Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 926–930, February 1991
Cell Biology

Guanosine 5′-[β-thio]triphosphate selectively activates calcium signaling in mast cells

(calcium oscillations/G proteins/exocytosis/guanosine nucleotides/mast cells)

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Communicated by Erwin Neher, October 29, 1990 (received for review August 23, 1990)

ABSTRACT **In rat peritoneal mast cells, the activation of GTP-binding proteins (G proteins) by guanosine 5′-[γ-thio]triphosphate GTP[γS] has been found to induce a transient rise in intracellular calcium as well as degranulation. A G protein that couples to phospholipase C (G_p) is thought to mediate the calcium response, whereas degranulation is mediated by a different G protein, termed G_e. In an attempt to activate mast-cell G proteins more selectively, the GTP analogues guanosine 5′-[α-thio]triphosphate (GTP[αS]) and guanosine 5′-[β-thio]triphosphate (GTP[βS]) (R_P and S_P diastereomers) were introduced into mast cells by means of patch pipettes. Degranulation and free intracellular calcium were monitored by cell capacitance and fura-2 measurements, respectively. It was found that R_P-GTP[αS], like GTP[γS], induced both calcium release and exocytosis. In contrast, R_P-GTP[βS] induced repetitive calcium spikes that were not regularly accompanied by exocytosis. These results suggest that R_P-GTP[βS] selectively activates calcium signaling in mast cells. The R_P-GTP[βS]-induced oscillations were independent of extracellular calcium. They were absent in the presence of heparin or high concentrations of inositol 1,4,5-trisphosphate and modulated by compound 48/80, suggesting the involvement of the inositol phospholipid signaling pathway. Latency of appearance and spiking frequency were markedly modulated by varying the intracellular ATP concentration. The differential activation of intracellular calcium signaling and exocytosis by GTP[βS] confirms the presence of independent signal-transduction pathways for the two cell responses. R_P-GTP[βS] may prove helpful in the biochemical and molecular characterization of G_p, the as-yet-unidentified G protein that couples receptors to intracellular calcium release.**

The elevation of intracellular calcium $[Ca]_i$ is a common signal in cellular stimulus–response coupling. Upon hormonal stimulation, a number of nonexcitable cells exhibit repetitive calcium release from intracellular stores (1). Such calcium oscillations accompany cellular events like differentiation in B lymphocytes (2), secretion in epithelial and glandular cells (3, 4), and fertilization in oocytes (5). This calcium release from internal stores is thought to be induced through the inositol phospholipid signaling pathway, where receptor-mediated activation of phospholipase C (PLC) leads to the breakdown of phosphoinositol lipids. Two second messengers are generated: inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. The former binds to receptors on intracellular calcium-containing pools and induces the release of calcium into the cytosol (6).

The use of the nonhydrolyzable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[γS]) has provided strong evidence that the coupling of receptors to PLC involves a G protein, termed G_p (7). However, the molecular characteristics of this G protein or family of G proteins remain unresolved. A selective probe for G_p is lacking, as GTP[γS] activates G proteins with no apparent selectivity, and other nucleotides, such as GppNHp, inosine triphosphate, and xanthosine triphosphate, stimulate secretion more efficiently than $InsP_3$ production (8).

In mast cells, different G protein-mediated processes have been studied at the single-cell level by using the patch-clamp technique. Upon stimulation, mast cells transiently release calcium into the cytosol, and they degranulate (9, 10). The G proteins that mediate these responses have been termed G_p and G_e, respectively (11, 12). One difference between them is their requirement for GTP. Although G_e-mediated exocytosis depends absolutely on GTP, activation of the G protein that couples to PLC, G_p, by exogenous agonists, such as the polyamine compound 48/80, induces calcium-release transients long after cellular GTP has washed out through the patch pipette (13). Together with previous evidence (8), this suggests that G_e and G_p differ concerning the binding and/or hydrolysis of guanine nucleotides. We have, therefore, examined the effects of the phosphorothioate analogues of GTP, GTP[αS] and guanosine 5′[β-thio]trisphosphate (GTP[βS]), on G protein-mediated effects in rat mast cells.

MATERIALS AND METHODS

Mast-Cell Purification and Culture. Rat peritoneal mast cells were obtained as described (13). Briefly, male Wistar rats were ether-anesthetized, decapitated, and exsanguinated. A cell mixture was obtained by peritoneal lavage, and mast cells were purified by Percoll-gradient centrifugation. They were incubated at 37°C on glass coverslips in modified M199 medium and used within 6 hr, or they were cocultured with Swiss 3T3 fibroblasts (14) and used within 1 week. No differences were detected between the two culture methods, except for a slightly larger cell size of the cocultured mast cells.

Synthesis of GTP Analogues. The R_P and S_P diastereomers of GTP[αS] and GTP[βS] were synthesized as described (15).

Whole-Cell Patch-Clamp Experiments. For experiments, coverslips were transferred to a recording chamber containing mast-cell Ringer's solution (140 mM NaCl/2.5 mM KCl/2 mM $CaCl_2$/5 mM $MgCl_2$/11 mM glucose/10 mM Hepes·NaOH, pH 7.2). Experiments were done at 23–26°C in the tight-seal whole-cell configuration of the patch-clamp technique. Cells were held at 0 to +20 mV. The pipette solution contained 145 mM potassium glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes·KOH, 0.1 mM fura-2 pentapotassium salt (Molecular Probes), and Mg-ATP (0.5 mM, unless indicated otherwise), pH 7.2. This solution was

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Abbreviations: PLC, phospholipase C; $InsP_3$, inositol 1,4,5-trisphosphate; GTP[αS], guanosine 5′-[α-thio]triphosphate; GTP[βS], guanosine 5′-[β-thio]triphosphate; GTP[γS], guanosine 5′-[γ-thio]triphosphate; $[Ca]_i$, intracellular calcium concentration.

supplemented with GTP analogues and in some experiments with different concentrations of heparin (Sigma) or $Ins P_3$ (Amersham) (see figure legends). Cell-membrane capacitance was measured with a two-phase lock-in amplifier as detailed (16). Fura-2 (17), loaded into the cell via the patch pipette, was excited alternately at 360 and 390 nm. The emitted fluorescence was measured with a photomultiplier. The fluorescence ratio, indicating $[Ca]_i$, was calculated as described (10). Data collection was made at 2 Hz.

RESULTS

Differential Effects of GTP Analogues on Secretion and Calcium Release. We first compared the relative potency of GTP analogues in stimulating G protein-mediated events in mast cells; this was done by loading cells in the whole-cell configuration of the patch-clamp technique with pipette solutions that included the various phosphorothioate analogues of GTP (15). We monitored both cell-membrane capacitance as a measure of secretory activity (16) and fura-2 fluorescence as an indicator of $[Ca]_i$ (17).

Intracellular application of the nonhydrolyzable GTP analogue, GTP[γS], mimicks the response to mast-cell secretagogues by irreversibly activating G proteins (18). Fig. 1*A* shows a typical response to 100 μM GTP[γS]: shortly after establishment of the whole-cell configuration (cell penetration), one or two transient elevations of $[Ca]_i$ are visible. Degranulation, as evidenced by an increase in membrane capacitance, begins with a similar latency and proceeds until the capacitance has tripled.

When the cells were loaded with the R_P isomer of GTP[αS], they exhibited changes in $[Ca]_i$ and capacitance qualitatively similar to those evoked by GTP[γS] (Fig. 1*A*). The relative degranulation amplitude (capacitance at 400 s versus capacitance at break-in) dose-dependently increased over the range of concentrations tested (Fig. 1*B*). The S_P isomer of GTP[αS], on the other hand, had no effect on $[Ca]_i$. At high concentrations, there was a small stimulatory effect on secretion. In the presence of this analogue, moreover, exocytosis can be induced by the mast-cell secretagogue, compound 48/80. Thus, although S_P-GTP[αS] is not stimulatory by itself, it can substitute for GTP in the highly GTP-dependent process of mast-cell exocytosis (13).

Introducing the β-phosphorothioate analogue of GTP, R_P-GTP[βS], into the cells induced large, repetitive calcium spikes that were not regularly accompanied by degranulation (Fig. 1*A*). At 300 μM R_P-GTP[βS], 41 of 46 cells showed more than one calcium transient within 600 s. The number of calcium transients per cell increased dose-dependently (Fig. 1*C*). Fig. 2 shows some measurements obtained in the presence of 300 μM R_P-GTP[βS]. The S_P isomer showed effects qualitatively similar to, but weaker than, those of R_P-GTP[βS] (Fig. 1 *B* and *C*). In most cells loaded with R_P-GTP[βS], total capacitance decreased slightly over the measured time period (600 s), as it did in control cells. In some cells, each calcium spike was accompanied by a small (1–3 pF), fast capacitance increase. Of 75 cells measured over 600 s, only 8 showed a smooth, gradual capacitance increase comparable to, but slower than, that following GTP[γS] stimulation. Thus, R_P-GTP[βS] induced oscillations of $[Ca]_i$ in the absence of secretion.

The Inositol Phospholipid Pathway Is Involved in the R_P-GTP[βS]-Induced Oscillations. Several lines of evidence point to an involvement of the inositol phospholipid signaling pathway in the GTP[βS]-induced calcium oscillations. (*i*) The calcium transients could be generated in the absence of extracellular calcium (Fig. 3*A*). A total of eight cells were tested, all of which generated calcium transients during the application of calcium-free Ringer's solution. This result shows that the changes in $[Ca]_i$ are due to release from

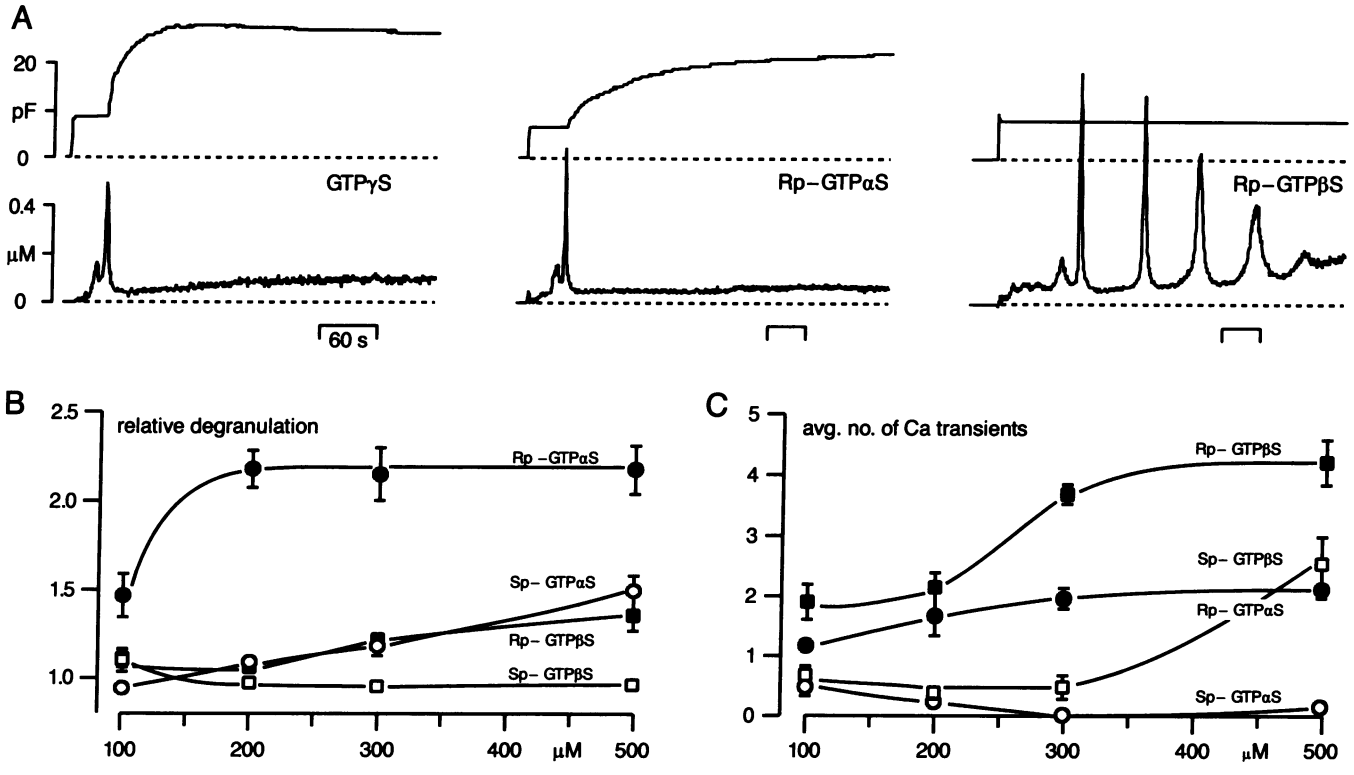

FIG. 1. Effects of GTP analogues on secretion and $[Ca]_i$. (*A*) Traces obtained with GTP[γS] (100 μM), R_P-GTP[αS] (500 μM), and R_P-GTP[βS] (200 μM). Upper traces show membrane capacitance, and lower traces represent $[Ca]_i$. The initial jump in the capacitance corresponds to cell penetration. (*B*) Comparison of the secretory efficiency of the GTP analogues: relative degranulation amplitude (means capacitance at 400 s versus capacitance at penetration) versus concentration of the GTP analogue. (*C*) Calcium mobilization by GTP analogues: average number (avg. no.) of transients per cell per 600 s versus concentration of the GTP analogue. Data points (±SEM, n = 5 to 45 cells) were connected by eye.

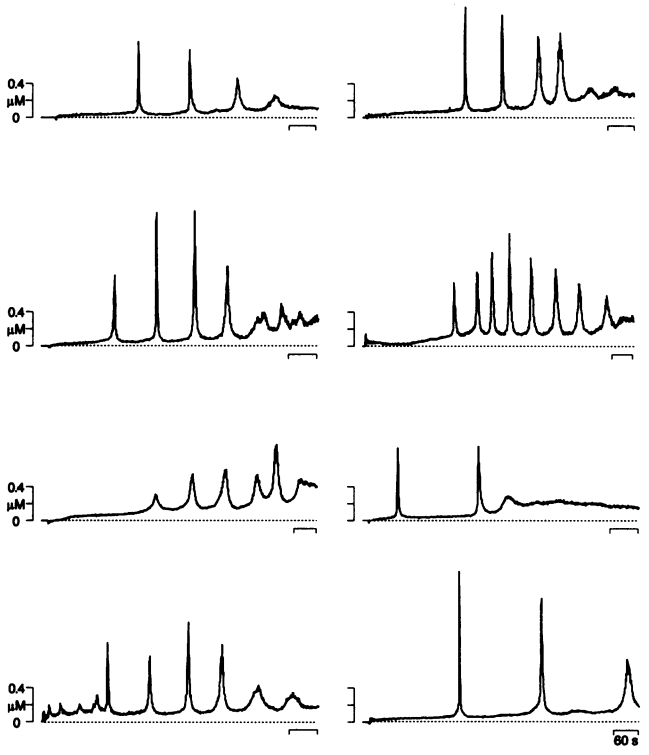

FIG. 2. Examples of calcium responses seen in the presence of 300 μM R_P-GTP[βS].

internal stores. (*ii*) InsP_3 and GTP[βS] appear to mobilize calcium from common pools because coapplication of a high concentration of InsP_3 with GTP[βS] abolished the R_P-GTP[βS]-induced calcium oscillations (Fig. 3*B*). In mast cells, InsP_3 releases calcium within a few seconds, so that usually only the falling phase of the release transient can be resolved. Of 13 cells examined with 10 μM InsP_3 and 300 μM R_P-GTP[βS], 10 responded with a fast initial calcium release and no subsequent transients, whereas the remaining three cells showed one to three small (<400 nM) elevations of $[Ca]_i$. (*iii*) Further evidence for the role of InsP_3 in the calcium spikes is provided by the inhibitory effects of heparin. Heparin is known to block InsP_3 effects by acting at the InsP_3 receptor (19). Fig. 3*C* shows an experiment done in the presence of 300 μM R_P-GTP[βS] and heparin at 250 μg/ml. At this or a higher dose, no changes in $[Ca]_i$ could be observed (eight cells). At lower doses, the calcium transients were reduced in peak amplitude relative to R_P-GTP[βS] controls (data not shown). (*iv*) In addition, R_P-GTP[βS]-loaded cells were stimulated with compound 48/80. When applied alone extracellularly, this agonist induces a transient increase in $[Ca]_i$ by activating inositol phospholipid turnover. When compound 48/80 was applied after the first or second R_P-GTP[βS]-induced calcium transient, it shortened the times between successive oscillatory transients (Fig. 3*D*). Application of compound 48/80 reduced the average spike interval of 100 ± 6 s (n = 126 spikes) to 68 ± 5 s (n = 28 spikes). Compound 48/80 did not increase the average number of transients per cell.

ATP Modulates the Oscillations. There was generally a long latency to the appearance of the first transient (202 ± 12 s for 300 μM R_P-GTP[βS], n = 52). The latency to the first transient did not correlate significantly with the average period of the following oscillations, which averaged 125 ± 9 s (41 cells). The transient oscillations usually damped out after 600–800 s.

The latency to the first transient as well as the frequency of the oscillations depended on the intracellular ATP concentration (Fig. 4). With no ATP present in the patch pipette, there was maximally one transient induced by 300 μM R_P-GTP[βS]. When 3.8 mM Mg-ATP was added to the pipette solution, the average number of transients increased to 6.4 ± 0.7 per 600 s, whereas the average latency decreased to 120 ± 21 s.

DISCUSSION

This study made use of the phosphorothioate analogues of GTP, GTP[βS] and GTP[αS], to examine G protein-mediated events in mast cells. It could be shown that the GTP analogues differ widely in their effects on $[Ca]_i$ and exocytosis in these cells. Of the α analogues, R_P-GTP[αS] resembles GTP[γS] in its stimulatory effects, inducing both calcium release and degranulation. S_P-GTP[αS], like GTP, is not stimulatory by itself but can support the GTP-dependent process of exocytosis.

These observations are consistent with earlier *in vitro* studies of G protein binding and activation. In one study (20), it was shown that the G protein of photoreceptors, transducin, bound both S_P-GTP[αS] and R_P-GTP[αS]. While S_P-GTP[αS] was hydrolyzed with a time constant comparable to GTP, R_P-GTP[αS] was hydrolyzed as slowly as GTP[γS]. In another study (21), it was found that R_P-GTP[αS] could activate G_s and a G protein in olfactory cells, G_{olf}, whereas S_P-GTP[αS] was inactive.

R_P-GTP[βS], on the other hand, selectively stimulated calcium release, while not inducing degranulation. The S_P isomer of GTP[βS] had similar, but weaker effects on $[Ca]_i$. This result suggests that R_P-GTP[βS] does not activate the G protein implicated in exocytosis, G_e. R_P-GTP[βS] is also ineffective in activating G_s, G_{olf}, and transducin (20, 21). These G proteins are, therefore, not likely to be targets of R_P-GTP[βS]. Together with the results from these studies, the observed effects on mast cells lead us to the hypothesis that R_P-GTP[βS] interacts selectively with G_p, the G protein that couples receptors to PLC.

Our data suggest that the inositol phospholipid pathway is involved in mediating the effects of R_P-GTP[βS]. The coapplication of a high concentration of InsP_3 presumably emptied a large portion of the InsP_3-releasable calcium pools (22).

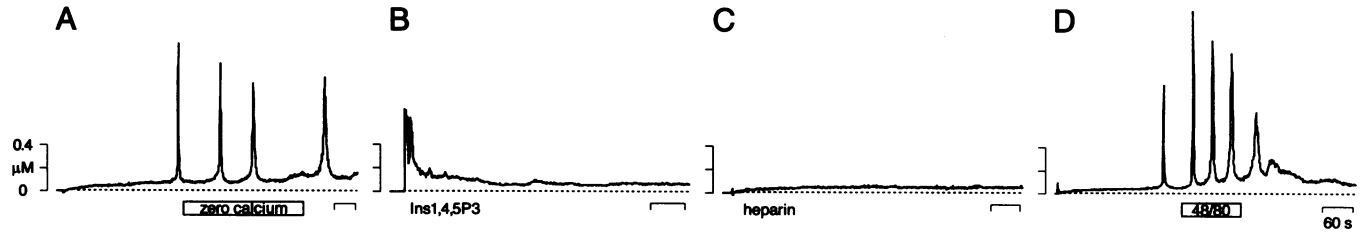

FIG. 3. Evidence for involvement of the PLC pathway in the oscillations. The R_P-GTP[βS] concentration was 300 μM. (*A*) Calcium-free mast-cell Ringer's solution/1 mM EGTA was applied from a puffer pipette during the time indicated by bar. (*B*) The pipette solution also contained 10 μM InsP_3. (*C*) Pipette solution also contained heparin at 250 μg/ml. (*D*) Application of compound 48/80 (5 μg/ml) from a puffer pipette during the time indicated by the bar.

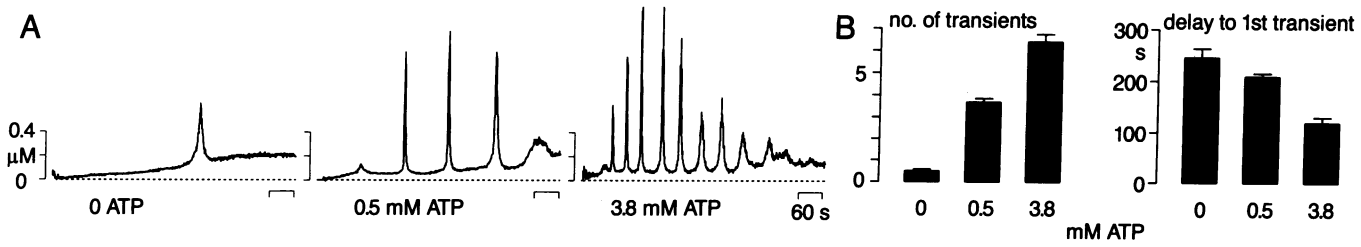

FIG. 4. Effect of internal ATP on oscillations induced by 300 μM R_P-GTP[βS]. (*A*) Left trace, no ATP in pipette; middle trace, 0.5 mM Mg-ATP added; right trace, 3.8 mM Mg-ATP added. (*B*) Dependence of the average number of transients per cell per 600 s and of the delay between cell penetration and first transient on the ATP concentration. The R_P-GTP[βS] concentration was 300 μM. Error bars represent the SEM of 5–45 cells.

The fact that this coapplication with R_P-GTP[βS] prevented the calcium oscillations is consistent with the idea that InsP_3 and R_P-GTP[βS] mobilize calcium from the same intracellular stores. This idea is further supported by the effects of heparin, which is known to inhibit the InsP_3 receptor and was found to prevent calcium oscillations in the present study.

The available evidence suggests that compound 48/80 causes calcium transients in mast cells by stimulating G_p (23). Because interactions of agonist–receptor complexes with G proteins promote the exchange of GTP for GDP (24), compound 48/80 might affect the oscillations by accelerating a step in the G protein cycle. This could also be the case if compound 48/80 interacts directly with G proteins, as has been suggested (23).

The calcium oscillations induced by R_P-GTP[βS] are reminiscent of those evoked by hormonal stimulation in a variety of nonexcitable cells (1). The models that have been suggested to account for repetitive calcium release invoke inositol phospholipid breakdown as a crucial step (25–27). Two mechanisms for generating calcium oscillations seem compatible with our results. (*i*) A constant, submaximal activation of G_p by GTP[βS] might generate low levels of InsP_3, which, together with appropriate cooperativity and feedback mechanisms, could result in cyclical calcium release (25, 26). The long latency to the first R_P-GTP[βS]-induced transient might point to such a model. The compound 48/80 effect would then be explained by an increased rate of InsP_3 formation.

(*ii*) Oscillatory calcium release could result from a cyclical generation of InsP_3 (27). Results in hepatocytes have implicated the activation of protein kinase C by diacylglycerol as the negative feedback step in this model (28). In our cells, however, preincubation with phorbol ester (phorbol 12-myristate 13-acetate, 100 nM, 30–360 min, 8 cells) had no obvious effect on the oscillations (data not shown).

The effects of ATP on the oscillations can be interpreted in several ways. No more than one calcium transient was evoked in the absence of ATP, showing that an ATP-dependent step is involved in the generation of the calcium oscillations. One such step would be the replenishment of $[Ca]_i$ stores by ATPase pumps. Enhanced calcium reuptake into releasable stores may thereby also affect the frequency of the oscillations. Besides reuptake, ATP may modulate release, as the InsP_3 receptor, like the ryanodine receptor in muscle, is thought to contain a modulatory ATP-binding site (29), and ATP has been found to dose-dependently increase InsP_3-mediated calcium release from intracellular organelles (30).

The modulation by ATP could also indicate that a phosphorylation/dephosphorylation reaction is involved in the generation of the calcium oscillations. One might speculate that R_P-GTP[βS], after getting hydrolyzed by G_p, is rephosphorylated by a G protein-associated nucleoside diphosphokinase. Results in liver (31) and platelet (32) membranes suggest the existence and agonist-dependent stimulation of such an enzyme. This scheme would entail that the hydrolysis product of R_P-GTP[βS], GDP[βS], is a substrate for that kinase. Although GDP[βS] has thus far been used as an inhibitor of cellular G proteins, recent evidence does suggest a stimulatory role of this analogue in fibroblasts (33). Preliminary experiments show that GDP[βS], in the presence of millimolar concentrations of ATP, induces calcium oscillations in mast cells (data not shown).

The evidence presented here shows a selective activation of calcium release by R_P-GTP[βS], whereas exocytosis, the other G protein-mediated process under investigation, is not activated by this GTP analogue. Our results suggest that R_P-GTP[βS] can stimulate inositol phospholipid turnover and calcium release from InsP_3-sensitive pools. The most obvious explanation of these findings is that R_P-GTP[βS] selectively activates G_p in mast cells. This G protein has so far remained elusive to biochemical characterization. R_P-GTP[βS] might be a valuable tool for clarifying its structure and function. By virtue of its selectivity towards G_p, it should be helpful in the study of calcium release and calcium oscillations.

We thank Erwin Neher and Michael Cahalan for helpful comments on the manuscript. This work was supported, in part, by the Deutsche Forschungsgemeinschaft (Grant 243/3-1).

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