylinositol from permeabilized cells labelled with [^{32}P]P_i or [^{3}H]palmitate.

Because of the relatively small amounts of cells that were available, it was not possible to measure phospholipid mass to determine whether the large loss of arachidonate from phosphatidylcholine was represent-

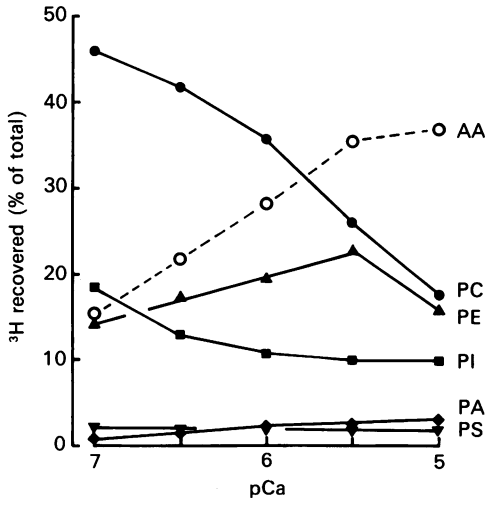

Fig. 3. Effect of Ca^{2+} on the distribution of [^{3}H]arachidonate radioactivity in lipids of permeabilized mast cells

[^{3}H]Arachidonate-labelled mast cells were permeabilized in the presence of 10 μM-GTP[S], 1 mM-ATP and Ca^{2+} as indicated and incubated for 10 min. The cellular phospholipids were extracted and isolated by t.l.c. Key: ○, arachidonate (AA); ●, phosphatidylcholine (PC); ▲, phosphatidylethanolamine (PE); ■, phosphatidylinositol (PI); ◆, phosphatidate (PA); ▼, phosphatidylserine (PS).

Table 1. Distribution of radioactivity in phospholipids of mast cells labelled with [^{3}H]arachidonate, [^{3}H]palmitate, [^{32}P]P_i or [^{3}H]choline, then permeabilized in the absence or presence of GTP[S] and Ca^{2+}

In experiments where we separated PI and PS [25], 80–90% of the total counts in each case were in PI. Results are presented as means ± S.D., from three separate experiments: * significantly different from controls ($P < 0.001$). Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine, LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; FA, fatty acids.

	Distribution (%)			
Label ...	[^{3}H]Arachidonate		[^{3}H]Palmitate	
	Control	Stimulated	Control	Stimulated
LPC	0.6±0.1	0.4±0.1	0.3±0.2	0.5±0.3
SM	0.6±0.3	0.3±0.2	8.4±0.9	8.4±0.4
PC	68.3±4.1	*27.6±5.2	56.4±5.1	57.4±6.4
LPE	0.6±0.1	0.4±0.1	0.5±0.2	0.4±0.2
PI/PS	12.1±0.1	8.5±0.5	3.1±0.2	2.8±0.2
PE	10.0±1.7	8.3±1.5	6.6±1.4	5.4±0.9
PA	3.5±0.2	4.0±0.2	2.9±0.9	2.6±0.9
FA	5.1±1.1	*44.4±4.5	22.2±2.7	22.8±3.1
	Total c.p.m. 86528±7320		72277±10620	

	Distribution (%)			
Label ...	[^{32}P]P_i		[^{3}H]Choline	
	Control	Stimulated	Control	Stimulated
LPC	1.7±0.1	1.8±0.2	0.97±0.4	0.9±0.3
SM	2.3±0.2	1.2±0.7	0.61±0.2	0.6±0.2
PC	34.4±5.3	34.2±6.2	97.30±0.5	97.5±0.4
LPE	0.7±0.4	0.2±0.5	< 1% of total	
PI/PS	24.8±3.8	22.4±5.1		
PE	27.4±5.2	31.1±3.5		
PA	9.5±2.9	8.2±3.8		
	Total c.p.m. 14471±2229		126448±23798	

ative of a general breakdown of this lipid class or of only a limited pool of arachidonyl species. However, when the cells were labelled with other radioactive precursors, including palmitate, choline or [^{32}P]P_i, no significant breakdown of phosphatidylcholine was observed (Table 1), and the increase in lysophosphatidylcholine in each case was only about 1% of total choline lipid labelling. Assuming that these precursors label the major pools of phosphatidylcholine in the cells, it appears that only a small pool of arachidonyl phosphatidylcholine, perhaps accounting for no more than 1% of the total choline lipid, is susceptible to attack by the endogenous phospholipase A_2. It is unlikely that this low value is due to reacylation of larger amounts of lysophosphatidylcholine formed originally, since in these permeabilized cells the enzymes and low-molecular-mass cofactors necessary for reacylation are largely washed out and greatly diluted in the medium. Furthermore, there was no indication of a fall in any labelled pool of lipid which could potentially donate fatty acid to lysophosphatidylcholine.

We have tested the following nucleotides as alternatives to GTP[S]: guanosine 5′-[$\beta\gamma$-imido]triphosphate, oxidized GTP, *R*P- and *S*P-guanosine 5′-[α-thio]-triphosphate (GTP[αS]), guanosine 5′-[$\beta\gamma$-methylene]-diphosphate, 2′-deoxy-GTP, guanosine 5′-γ-phenyl-triphosphate. All these nucleotides are capable of stimulating both exocytosis and release of arachidonate in the absence of ATP, and their ED_{50} (which is in the order listed) ranges from 1 μM (GTP[S]) to 1 mM (γ-phenyl analogue). There was little apparent difference in the affinities of the two diastereoisomers of GTP[αS].

From the results so far described, the secretion of lysosomal enzymes and the release of arachidonate can be seen to be closely related in terms of their requirements for Ca^{2+} and guanine nucleotide and ATP, although it should be noted that the secretory reaction occurs at lower concentrations of Ca^{2+} and GTP[S] than does arachidonate release. This latter point is made explicit in Fig. 2(*c*), in which averaged normalized data relating to secretion and arachidonate release are presented as functions of Ca^{2+} and GTP[S] concentrations (at 10 μM-GTP[S] and pCa 5, respectively). The effector concentrations required to activate arachidonate release are consistently higher than those which activate exocytosis. Note that secretion is first discernible at 4.6 nM-GTP[S] ($10^{-8.33}$ M), but that in four experiments we failed to detect release of arachidonate at this concentration.

Fig. 4 illustrates the time course of exocytosis and arachidonate generation from the permeabilized cells. In these experiments the cells were permeabilized in the presence of GTP[S] and incubated for a further 2 min in order to ensure equilibration of the guanine nucleotide, and only then triggered by addition of Ca^{2+} (pCa 5). For

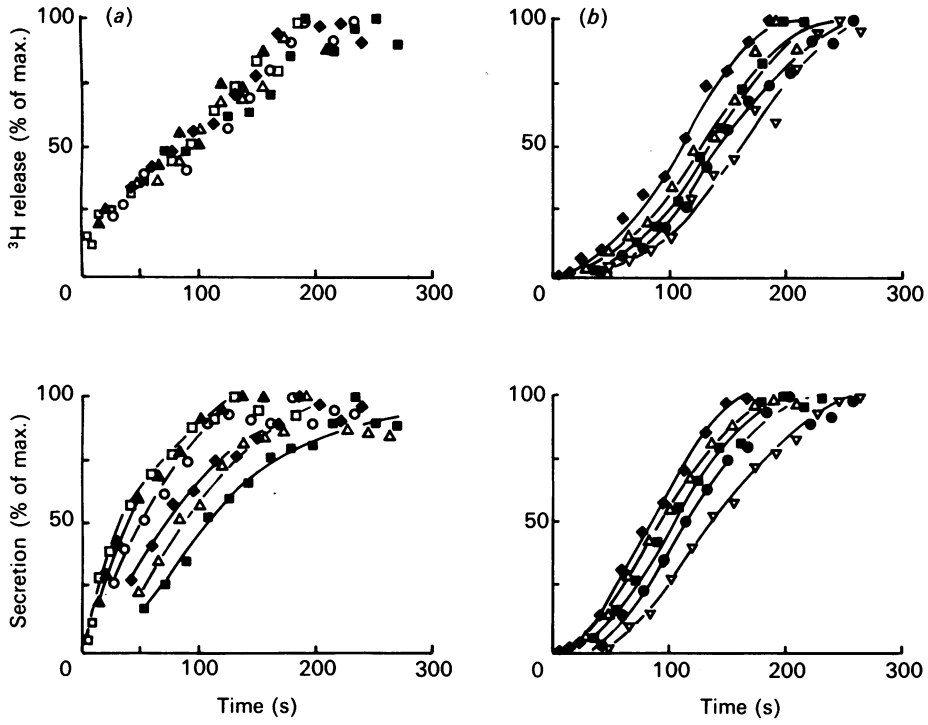

Fig. 4. Time course of release of arachidonate and hexosaminidase from cells permeabilized in the absence and presence of 1 mM-ATP

Mast cells, labelled with [^{3}H]arachidonate and treated with metabolic inhibitors, were permeabilized in the presence of 0.2 mM-EGTA buffer (to regulate pCa 7), in the absence (*a*) or presence (*b*) of MgATP (1 mM) and GTP[S] as indicated. After incubation for 2 min, 3 mM-EGTA buffer was added to regulate pCa 5, and sampling was commenced. The ordinates represent percentage of the maximum reaction which occurred during the ensuing 5 min. [GTP[S]]: □, 31.6 μM; ▲, 17.7 μM; ○, 10 μM; ◆, 5.61 μM; △, 3.16 μM; ■, 1.77 μM; ●, 1.0 μM; ▽, 0.561 μM.

cells stimulated in the presence of ATP, the appearance of extracellular hexosaminidase and arachidonate occurred after a delay the duration of which is inversely related to the concentration of GTP[S]. We have previously discussed such onset delays in terms of a sequence of reactions preceding the activation of the terminal stage of exocytosis. The manifestation of similar delays preceding arachidonate release suggests that this too is preceded by an enabling reaction. When ATP is omitted, both cell functions are initiated promptly upon completion of the full effector system. Thus, for the initiation of both secretion and arachidonate generation, ATP acts as an inhibitor, even though it increases the apparent affinity for Ca^{2+} and GTP[S] in the exocytotic reaction and enhances the extent of arachidonate release (see above).

We have previously noted that the extent of secretion that can be elicited declines rapidly when the effectors are provided to the cells after, instead of at the time of, permeabilization [27]. Fig. 5 illustrates GTP[S]-dependence of secretion and arachidonate release from cells stimulated by addition of the dual effectors (Ca^{2+} at pCa 5) at times ranging up to 1 h after treatment with streptolysin-O. During this preincubation period (the permeabilization interval) the cells were maintained at 0 °C in the presence of ATP (1 mM), and Ca^{2+} was buffered at pCa 7 by 0.2 mM calcium buffer. No release of hexosaminidase occurs under these conditions, though there is a slow release of arachidonate. Both cellular responses to stimulation with Ca^{2+} plus GTP[S] decline with time, but it is clear that the phospholipase A_2 function is more labile. At 60 min we were unable to detect any stimulation of arachidonate release at concentrations of GTP[S] up to 1 μM, although this still reliably induces 15% enzyme secretion.

DISCUSSION

GTP and its analogues can be expected to interact with many GTP-binding proteins (G-proteins) in the intracellular environment, including, for example, G_P, which mediates the receptor-controlled activation of inositide breakdown by phospholipase C in many different kinds of cells [28]. However, we have previously demonstrated that it is possible to induce exocytosis from permeabilized mast cells under conditions in which the G_P-linked activation of phospholipase C is prevented by neomycin [17]. This indicates that activation of phospholipase C is not essential for the secretory response in mast cells, and thus the G-protein required for secretion must involve some other cellular component. This could be G_A, the hypothetical G-protein which activates phospholipase A_2 [18].

Here we wish to address two main questions: (1) is activation of phospholipase A_2 essential for exocytosis from permeabilized mast cells triggered by introduction of Ca^{2+} and GTP[S]? (2) is the GTP-binding protein which stimulates exocytosis (G_E) identical with that which activates phospholipase A_2?

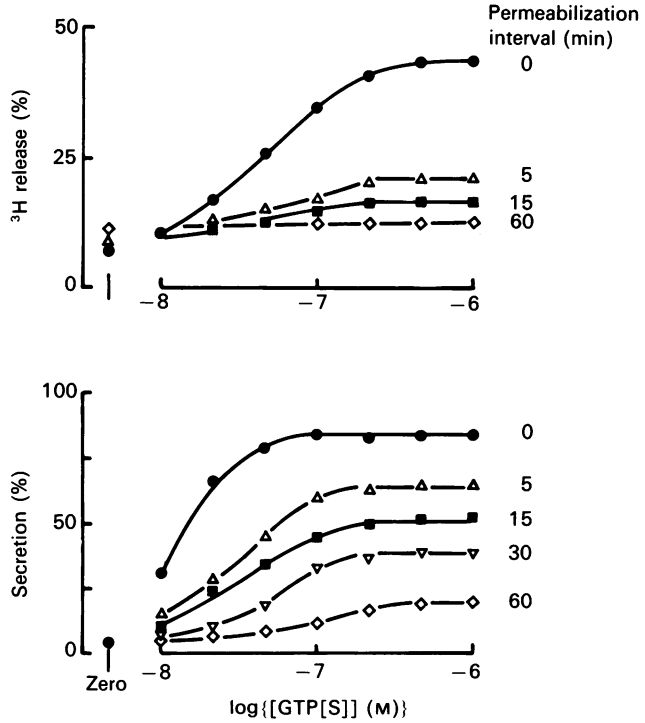

Fig. 5. Duration of responsiveness of permeabilized cells to delayed addition of Ca^{2+} plus GTP[S]

Mast cells, labelled with [^{3}H]arachidonate, were permeabilized in the presence of 0.2 mM-EGTA buffer (pCa 7) and 1 mM-ATP. After 2 min the cells were cooled to ice temperature. At the times indicated, the cells were returned to 37 °C and stimulated by addition of 10 μM-GTP[S] and Ca^{2+} (buffered with 3 mM-EGTA) as indicated.

The amount of arachidonate release induced by Ca^{2+} plus GTP[S] from permeabilized cells is certainly more extensive than that induced by a concentration of compound 48/80 which causes a similar extent of secretion from intact cells. There are a number of possible reasons why this should be. Firstly, the activation of the system by GTP[S] and Ca^{2+} is persistent, and none of the normal down-regulation mechanisms which operate in ligand-stimulated cells are likely to be operative. Second, as pointed out in reference to a similar investigation on permeabilized adrenal chromaffin cells [29], it is possible that a considerable proportion of the arachidonate that is normally reconverted into arachidonyl-CoA will leak from the permeabilized cells and thus escape recycling into membrane phospholipids. Furthermore, recycling will be suppressed, owing to the leakage of soluble acylating enzymes from the permeabilized cells.

The results presented here indicate that the conditions which promote exocytosis from permeabilized mast cells also have the effect of stimulating a phospholipase A_2 activity which releases arachidonate from phosphatidylcholine and perhaps also from phosphatidylinositol. The susceptible pool of arachidonyl phosphatidylcholine appears to represent only a very small proportion of the total phosphatidylcholine, since permeabilized cells labelled with [^{3}H]palmitate, [^{32}P]P_i or [^{3}H]choline showed little (< 1 %) change in their phospholipid radioactivity when exposed to GTP[S] and Ca^{2+} under the same conditions as for the arachidonate-labelled cells. At present we do not know the subcellular location of this small pool of arachidonyl phosphatidylcholine, but it seems likely to be close to the phospholipase A_2 which attacks it so specifically when the cells are activated.

Arachidonate release broadly parallels the secretory event not only in terms of responsiveness to guanine nucleotides and Ca^{2+} but also with respect to its kinetic characteristics. Thus, in the absence of ATP, the onset of exocytosis and of arachidonate release occurs within a few seconds of completing the pair of essential effectors, but a delay precedes the onset of both functions when MgATP is provided at the time of permeabilization. There is no indication that the onset of exocytosis is preceded by release of arachidonate. For the onset of secretion we have previously demonstrated that the duration of such ATP-induced delays (τ) is related to the concentrations of both essential effectors, such that [22,26]:

$$1/\tau = k\{[Ca^{2+}][GTP[S]]\}^{\frac{1}{2}}$$

and we reasoned that k represents a rate constant (having second-order dimensions) in a reaction leading to the generation of a new steady state which is permissive for exocytosis to proceed [26]. Since such delays are not manifest when ATP is replaced by its non-phosphorylating $\beta\gamma$-imido analogue, we have suggested that the rate-limiting step could be a dephosphorylation reaction. Although it has not been possible to examine the onset characteristics of arachidonate release in such detail, the present data show that these too are subject to ATP-induced delays and that with higher concentrations of GTP[S] their duration is decreased, indicating that the rate of reaction through the pre-release period is enhanced. From this we are led to conclude that the phospholipase A_2 reaction that we have observed, although dependent on the presence of a guanine nucleotide, is unlikely to be under direct regulation by a G-protein, but becomes activated only after the generation of an intermediate, itself the product of a G-protein-dependent enzyme.

There are a number of indications that release of arachidonate is not absolutely necessary for exocytosis to occur from the permeabilized cells. Thus measurable secretion is observed at low concentrations of GTP[S] (Fig. 4) or after protracted preincubation after permeabilization (Fig. 5), and under these conditions arachidonate release is not detectable. It is of course conceivable that only a minute fraction of the arachidonyl phosphatidylcholine pool needs to be degraded by phospholipase A_2 in order for secretion to occur, and that the much more extensive release of arachidonate which occurs in permeabilized cells activated with Ca^{2+} plus GTP[S] is due to the persistence of the stimulus and represents an uncoupling of the phospholipase A_2 from its normal physiological role.

On the basis of our data, it seems likely that phospholipase A_2 activation is not mandatory for exocytosis, and in view of the similarity (but non-identity) between the guanine-nucleotide- and Ca^{2+}-dependencies of the two processes we cannot exclude the possibility that phospholipase A_2 activation is a consequence of the membrane-fusion events implicit in the secretory mechanism. Furthermore, since metabolites of arachidonate (but not arachidonate itself) can activate mast cells [30], it is plausible that their production during stimulated secretion *in vivo* has a role in recruiting more cells which can enhance the effective response to a stimulus.

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