Mas-7, a mastoparan derivative, induces elevation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) along two independent pathways. The minor contribution occurs via phospholipase C activation and is negatively regulated by treatment with phorbol 12-myristate 13-acetate, a protein kinase C activator. The major contribution involves plasma membrane pores allowing not only Ca\(^{2+}\), Mn\(^{2+}\), and Na\(^+\) to enter but also the uptake of ethidium bromide (314 Da) and lucifer yellow (457 Da), but not fura-2 (831 Da), Evans blue (961 Da), and fluorescein-conjugate phalloidin (1,175 Da). Mas-7-induced current, as measured in planar lipid bilayers, reveals that Mas-7-induced pores have two slope conductances, 290 and 94 pS, and that the pores are nonselective for cations. The results also indicate that Mas-7 can produce pores by direct interaction with the plasma membrane without the involvement of membrane proteins and cytosolic factors. Besides in human neuroblastoma cells, similar Mas-7 effects were also observed in other cell lines such as HL-60, 1321N1 human astrocytoma, and bovine chromaffin cells. The data suggest that the Mas-7-induced [Ca\(^{2+}\)]\(_i\) elevation is the combined result of Ca\(^{2+}\) release from stores via phosphoinositide turnover and prolonged Ca\(^{2+}\) influx through membrane pores.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbamylcholine (carbachol) chloride, thapsigargin, (±)-sulfinpyrazone, Triton X-100, EGTA, EDTA, Trizma base, trichloroacetic acid, bovine serum albumin, and IP\(_3\) were obtained from Sigma. Phorbol 12-myristate 13-acetate (PMA) and 4-α-PMA were purchased from Research Biochemicals Inc. (Natick, MA). \(^{3}H\)IP\(_3\) was obtained from DuPont NEN, and fura-2/AM was purchased from Molecular Probes (Eugene, OR). Mas-7 and Mas-17 were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Cell Culture**—Human neuroblastoma clone SK-N-BE(2)C, human promyelocytic leukemia HL-60, and human astrocytoma 1321N1 cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated bovine calf serum (HyClone, Logan, UT) and 1% antibiotics (Life Technologies, Inc.) in a humified atmosphere of 0.5% CO\(_2\).
Facilitation of Membrane Pores by Mas-7

RESULTS

PMA-sensitive and -insensitive Effects on the $[\text{Ca}^{2+}]_i$—The ability of Mas-7 to alter $[\text{Ca}^{2+}]_i$ in SK-N-BE(2)/C neuroblastoma cells was examined using the fluorescent Ca$^{2+}$ indicator fura-2. We found that, in the presence of extracellular Ca$^{2+}$, Mas-7 caused a sustained elevation of the cytosolic Ca$^{2+}$ concentration, as illustrated in Fig. 1A. A high concentration of Mas-7 (50 $\mu$M) increased the $[\text{Ca}^{2+}]_i$, rapidly (Fig. 1A), while treatment with a submaximal concentration (20 $\mu$M) of Mas-7 led to a slow increase in $[\text{Ca}^{2+}]_i$ (data not shown). Interestingly, the Mas-7-induced response was partially inhibited by pretreatment with PMA, the PKC activator. Addition of Mas-17, an analog of mastoparan that does not activate G-proteins (34), was ineffective in raising the $[\text{Ca}^{2+}]_i$. In the absence of extracellular Ca$^{2+}$, exposure of the cells to Mas-7 resulted in a transient increase in $[\text{Ca}^{2+}]_i$, which was completely abolished by PMA pretreatment (Fig. 1B). The concentration dependence of the $[\text{Ca}^{2+}]_i$ rise induced by Mas-7 with or without PMA treatment is presented in Fig. 1C. In the presence of extracellular Ca$^{2+}$, maximal effective concentrations of Mas-7 raised the $[\text{Ca}^{2+}]_i$ to over 1600 nm from a resting level of ~80 nm. The maximal and half-maximal ($E_{50}$) effective concentrations were 80 ± 15 $\mu$M and 28 ± 6 $\mu$M, respectively. It is noteworthy that, under these conditions, the elevated $[\text{Ca}^{2+}]_i$ was sustained at all concentrations of Mas-7 tested. 4-40-PMA, the inactive analog of PMA, did not inhibit
the Mas-7-induced [Ca^{2+}]_{i} rise, indicating that the effect is mediated by PKC (data not shown).

We performed a similar analysis with regard to the effect on IP_{3} generation. We found that the peak IP_{3} generation is obtained 1 min after the addition of Mas-7 and that the effect is maintained for more than 20 min (Fig. 2A). Mas-17 was ineffective in eliciting IP_{3} production. Fig. 2B shows that Mas-7 produced IP_{3} in a concentration-dependent manner, the maximal response and half-maximal response being obtained at 78 ± 25 and 28 ± 7 μM, respectively. The IP_{3} generation in response to variations in Mas-7 concentration occurred in a pattern similar to that observed for the [Ca^{2+}]_{i} rise. However, removal of extracellular Ca^{2+} decreased the Mas-7-induced IP_{3} production, and PMA treatment completely blocked the response. These data suggest that Mas-7 induces [Ca^{2+}]_{i} rise and IP_{3} production and that PMA treatment inhibits both responses. However, PMA treatment inhibits the [Ca^{2+}]_{i} rise and the IP_{3} production only partially in the presence of extracellular Ca^{2+} (Figs. 1C and 2B), while in the absence of extracellular Ca^{2+}, it does so completely (Fig. 1B and 2B).

**Ca^{2+} Influx Induced by Mas-7 in Thapsigargin-treated Cells**—To look into the mechanism of the [Ca^{2+}]_{i} rise during Mas-7 stimulation, we depleted the internal Ca^{2+} stores by pretreating the cells with thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPases. Incubation of the cells with 1 μM thapsigargin for 10 min increased [Ca^{2+}]_{i}, and then the [Ca^{2+}]_{i} was slowly decreased and sustained to a slightly elevated level as we previously demonstrated (47). The above thapsigargin treatment abolished a subsequent [Ca^{2+}]_{i} rise induction by carbachol treatment (Fig. 3A), indicating that the IP_{3}-sensitive Ca^{2+} stores were depleted by the thapsigargin treatment. However, addition of Mas-7 after the thapsigargin treatment pro-

**Fig. 1. Effect of Mas-7 on [Ca^{2+}]_{i} rise.** Human neuroblastoma SK-N-BE(2)C cells were loaded with fura-2 as described under “Experimental Procedures.” Mas-7-induced [Ca^{2+}]_{i} rise was monitored in the presence (panel A) or absence (panel B) of extracellular Ca^{2+} after the cells were treated with vehicle or 1 μM PMA for 5 min. A, 50 μM Mas-7 and 50 μM Mas-17 were added at the time point indicated by the arrowhead. B, 20 μM Mas-7 was added. C, Concentration-dependent effect of Mas-7 (○ and ●) or Mas-17 (▲) on the peak elevation of [Ca^{2+}]_{i} measured in the cells incubated with vehicle (□) or PMA (●). Basal level (□) is presented. Data are the means ± S.E. of four independent experiments.

**Fig. 2. Time course and concentration dependence of IP_{3} formation.** A, SK-N-BE(2)C cells were stimulated with 50 μM Mas-7 (○) and 50 μM Mas-17 (●) for the designated times. B, variable concentrations of Mas-7 were applied for 1 min to vehicle-treated (○, △) or PMA-treated cells (●, ▲) in the presence (circle) or absence (triangle) of extracellular Ca^{2+}. The basal level (□) is presented. The reaction was stopped by treatment with 15% TCA. IP_{3} production was measured by competition assay as described under “Experimental Procedures.” Three independent experiments showed similar results, and the values shown are means ± S.E.
trigger the Mn$^{2+}$ quenching effect at all (Fig. 4B). The concentration dependence of the Mas-7 effect seen in Fig. 4C shows that maximal and half-maximal stimulation of the Mn$^{2+}$ influx occurs at 72 ± 12 and 24 ± 5 μM Mas-7, respectively.

To test the selectivity for Ca$^{2+}$ in the Mas-7-induced ion current, Na$^{+}$ influx upon Mas-7 treatment was measured in cells loaded with SBFI/AM, a Na$^{+}$-specific fluorescent dye. The treatment of cells with Mas-7 resulted in an increase of the [Na$^{+}$], in the SBFI-loaded cells (Fig. 5), indicating that Mas-7 induces the influx of Na$^{+}$ in addition to that of Ca$^{2+}$. The Mas-7-induced influx of Na$^{+}$ was also sustained, similar to the [Ca$^{2+}$], rise. Mas-17 did not change the [Na$^{+}$] (data not shown). Also, the Mas-7-induced influx of Na$^{+}$ was not obtained when extracellular Na$^{+}$ was substituted with choline (dotted trace in Fig. 5). Monensin, a Na$^{+}$-specific ionophore, was used as a positive control in the test of the [Na$^{+}$], elevation. These results reflect the ability of Mas-7 to regulate membrane permeability for a variety of ions.

**Formation of Membrane Pores by Mas-7**—To test whether Mas-7 makes membrane pores, we examined the effect of Mas-7 on the uptake of EtBr. Fig. 6A shows that submaximal concentration (30 μM) of Mas-7 evoked rapid uptake of EtBr (314 Da). Digitonin, as a positive control, also induced EtBr uptake. The maximal effective concentration (80 μM) increased the fluorescence intensity to the digitonin-induced level (data not shown). The concentration dependence, as seen in Fig. 6B, strongly supports the conclusion that membrane pores are formed upon Mas-7 stimulation.

To assess the pore size formed by Mas-7, we measured the permeability of various fluorescent dyes with different molecular weights. Mas-7 induced the uptake of lucifer yellow (457 Da) in a concentration-dependent manner with maximal and half-maximal concentrations seen at 75 ± 18 and 22 ± 5 μM, respectively (data not shown). However, Mas-7 did not induce the uptake of fura-2 (831 Da), Evans blue (961 Da), and fluo-rescein-conjugated phalloidin (1,175 Da) even at the maximal effective concentration of 80 μM Mas-7 (data not shown). These results indicate that Mas-7 forms specific pores permeable to molecules at least up to 457 Da in size.

**Mas-7 Forms a Channel in a Planar Lipid Bilayer**—The formation of membrane pores by Mas-7 was further investigated in a planar lipid bilayer in an effort to explain the entry...
of various ions and molecules into Mas-7-treated cells. Fig. 7A shows the planar bilayer recordings of Mas-7-induced channels in the cis solution containing Locke’s solution and in the trans solution containing the modified Locke’s solution with high K\(^+\). In three separate experiments, we observed two types of channels with different conductances. The current-voltage relationships of these two channels appeared linear with little voltage dependence, and the slope conductances were 290 and 94 pS (Fig. 7B). The arrows in Fig. 7A represent the current level contributed by both the closed state of the large conductance channel and the open state of the small conductance channel. The open probability of the 290 pS channel was 0.25 (\(n = 3\)), however, the small conductance channel remained open most of the time and its closure is marked with an asterisk.

**Mas-7-mediated Pore Formation in Various Cells**—We also looked at the effect of Mas-7 on \([Ca^{2+}]\), rise and EtBr uptake in other cell types beside SK-N-BE(2)C. We tested the Mas-7 effect also on HL-60, 1321N1 human astrocytoma, and bovine chromaffin cells. As Fig. 8 shows, Mas-7 increases the \([Ca^{2+}]\) in the presence of extracellular \(Ca^{2+}\) and EtBr uptake in all the tested cells. This suggests that Mas-7 regulates membrane permeability by a mechanism common to a variety of cell types.

**DISCUSSION**

Mastoparan has been widely used to prime G-protein activation in a receptor-independent manner with various cell types (9, 17, 30, 48). Although the priming mechanism is unclear, this agent appears to activate phosphoinositide-specific PLC via G-protein and to produce inositol phosphate (49, 50). To further characterize cytosolic calcium elevation induced by mastoparan, we applied Mas-7, a highly active form of mastoparan (34), to human neuroblastoma SK-N-BE(2)C and other cell types. The treatment of SK-N-BE(2)C cells with Mas-7 evoked IP\(_3\) production and \([Ca^{2+}]\) rise in a concentration-dependent manner. Both responses increased gradually and stayed steady for a long time. In the [Ca\(^{2+}\)]\(_{i}\) rise induced by Mas-7, the major event was a prolonged Ca\(^{2+}\) influx from extracellular spaces while a minor contribution came from a transient IP\(_3\)-sensitive Ca\(^{2+}\) release from intracellular stores.

It has been suggested that IP\(_3\) can directly activate plasmalemmal Ca\(^{2+}\)-conductive channels in lymphocytes (51). However, in the cells we tested, such channels do not appear to contribute significantly to the Mas-7-generated [Ca\(^{2+}\)]\(_{i}\) rise since the inhibition of IP\(_3\) production by treatment with PMA had little effect on blocking the [Ca\(^{2+}\)]\(_{i}\) rise. In addition, thapsigargin, the agent used to deplete internal Ca\(^{2+}\) stores, has also little effect on the Mas-7-induced [Ca\(^{2+}\)]\(_{i}\) rise. These results indicate that depletion of Ca\(^{2+}\) stores is not the main cause for the Ca\(^{2+}\) permeability and that most of the Ca\(^{2+}\) entry is not from a capacitative entry pathway through Ca\(^{2+}\)-release activated channel (52). Instead, our experiments with EtBr and fluorescent dyes suggest that Ca\(^{2+}\) influx from the extracellular space occurs through membrane pores. These pores are permeable to Na\(^+\), as well as Ca\(^{2+}\) and Mn\(^{2+}\), and also to EtBr and lucifer yellow. Previous studies suggested that the enhancement of membrane permeability by mastoparan might possibly be evoked by a cytotoxic mechanism independent of G-protein activation (53). Our findings indicate that this permeability is not a nonspecific membrane perturbation since the pores are not permeable to bigger molecules such as fura-2 (831 Da). In addition, removal of Mas-7 allowed the cells to restore their responsiveness to a subsequent muscarinic stim-
ulation, indicating that the action site of Mas-7 is the plasma membrane rather than the internal membrane. Mas-17, which has an amino acid composition almost identical to Mas-7 except for two amino acids, has no effect in the formation of membrane pores. The data, therefore, suggest that Mas-7 forms membrane pores by a specific process and that a specific sequence in Mas-7 is essential for the interaction with the membrane.

The formation of membrane pores by Mas-7 was demonstrated in a lipid bilayer. The Mas-7-induced channels appeared to be of two types with slope conductances of 290 and 94 pS when we used the Locke’s solution in cis and modified Locke’s solution containing high K⁺ and low Na⁺ in trans to mimic the extracellular and intracellular ion composition. These two channels seemed to behave independently because we were able to observe only small conductance channels in four experiments. The reversal potentials of these two types of channels were observed at near 0 mV, suggesting that the conductance was not contributed by the passage of a single kind of cation. These results support the hypothesis that the Mas-7-induced channels are nonselective for cations. Although we still have to explain the permeability of the Mas-7-induced channels to large molecules, such as EtBr and lucifer yellow, the large conductance channel is likely to form a large pore that could allow these molecules to enter. The situation is reminiscent of the ryanodine receptor of muscle sarcoplasmic reticulum, which has a comparable conductance under similar ionic conditions and can let a molecule of the size of glucose pass through (54). The results clearly show that the partitioning of Mas-7 into the lipids is sufficient to make membrane pores without involvement of membrane protein and cytosolic components.

Previous studies suggested that mastoparan forms α-helical structures that are highly amphiphilic in a lipid environment, with hydrophobic residues within the lipid bilayer, and with hydrophilic, positively charged residues facing outward (27, 28, 55). Our results indicate that the alignment of mastoparan makes pores in the membrane permeable to molecules of a specific molecular size although the mechanism of pore formation remains unclear. Recently, maganin, an antimicrobial peptide isolated from frog skin, has been found to form α-helical structures and pores in membranes, thus altering the permeability of the pores by the aggregation of a number of transmembrane peptide subunits within the bilayer (56). The effect of small peptide fragments on the lipid bilayer conductance was also explored with certain antibiotics, such as alamethicin and monazomycin, derived from fungi. Here, water-filled pores spanning the bilayer were formed via the aggregation of variable numbers of individual molecules (57, 58). These reports suggest the possibility that mastoparan too may form pores by a mechanism similar to that of the peptide fragments.

The cation permeability of the Mas-7-induced pores indicates that this bilayer conductance does not lend itself to the simple speculation that anions are favored due to the high density of positive charges lining the pore. Rather, the phenomenon is similar to that of maganin, peptide fragments of the sodium channel, and other peptide antibiotics (56, 59). All these peptides are positively charged but generate cation-selective channels when incorporated into artificial lipid bilayers. It has also been reported that the presence of the negatively charged lipid phosphatidylycerine is important to the peptides forming the pores in the bilayer (60). Interestingly, mastoparan is also known to penetrate into membranes that contain mainly acidic phospholipids (32). The maximal responsive concentration of Mas-7 differs from cell type to cell type, indicating cell-specific variations in the Mas-7-induced permeabilization. The difference may reflect variations in the affinity of Mas-7 for each particular cell membrane and/or the lipid composition of the plasma membranes.

The maximal and half-maximal responsive Mas-7 concentrations for the [Ca²⁺]i rise, the Mn²⁺ influx, the IP3 generation, and the uptake of EtBr and lucifer yellow are almost the same. The results indicate that Mas-7 is of similar potency with regard to G-protein activation as well as pore formation. However, the Mas-7-induced IP3 production and [Ca²⁺]i rise are dramatically decreased in the absence of extracellular Ca²⁺. This finding may reflect a Ca²⁺ requirement for the interaction of Mas-7 with the plasma membrane or for the activation of PLC. Removal of external Ca²⁺ is expected to result in a stoppage of Ca²⁺ influx, which then would in turn block the phosphoinositide turnover. This interpretation does not, however, rule out an additional direct modulatory effect of Ca²⁺ on Mas-7-mediated G-protein activation. On the contrary, in human neutrophils the G-protein modulating and membrane disrupting activities of mastoparan appear to be separate and not to effect each other (53).

The experiments with PMA suggest that PKC activation reduces the effectiveness of Mas-7. This inhibitory effect of PKC may be a result of a reduction in Mas-7-induced IP3 generation. Since the depletion of the internal Ca²⁺ stores after treatment with thapsigargin abolishes the inhibitory effect of PKC on the Mas-7-induced [Ca²⁺]i rise and since the Mas-7-induced Ca²⁺ release is blocked by PMA treatment, the regulation of Mas-7-induced [Ca²⁺]i rise by PKC may be limited to PLC signaling. This is even more strongly supported by the fact that the Ca²⁺ influx from the extracellular space via membrane pores formed by Mas-7 is not effected by PMA treatment.

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