The most abundant and widely expressed mammalian phosphoinositide kinase activity is contributed by phosphatidylinositol 4-kinase IIα (PI4KIIα). In this study we demonstrate that PI4KIIα is a novel GTP-independent target of the wasp venom tetradecapeptide mastoparan and that different mechanisms of activation occur in different subcellular membranes. Following cell membrane fractionation mastoparan specifically stimulated a high activity Golgi/endoosomal pool of PI4KIIα independently of exogenous guanine nucleotides. Conversely, GTPγS stimulated a low activity pool of PI4KIIα in a separable dense membrane fraction and this response was further enhanced by mastoparan. Overexpression of PI4KIIα increased the basal phosphatidylinositol 4-kinase activity of each membrane pool, as well as the mastoparan-dependent activities, thereby demonstrating that mastoparan specifically activates this isozyme. Both mastoparan and M7, at concentrations known to invoke secretion, stimulated PI4KIIα with similar efficacies, resulting in an increase in the apparent Vₘₐₓ and decrease in Kₘ for exogenously added PI. Mastoparan also stimulated PI4KIIα immunoprecipitated from the raft fraction, indicating that PI4KIIα is a direct target of mastoparan. Finally we reveal a striking dependence of both basal and mastoparan-stimulated PI4KIIα activity on endogenous cholesterol concentration and therefore conclude that changes in membrane environment can regulate PI4KIIα activity.

The wasp venom peptide mastoparan stimulates signaling by Gi and G0 heterotrimeric G-proteins by enhancing the rate of dissociation of bound GDP, thereby allowing GTP to bind. Increased Gi and G0 signaling then activates a variety of cell-type-dependent events, including secretion and ion transport. However the targets of mastoparan are by no means limited to heterotrimeric G-proteins. Mastoparan is also known to affect the activity of phospholipase D2 (1), Rho (2), nucleotide diphosphate kinase (3), calmodulin (4), glycogen phosphorylase (5), phospholipase A2 (6), p67-phox (7), and the type II phosphatidylinositol 4-kinase (PI4KII)² (8, 9).

PI4KIIα localizes to membranes of the Golgi-endoosomal system (10, 11) where it provides the phosphatidylinositol 4-phosphate (PI₄P) that is required to recruit the AP-1 clathrin adaptor complex to membranes of the trans-Golgi network (TGN) (11). Two membrane fractions containing PI4KIIα activity have been identified using sucrose density gradient ultracentrifugation (12) and found to possess differing levels of intrinsic kinase activity both in intact membranes and in immunoprecipitates prepared from detergent lysates (13). The higher activity pool is found in membranes that possess the raft-like properties of high buoyancy in sucrose density gradients and resistance to detergent solubilization (12–14). As reported by Barylko et al. (15), rafting of PI4KIIα is likely to arise from palmitoylation of a cysteine-rich sequence within the kinase domain (residues 174–178, CCPC), but whether or not a particular raft lipid environment is required for high PI4KIIα activity is not known. The lower activity pool is less buoyant and contains the bulk of the enzyme (12, 13). Previous studies of the activation of PI4KII by mastoparan have assumed a dependence on G-protein activation (8, 9); however experiments have hitherto not addressed whether mastoparan activates either or both enzyme pools nor whether activation of either pool by mastoparan can occur directly.

The established mechanisms by which mastoparan activates signaling involve the formation of an amphipathic α-helix from the random conformation present in free solution. The basic, helical peptide mimics the effector region of cognate G-protein-coupled receptors (16) and of calmodulin-binding proteins (17), leading to activation of Gₐα and calmodulin, respectively. Two distinct modes of binding have been identified, one in which the peptide binds only to the target protein and another in which the peptide binds concomitantly to a membrane and a target protein. For example, in aqueous solution mastoparan binds directly to p67-phox (7) and to calmodulin. Mastoparan binds to calmodulin in a helical conformation with low nanomolar affinity (18). In contrast binding to purified Gₐα-proteins is weak, but micromolar affinities are afforded by the presence of membrane lipids (19); this phenomenon arises from the fact that mastoparan adopts an amphipathic helical conformation at the lipid-aqueous interface (20, 21).

The mechanism by which mastoparan binds to PLD2, Rho, NDPK, glycogen phosphorylase, PLA2, and PI4KII has not yet been characterized; although NDPK, PLA2, and PI4KII may be activated indirectly, as a direct interaction with mastoparan has not been established. As with calmodulin and p67-phox, the interaction between mastoparan and Rho and between mastoparan and glycogen phosphorylase occurs in solution (5), whereas binding to PLD2, like the interaction between heterotrimeric G-proteins, has only been observed for the membrane-bound protein (1, 22).

This investigation began with the question of whether mastoparan and GTP regulate the two main pools of PI4KIIα activity via a common G-protein-dependent mechanism. Our results indicated that activation of PI4KIIα by mastoparan occurs via both GTP-dependent and GTP-independent mechanisms in different membrane fractions. We also found that GTP-independent activation of PI4KIIα in buoyant mem-
branes occurs directly. Finally we reveal for the first time that the PI4KIIα activity in buoyant membranes shows a striking dependence on cholesterol concentration. The results are discussed in terms of the role of rafting in the regulation of PI signaling.

EXPERIMENTAL PROCEDURES

Materials—G-protein agonists, pertussis toxin, wortmannin, and mastoparans were purchased from Calbiochem (Nottingham, UK). GDP, cGMP, and PI were bought from Sigma. Anti-PI4KII antiserum was prepared and purified as previously described by us (12). Anti-Gαi/1/2 and anti-Gαo were from Santa Cruz. [γ-32P]ATP was obtained from Amersham Biosciences. Dulbecco’s modified Eagle’s medium, fetal calf serum, and penicillin/streptomycin were purchased from Gibco. Dulbecco’s modified Eagle’s medium containing Glutamax, 10% fetal calf serum, 50 IU/ml penicillin, and 50 gg/ml streptomycin. Separation of A431 and HT1080 cell post nuclear supernatants on a continuous 10–40% (w/v) sucrose gradient was performed as previously described (13). The effects of M7 and GTPγS did not significantly affect the PI4K activity in the absence of G-protein activation, mastoparan (10 μM) was added to each membrane fraction in the absence of GTP. PI4K assays revealed that mastoparan elicited a highly reproducible 3–4-fold increase in PI4P generation in the more buoyant region of the gradient, which contained late endosomes, TGN and also a pool of invaginated membranes. The distributions of PI4K activity by GTPγS and GTPβS in denser membranes indicated the presence of a PI4KIIα/Gi/0-cyclodextrin (MβCD) complex at a ratio of 10:1 cholesterol:MβCD prepared as previously described (23).

RESULTS

Regulation of PI4KIIα by Mastoparan and GTP—The distribution of PI4P synthesis using endogenous PI as substrate was examined in subcellular fractions prepared from A431 cells and separated as previously reported on a 10–40% continuous sucrose gradient (12, 13). For the purposes of this study we concentrated on fractions 7–12 of the gradient, which contain all the PI4KII activity. As we previously reported, PI4KII activity was found to peak in the buoyant membrane fractions 8–10 (12, 13), which contain late endosomes, TGN and also a pool of activated PI4KIIα (12, 13). To investigate whether or not mastoparan (Fig. 1A) could enhance PI4KII activity in the absence of G-protein activation, mastoparan (10 μM) was added to each membrane fraction in the absence of GTP. PI4K assays revealed that mastoparan elicited a highly reproducible 3–4-fold increase in PI4P generation in the more buoyant region of the gradient that contained activated PI4KIIα (Fig. 1, B and C) but had little effect on denser fractions that contained the bulk of PI4KIIα and PI4KIIβ protein (12, 13). The M7 analogue of mastoparan (Fig. 1A) is a more potent activator of Gβγ-proteins (24) but gave a similar amount of activation to mastoparan. In contrast to the extra cellular fraction of the peak of PI4K activity by mastoparan, the addition of GTPγS (100 μM) did not significantly affect the PI4K activity in this region of the gradient (Fig. 1C). However GTPγS did enhance PI4P generation in denser fractions, which contained secretory vesicles, plasma membrane, rough endoplasmic reticulum, lysosomes, and PI4KIIβ (12, 13). Compared with the mastoparan response, the stimulatory effect of GTPγS was found to be more variable (range 0.2–2-fold) and labile, as it was lost more rapidly on storage than the mastoparan responsiveness. Consequently, in response to the starting question concerning the mechanism by which PI4KIIα is regulated by mastoparan and GTP, these data indicate that different modes of regulation occur in different subcellular membranes.

Activation of PI4KIIα in buoyant membranes by mastoparan in the absence of exogenous GTP suggested that the previously assumed mediators of PI4KII regulation by mastoparan, namely the G12 heterotrimeric G-proteins (8, 9) and Rho family small G-proteins (8), are not in fact required in these membranes. In contrast the stimulation of PI4KII activity by GTPγS in denser membranes indicated the presence of a G-protein-activated PI4KII activity in this region of the gradient, which was maintained in the presence of wortmannin (Fig. 1D). Unexpectedly however, co-addition of M7 and GTPγS gave rise to a level of PI4K activation in fraction 12 that was only equivalent to the sum of the responses observed when each reagent was added independently (Fig. 1D). The effects of M7 and GTPγS on PI4KII in fraction 12 did not
levels of PI4P generation were reduced as would be expected (25, 26) (data not shown). Furthermore GDPβS, a potent G-protein antagonist did not affect mastoparan-dependent PI 4-kinase activity (Fig. 2), thereby confirming G-protein-independent activation in the buoyant fraction.

Overexpression of PI4KIIα Leads to Increased Basal and Mastoparan-stimulated Activity—To verify that mastoparan was activating the PI4KII isoforms and not activating a secondary pool of the PI4KIIβ enzyme or inhibiting a PI4P phosphatase, we investigated the effect of increasing the expression of this enzyme. HT1080-PI4KIIα cells stably expressed a GFP-PI4KIIα fusion protein and were chosen because they afforded a measurable increase in PI4KII expression without the disruption of the Golgi body that has been observed in other cells (11) or any measurable change in Gαo levels as shown by Western blotting (Fig. 3A). The level of GFP-PI4KIIα in these cells was typically 2–4-fold higher than endogenous PI4KII as determined by Western blotting with anti-PI4KIIα antisera (Fig. 3B). If mastoparan did activate PI4KIIα we expected to find increases in the control and mastoparan-stimulated activities. There was indeed an increase in the effects of mastoparan on the profile of PI4K activity in subcellular fractions obtained from these cells, and typically, HT1080-PI4KIIα cells had a 2–3-fold increase in the peak of PI4K activity relative to non-transfected cells. Furthermore, in line with our previous reports using different epithelial cell lines (12, 13), the peak of PI4K activity in this fibrosarcoma cell line was almost completely inhibited by the monoclonal antibody 4C5G but insensitive to wortmannin, thereby confirming the activity as PI4KII (Fig. 3C). As observed with A431 cells, addition of either mastoparan or its M7 analogue (10 μM) to membranes prepared from HT1080 or HT1080-PI4KIIα cells resulted in a similar 3–4-fold increase in the peak of PI4K activity (Fig. 3D). The observed changes in activity in response to mastoparan were therefore increased by PI4KII overexpression (Fig. 3E), thereby confirming that mastoparan and M7 stimulate PI4KIIα activity. Finally, with the basal activity, the mastoparan response was inhibited by the 4C5G monoclonal antibody (Fig. 3F).

Lack of Synergy between Mastoparan and Guanine Nucleotides—Although Gα10 proteins have previously been reported to stimulate PI4K activity (8, 9), G-protein-independent activation by mastoparans was a
novel finding. Subsequent experiments therefore focused on the effect of mastoparan on the buoyant, activated pool of PI4KIIα/H9251, which localizes to fractions enriched in late endosomal and TGN markers. We first returned to investigate more closely whether there was any evidence at all for synergy between mastoparan and guanine nucleotides by taking advantage of the higher activities observed with the HT1080-PI4KIIα/H9251 cells. We found that addition of GTPS (100 μM), GTP (100 μM), GDP (10 μM), or cGMP (10 μM) either on their own or in the presence of mastoparan had a slight inhibitory effect on PI4P generation by buoyant membranes (Fig. 4A). However the apparent inhibition by guanine nucleotides was found not to be statistically significant when assessed in further experiments. These results confirmed that neither GTP nor its metabolites are required for the observed stimulation of the PI4KII activity in buoyant membranes by mastoparan. Moreover, preincubation of cells with pertussis toxin had no apparent effect on the stimulation of PI4K activity by mastoparan in the buoyant membranes (Fig. 4B). However similar to heterotrimeric G-protein activation (19), the M17 analogue (Fig. 1A) did not significantly enhance PI4K activity (Fig. 3F).

Quantification: Mastoparan Dose Response and PI4KIIα Enzymatic Activity in Buoyant Membranes—Dose responses to both mastoparan and M7 were obtained for the PI4K activity in buoyant membranes prepared from HT1080-PI4KIIα cells (Fig. 4C). We found that mastoparan and M7 stimulated this PI4KIIα activity with EC₅₀ values of 12.9 ± 3.1 μM (n = 3) and 7.2 ± 4.1 μM (n = 3), respectively. The similar potencies of mastoparan and M7 observed in this study provide further evidence that these peptides do not stimulate PI4KIIα via Gᵢ₁₀ activation. It is also
guanine nucleotides were not significantly different. Responses to either mastoparan alone or co-addition of mastoparan with any of the toxin (100 ng/ml).

Quantitation of the GTP-independent stimulation of PI4KIIα by mastoparan. PI4P production in peak PI4KIIα-activity membrane fractions from HT1080 cells in the presence (●) and absence (■) of mastoparan (10 μM) in response to increasing concentrations of exogenously added PI. Data are representative of three separate determinations.

Activity Is Dependent on Cholesterol Concentration

The effects of mastoparan on the enzymatic properties of the activated pool of PI4KIIα were also quantified. By varying the concentration of exogenously added PI (0–200 μM) we were able to quantify changes in the rate of PI4P production by PI4KIIα in intact membranes (Fig. 5). In the presence of mastoparan (10 μM), the apparent Vmax for the reaction was enhanced 3.6-fold (545 ± 39 PhosphorImager units/h for control membranes versus 1967 ± 171 PhosphorImager units/h in the presence of mastoparan). The apparent Km for PI was also affected significantly by mastoparan (9.8 ± 3.1 μM for control membranes versus 1.7 ± 0.3 μM in the presence of mastoparan). Therefore, mastoparan appeared to enhance the rate of PI4P production at saturating and sub-saturating concentrations of PI.

Mastoparan Activates PI4KIIα Isolated from Membrane Rafts—The different responses of PI4KIIα in buoyant and dense membrane pools indicated that either a modification of the enzyme itself or a secondary molecule was localized to the buoyant membranes. Indeed M7 (50 μM) failed to elicit any increase in the kinase activity of a bacterially expressed GST-PI4KIIα fusion protein over the range of PI concentrations shown in Fig. 6A, supporting the notion that activation of PI4KIIα by mastoparan requires either a particular modification of PI4KIIα itself (direct activation) or a particular membrane environment (indirect activation). These assays required the presence of Triton X-100 because the recombinant enzyme was otherwise completely inactive. In contrast PI4KIIα immunoprecipitated from the buoyant fraction was active in the absence of Triton X-100 and was stimulated by mastoparan (Fig. 6B). In the presence of Triton X-100 the basal activity was enhanced over 10-fold, and no response to mastoparan was detectable under these conditions.

PI4KIIα Activity Is Dependent on Cholesterol Concentration—The presence of PI4KIIα and PI in buoyant membrane rafts has been inferred from several previous studies (13, 14, 28, 29). To establish whether or not the organization of PI4KIIα and PI in rafts is required for the observed regulation by mastoparan, the effects of cholesterol depletion were investigated. Fig. 7A illustrates that treatment of the buoyant membrane fraction with MβCD, which selectively removes endogenous cholesterol from membranes, caused a drastic inhibition of mastoparan-stimulated PI4KIIα activity (IC50 1.9 ± 0.1 mM, n = 3). However proportionally similar amounts of inhibition by MβCD were also observed using unstimulated preparations (IC50 4.2 ± 0.1 mM, n = 3).
Regulation of PI4KIIα

and using endogenous or exogenous PI substrate, indicating that cholesterol is a direct regulator of the enzyme itself rather than operating via a secondary mastoparan target or PI. This idea was further substantiated by using cholesterol-loaded MβCD to replenish cholesterol in membranes. In these experiments the inhibition caused by cholesterol depletion was reversed (Fig. 7 B). Furthermore cholesterol also seemed to augment the basal level of PI4P synthesis indicating that cholesterol levels may regulate the concentration of PI4P in rafts.

**DISCUSSION**

The regulation of cellular PI4P synthesis is of central importance to many events in the cell including the provision of PI4P for phospholipase C signaling, formation of secretory vesicles from the TGN and endocytosis. The major PI4P biosynthetic activity in most mammalian cells is provided by PI4KIIα. In this study we show that the amphiphilic peptides mastoparan and M7 can activate a pool of PI4KIIα associated with a buoyant subcellular fraction enriched in TGN-derived vesicles, late endosomes, and p97/valosin-containing protein-rich endoplasmic reticulum (12). Although mastoparan has been shown previously to enhance PI4KII activity in preparations of secretory vesicles (8, 9), in these studies the authors concluded that mastoparan was acting to regulate PI4KII through G11α activation. Despite this conclusion, in these previous studies there was no reported addition of guanine nucleotides with the mastoparan, a condition that would be necessary if mastoparan was acting as a promoter of GTP exchange on the α-subunit of G11α. The results presented here demonstrate that the mastoparan and M7 stimulation of PI4KIIα can occur completely independently of G-protein activation. In addition, GTP, GTPγS, nor the metabolites GDP and cGMP affected PI4KII activity on membranes either on their own or in conjunction with mastoparan. Furthermore, preincubation of cells with pertussis toxin, a bacterial enzyme that inactivates the α-subunit of G11α through ADP-ribosylation, also had no affect on PI4KII activity in this membrane fraction. These results demonstrated that mastoparan does not activate PI4KIIα on late endosomal and TGN membranes via G11α activation.

In contrast to the behavior of the activated pool of PI4KIIα present in buoyant membranes, a weakly GTP-sensitive PI4K activity was associated with a dense membrane fraction that contained plasma membrane, rough endoplasmic reticulum, and secretory vesicles. It is important to stress that the wortmannin insensitivity of the mastoparan and GTPγS responses allowed contributions by G-protein-dependent type III PI 4-kinases that are wortmannin-sensitive (30) to be excluded. However we have shown previously that this gradient fraction is enriched in PI4KIIβ (13); hence the GTP-sensitive response could represent an effect on this particular isoform. Consistent with this view Wei et al. (31) have shown that PI4KIIβ can be regulated by the small G-protein Rac. Nonetheless, overexpression of PI4KIIα increased the GTP-independent and GTP-dependent response across the whole density gradient, indicating that the PI4KIIα isozyme is predominately responsible for the observed GTP-dependent activity in dense membranes. The unexpected inability of M7 to induce a synergistic activation of the GTPγS response in this region of the gradient (fraction 12), suggests that in dense membranes the mastoparan and GTP-dependent responses occur via biochemically independent pathways.
Analysis of the dose-responses for mastoparan and M7-stimulated PI4P synthesis by the active PI4KIIα fraction also points to the importance of a mechanism other than Gβγ activation. It is important to note that the EC₅₀ values for mastoparan and M7 (12.9 ± 3.1 and 7.2 ± 4.1 µM, respectively) are also in the physiological range reported for mastoparan-induced secretion obtained in other studies, an effect that had been attributed to heterotrimeric G-protein activation (27).

We found that both the maximal responses and EC₅₀ values for mastoparan and M7 are not significantly different. This is not the case for Gβγ regulation, where the EC₅₀ for M7 is 4-fold lower than for mastoparan and where maximal stimulation is twice that of mastoparan. M7 has one less positive charge than mastoparan because the Lys-Ile residues at positions 12 and 13 in mastoparan are substituted by Ala-Leu in M7. For less positive charge than mastoparan because the Lys-Ile residues at positions 12 and 13 in mastoparan are substituted by Ala-Leu in M7. For

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Consequently, it is possible that the observed increased efficacy of mastoparan stimulation in the TGN/late endosomal fraction may require the presence of PI4KIIα-rich lipid rafts on the surface of these organelles.

Finally, although PI4KIIα is intrinsically activated in the buoyant membranes, presumably via one or more specific covalent modifications, we have found a striking dependence of kinase activity on the endogenous cholesterol level. The virtual ablation of activity following cholesterol depletion indicates that PI4KIIα activity is critically dependent on its immediate membrane environment. This observation is consistent with the existence of PI4KIIα in cholesterol-rich rafts, and with studies on intact cells in which decreased Pip levels were found in buoyant membrane fractions prepared after treatment of A431 cells with MβCD (33) or after treatment of Madin-Darby canine kidney cells with filipin (34). These results lead to the exciting hypothesis that cholesterol, which is known to affect vesicle trafficking in the Golgi-endosomal system, is a physiological regulator of PI4KIIα activity.

ACKNOWLEDGMENT—We acknowledge the helpful advice received from Dr Nicholas Beaumont.

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