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Different Modes of Regulation for Receptors Activating Phospholipase C in the Rat Pancreatoma Cell Line AR4-2J

FRANK S. MENNITI, HARUO TAKEMURA, KERRY G. OLIVER, and JAMES W. PUTNEY, JR.

Calcium Regulation Section, Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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SUMMARY

The inositol phosphate responses to substance P, bombesin, cholecystokinin, and the muscarinic cholinergic agonist methacholine were examined in the rat pancreatoma cell line AR4-2J. It was found that each agonist produced a distinct temporal pattern of inositol phosphate formation. Furthermore, these different response patterns resulted, at least in part, from different patterns of homologous receptor desensitization. The response to substance P desensitized rapidly and completely within 90 sec. After a 10–15-min refractory period, the response recovered with a $t_{1/2}$ of approximately 1 hr. The response to methacholine also completely desensitized. However, in this case desensitization developed slowly over the course of 40 min, and no recovery of responsiveness was detected for up to 45 min after the cessation of stimulation. The inositol phosphate responses to bombesin and cholecystokinin were similar to one another and appeared to be composed of two phases. Initially, there was a robust activation of phospholipase C. This initial phase was followed within 20 sec by a second phase of lesser magnitude. For bombesin, attenuation of the initial phase was due to rapid, but only partial, desensitization of the response. Furthermore, the concentration of bombesin required to maintain the second phase of the response was about 100-fold lower than that required to maximally activate the initial phase of the response. These results may indicate multiple mechanisms for the regulation of different phospholipase C-linked receptors in this cell line.

It is now well established that stimulation of certain cell surface receptors is transduced to the cell interior via the activation of phospholipase C, resulting in the generation of two intracellular messenger cascades. Phospholipase C catalyzes the breakdown of the minor membrane lipid PIP_2[1] (1, 2). The soluble product of this reaction, $(1,4,5)IP_3$, causes the release of Ca^{2+} from specialized intracellular stores and initiates the process of Ca^{2+} mobilization (3). Furthermore, the metabolism of $(1,4,5)IP_3$ results in the formation of numerous soluble inositol phosphates (4), which may also be involved in the regulation of intracellular calcium or other intracellular processes. The second product of PIP_2 breakdown, diacylglycerol, activates protein kinase C (5), resulting in phosphorylation of specific intracellular proteins. Receptor activation of these two intracellular messenger systems has been described in a wide variety of cell types of vastly divergent physiological functions; thus, the mechanisms underlying the regulation of these systems are of fundamental interest.

Many cell types have more than one type of phospholipase C-linked receptor, and activation of different receptors on a given cell type often results in different patterns of inositol phosphate formation (see, for examples, Refs. 6–11). Thus, a critical site for the regulation of the inositol phosphate/Ca^{2+}/protein kinase C cascade may be at the level of the cell surface receptor. The present study was undertaken to investigate the regulation of phospholipase C-linked receptors of AR4-2J cells. This rat pancreatoma cell line has proved to be particularly useful for the study of the inositol phosphate-Ca^{2+} signaling system (12–22) and appears to be an excellent model of acinar cell physiology. The AR4-2J cells express four phospholipase C-linked receptor types found on nonimmortalized pancreatic acinar cells, i.e., receptors for cholecystokinin, bombesin, substance P, and acetylcholine (muscarinic subtype). We report that the activation of these receptors causes distinct temporal patterns of phospholipase C activation and that this phenomenon is due, at least in part, to differences in the time course and extent to which each receptor undergoes homologous desensitization. A preliminary report of these results has been presented (23).

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium, qualified fetal bovine serum, and glutamine were from GIBCO BRL (Grand Island, NY). *myo*-[^{3}H]

[1] The inositol phosphates and lipids are abbreviated according to the "Chilton Convention" (50), as, for example, $(1,4,5)IP_3$ for D-*myo*-inositol (1,4,5)-trisphosphate and PIP_2 for phosphatidylinositol (4,5)bisphosphate.

ABBREVIATIONS: KRH, Krebs/Ringer/HEPES solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL, total inositol lipids; HPLC, high performance liquid chromatography.

Inositol (10–20 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Cholecystokinin, substance P, and bombesin were from Peninsula Laboratories, Inc. (Belmont, CA). Methacholine and atropine were from Sigma Chemical Co. (St. Louis, MO). Dowex AG 1-X8 formate form (200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest purity available.

Cell Culture

AR4-2J cells were subcultured from cells generously provided by Dr. Craig Logsdon (University of California, San Francisco, CA). Cultures were maintained as described previously (18, 19). Briefly, cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and supplemented with 10% qualified fetal bovine serum and 2 mM glutamine, at 37° in a humid 5% CO_2/95% air atmosphere. For cells in which [^{3}H]inositol phosphate formation was to be measured, inositol phospholipids were labeled by addition of *myo*-[^{3}H]inositol (10–50 μCi/ml) to the culture medium at the time of passage. For experiments, cells were passed onto 150-mm or 35-mm plastic tissue culture dishes, and experiments were carried out 3 days later.

[^{3}H]Inositol Phosphate Formation in Intact Cells

All experiments were carried out at 37°. Cells labeled for 3 days with *myo*-[^{3}H]inositol were preincubated for approximately 20 min in KRH (115 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 1.35 mM $CaCl_2$, 0.6 mM $MgSO_4$, 11 mM glucose, 25 mM HEPES, pH 7.4). Stimulation of [^{3}H]inositol phosphate formation was initiated by replacement of the preincubation solution with fresh KRH containing agonist. Other manipulations are described below for each experiment. Reactions were stopped in one of two ways. For experiments with cells on 150-mm plates, the medium was aspirated and the cells were covered with 5 ml of ice-cold 7.5% trichloroacetic acid containing 250 μg of phytate (24). For cells plated on 35-mm dishes, the reaction was stopped by addition of a volume of ice-cold 12% perchloric acid, containing 250 μg/ml or 1 mg/ml phytate, equal to the volume of the incubation buffer (0.75 or 1.0 ml). In both cases, the plates were incubated at 4° for at least 20 min and then the acidic supernatant was removed for neutralization and [^{3}H]inositol phosphate assay.

Cellular levels of [^{3}H]inositol phosphates are expressed as a percentage of radioactivity in [^{3}H]IL. [^{3}H]IL were extracted, according to the method of Schacht (25), from the cell matrix remaining attached to the tissue culture plates after removal of the acidic supernatant of stopped reactions. Radioactivity was determined by liquid scintillation counting.

[^{3}H]Inositol Phosphate Assays

Acidic extracts from *myo*-[^{3}H]inositol-labeled cells were neutralized, and the [^{3}H]inositol phosphates were separated by anion exchange chromatography or HPLC, as described below.

Anion exchange chromatography. Acidic supernatants from perchloric acid-stopped reactions were neutralized by the addition of 1.25 volumes of 0.5 M KOH, 9 mM $Na_2B_4O_7$. After storage overnight at 4°, the samples were centrifuged at 1000 × *g* to remove precipitated potassium perchlorate, and the supernatants were applied to gravity-fed columns containing Dowex anion exchange resin (1 ml). Total [^{3}H]IP_3 was eluted from the columns as previously described (26); lower inositol phosphates were sequentially eluted, after which [^{3}H]IP_3 was eluted with 8 ml of 0.8 M ammonium formate, 0.1 M formic acid. It is noted that, in the AR4-2J cells, (1,4,5)IP_3 is the principal IP_3 accumulated, and only low levels of (1,3,4)IP_3 are formed (18, 19). In some experiments, total [^{3}H]inositol phosphates (IP_1 plus IP_2 plus IP_3 plus IP_4) were collected by washing of the anion exchange columns with 10 × 10 ml of H_2O before elution with 10 ml of 1.2 M ammonium formate, 0.1 M formic acid. In each case, radioactivity in collected fractions was determined by liquid scintillation counting.

HPLC. Acidic supernatants from perchloric acid-stopped reactions prepared for HPLC were neutralized by the freon/tri-*n*-octylamine extraction method of Downes *et al.* (27), as modified by Shears *et al.* (28). Acidic supernatants from trichloroacetic acid-stopped reactions were neutralized by ether extraction, as described by Horstman *et al.* (18). No differences between these two methods were noted. Isomers of [^{3}H]IP_3 were separated by HPLC, using a protocol based on that of Dean and Moyer (29), as described by Horstman *et al.* (18). Radioactivity was monitored using a flow-type scintillation counter (Flo-one; Radiomatic Instruments, Tampa, FL).

Results

[^{3}H](1,4,5)IP_3 formation induced by different receptor agonists. *myo*-[^{3}H]Inositol-labeled AR4-2J cells accumulated [^{3}H](1,4,5)IP_3 on exposure to bombesin (0.2 μM), cholecystokinin (1 μM), substance P (1 μM), or the muscarinic acetylcholine receptor agonist methacholine (100 μM) (Fig. 1) (maximally effective concentrations for each agonist). For the peptide agonists, the peak level of [^{3}H](1,4,5)IP_3 was attained within 10–15 sec. The peak [^{3}H](1,4,5)IP_3 level was similar after bombesin or cholecystokinin but was typically 50–70% lower after substance P. Within 90 sec in the continued presence of bombesin or cholecystokinin, [^{3}H](1,4,5)IP_3 levels declined from the peak to a steady state several times greater than that before stimulation. In contrast, the [^{3}H](1,4,5)IP_3 level returned to the prestimulation level in the continued presence of substance P, as previously reported (18). After exposure of AR4-2J cells to methacholine, [^{3}H](1,4,5)IP_3 levels rose slowly to a peak at 90 sec (Fig. 1). Thereafter, in the continued presence of this agonist, [^{3}H](1,4,5)IP_3 levels declined slowly and returned to basal levels within 40–60 min (data not shown).

Desensitization of the substance P response. In substance P-challenged cells, the rapid decline in [^{3}H](1,4,5)IP_3 to prestimulation levels is consistent with a rapid desensitization of the response to substance P. Desensitization is also demonstrated by the finding that brief exposure to substance P rendered the AR4-2J cells refractory to a second exposure to

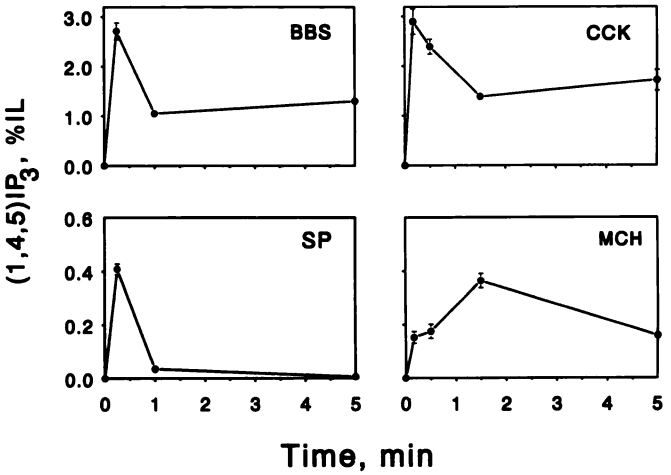

Fig. 1. Formation of [^{3}H](1,4,5)IP_3 in AR4-2J cells stimulated with bombesin, cholecystokinin, substance P, or methacholine. [^{3}H]Inositol-labeled AR4-2J cells were exposed to 0.2 μM bombesin (*BBS*), 1 μM cholecystokinin (*CCK*), 1 μM substance P (*SP*), or 100 μM methacholine (*MCH*) for 10–300 sec. [^{3}H](1,4,5)IP_3 was quantified by HPLC. Basal values for [^{3}H](1,4,5)IP_3 (time 0 sec) were determined with cells briefly exposed to KRH only and were subtracted from each time point. For these experiments, the basal [^{3}H](1,4,5)IP_3 level was 0.13 ± 0.02% of IL (mean ± standard error; 13 determinations). Each *point* is the mean ± standard error of three or four dishes from one of at least three similar experiments for each agonist.

this agonist. In the experiment depicted in Fig. 2, AR4-2J cells were incubated for 120 sec with 1 μM substance P, washed extensively, and then incubated for 10 min in the absence of substance P. Upon a second exposure to 1 μM substance P, no [^{3}H]IP_3 accumulation was observed. Complete desensitization occurred with as little as 60 sec of initial exposure to substance P and was not immediately reversed by extensive washing of the cells after exposure to this agonist. AR4-2J cells exposed to a maximal concentration of substance P remained completely refractory to this agonist for 10–15 min. Thereafter, the substance P response recovered with a half-time of approximately 1 hr (Fig. 3). However, exposure of AR4-2J cells to a maximal concentration of substance P did not inhibit a subsequent response to bombesin, cholecystokinin, or methacholine. For example, in cells pretreated for 1 min with 1 μM substance P, washed, and then challenged for 10 sec with 1 μM cholecystokinin, the cholecystokinin response was 91.2 ± 2.8% (mean ± standard error; four determinations) of the response in naive cells. This indicates that the desensitization to substance P is homologous.

Exposure of the AR4-2J cells to the NK_1 receptor agonist substance P methyl ester (30) also induced a transient increase in IP_3 and inhibited a subsequent response to substance P (Table 1). Cells were exposed to different concentrations of substance P methyl ester for 60 sec and then washed free of the agonist. After 10 min, during which time IP_3 had returned to basal level, the cells were challenged for 10 sec with substance P. It was found that the response to the substance P challenge was diminished in proportion to the concentration of substance P methyl ester to which the cells were first exposed (Table 1). At the highest substance P methyl ester concentration (20 μM), the subsequent response to substance P was almost completely abolished. These data confirm that the substance P receptors of the AR4-2J cells are of the NK_1 (substance P-P) type, as previously reported (12).

Desensitization of the response to the muscarinic receptor agonist methacholine. In AR4-2J cells treated with methacholine, the slow decrease in [^{3}H](1,4,5)IP_3 from peak to basal level over the course of 40–60 min may be accounted for by a slowly developing desensitization of the response to muscarinic cholinergic receptor stimulation. In cells stimulated for 5 min with 100 μM methacholine, the elevated level of [^{3}H](1,4,5)IP_3 rapidly returned to basal level upon addition of 10 μM atropine or rapid washing of the cells to remove the agonist. However, in cells exposed to methacholine and then washed, the inositol phosphate response to a subsequent methacholine exposure was inhibited (Fig. 4). The degree of inhibition was dependent on the time of the initial exposure; after incubation of AR4-2J cells with 100 μM methacholine for 40 min, the cells

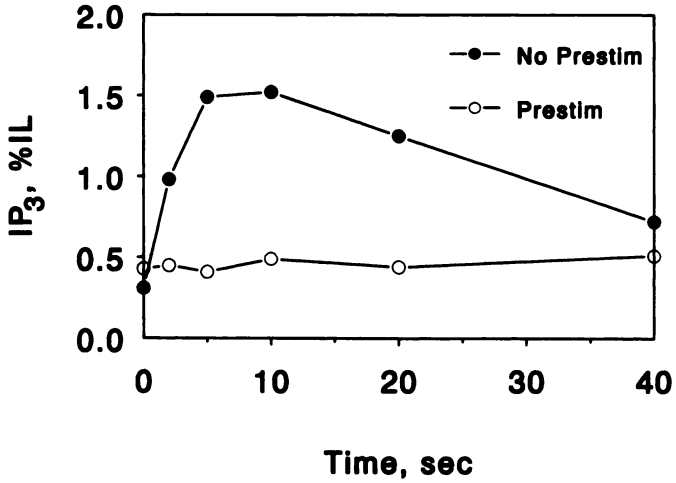

Fig. 2. Desensitization of [^{3}H]IP_3 formation in response to substance P in AR4-2J cells. [^{3}H]Inositol-labeled AR4-2J cells were incubated for 60 sec in KRH with (○) or without (●) 1 μM substance P. The incubation buffer was then rapidly aspirated, and fresh KRH was added. After an additional 10 min of incubation, all cells were challenged with 1 μM substance P for 2–40 sec. Total [^{3}H]IP_3 levels after the challenge with substance P were quantified using anion exchange columns. Each *point* is the mean ± standard error of triplicate dishes. Similar results were obtained in three other experiments.

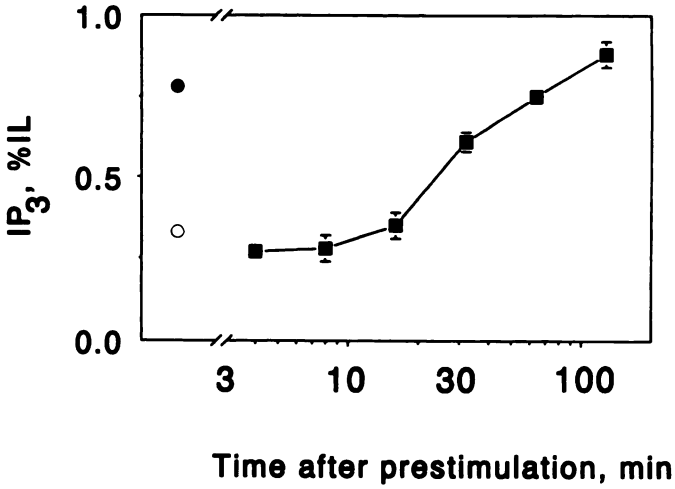

Fig. 3. Time course for recovery of the [^{3}H]inositol phosphate response to substance P after desensitization. [^{3}H]Inositol-labeled AR4-2J cells were exposed to 1 μM substance P for 60 sec. The incubation buffer was then rapidly aspirated, and the cells were further incubated in fresh KRH for 4–128 min. Cells were then challenged with 1 μM substance P for 10 sec (■). Also shown, for comparison, are two groups of cells that were not preexposed to substance P before challenge for 10 sec with 1 μM substance P (●) or KRH only (○). These latter groups represent the maximal substance P-stimulated increase and basal level, respectively, of [^{3}H]IP_3. [^{3}H]IP_3 levels were determined using anion exchange columns. Each *point* is the mean ± standard error of triplicate dishes from one of three similar experiments.

TABLE 1

Effect of substance P methyl ester on [^{3}H]inositol phosphate formation and the response to substance P in AR4-2J cells

One group of AR4-2J cells, plated on 35-mm dishes in the presence of *myo*-[^{3}H]inositol (10 μCi/ml), as described in Materials and Methods, was incubated for 10 sec in KRH containing different concentrations of substance P methyl ester (SP-ME). A second group was exposed to these same substance P methyl ester concentrations for 60 sec, after which the incubation buffer was aspirated and replaced with KRH containing no agonist. After an additional 10-min incubation, these cells were challenged with 1 μM substance P (SP). [^{3}H]IP_3 levels were quantified using gravity-fed anion exchange columns, as described. The basal [^{3}H]IP_3 level was 0.20 ± 0.01% of IL, and the level of [^{3}H]IP_3 10 sec after substance P addition in substance P methyl ester-naive cells was 0.63 ± 0.01% of IL. Each value is the mean ± standard error of triplicate dishes from one experiment representative of three similar experiments.

[SP-ME]	IP_3	
	Response to SP-ME	Response to SP after SP-ME
μM	% of IL	
0.02	0.34 ± 0.01	0.58 ± 0.02
0.2	0.43 ± 0.01	0.52 ± 0.02
2.0	0.50 ± 0.02	0.35 ± 0.02
20.0	0.51 ± 0.01	0.28 ± 0.03

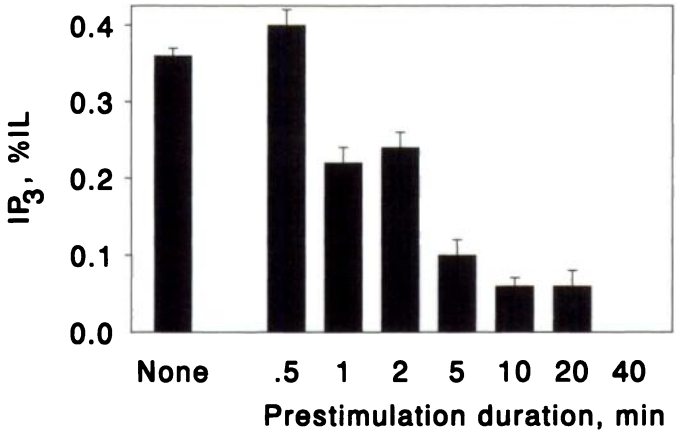

Fig. 4. Effect of time of preexposure to methacholine on the subsequent [^{3}H]inositol phosphate response to this agonist in AR4-2J cells. [^{3}H]Inositol-labeled AR4-2J cells were exposed to 100 μM methacholine for the times indicated. The incubation buffer was then rapidly aspirated, and the cells were further incubated in fresh KRH for 5 min. The cells were then challenged with 100 μM methacholine for 90 sec, and the accumulation of [^{3}H]IP_3 was determined with anion exchange columns. Basal values for [^{3}H]IP_3 were determined with cells briefly exposed to KRH only and were subtracted from each *bar*. For this experiment, the basal [^{3}H]IP_3 level was 0.28 ± 0.01% of IL (mean ± standard error; three experiments). Each *bar* is the mean ± standard error of triplicate dishes from one of two similar experiments.

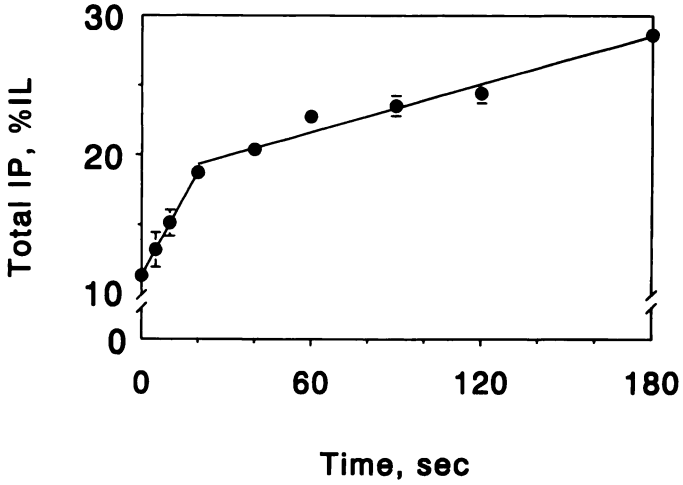

Fig. 5. Accumulation of total [^{3}H]inositol phosphates in bombesin-stimulated AR4-2J cells incubated with lithium. [^{3}H]Inositol-labeled AR4-2J cells were preincubated for 10 min in KRH supplemented with 10 mM LiCl. Bombesin (0.2 μM, final concentration) was added, and the cells were further incubated for 5–180 sec. Total [^{3}H]inositol phosphates were quantified using gravity-fed anion exchange columns, as described. Rates of [^{3}H]inositol phosphate accumulation during 0–20 and 20–180 sec were taken from the slopes determined by linear regression ($r >$ 0.95 in both instances). Incubation of cells in the presence of 10 mM LiCl but without added bombesin did not affect the basal level of total [^{3}H]inositol phosphates (data not shown). Each *point* is the mean ± standard error of triplicate dishes. Similar results were obtained in a separate experiment using cholecystokinin as agonist.

were completely refractory to a second exposure to this agonist (Fig. 4). Recovery of responsiveness did not occur within 45 min (the longest time interval examined). However, prior exposure of the AR4-2J cells to methacholine did not diminish the increase in [^{3}H]IP_3 in response to other agonists. For example, in cells pretreated for 1.5, 10, or 60 min with 100 μM methacholine, washed, and then challenged for 10 sec with 0.2 μM bombesin, the bombesin responses were, respectively, 100 ± 15, 107 ± 4, and 118 ± 10% (mean ± standard error; three determinations for each condition) of that in naive cells. This indicates that the desensitization of the inositol phosphate response to muscarinic cholinergic receptor stimulation is homologous.

Desensitization of the inositol phosphate response to bombesin. The inositol phosphate responses to bombesin and cholecystokinin were similar and appeared to be composed of two phases. With maximal stimulation of AR4-2J cells with bombesin or cholecystokinin, the initial peak in [^{3}H](1,4,5)IP_3 level was followed by a steady state increase in [^{3}H](1,4,5)IP_3 to several times the level before stimulation (Fig. 1). The biphasic nature of the inositol phosphate response to these agonists may be accounted for by a rapidly developing, but only partial, desensitization of this response. This possibility was analyzed in detail for the response to bombesin.

In a first experiment, total [^{3}H]inositol phosphate accumulation as a function of time was measured after bombesin stimulation in cells preincubated with LiCl. Lithium blocks the metabolism of IP_1 to inositol (31–33); thus, total [^{3}H]inositol phosphate accumulation in cells incubated with LiCl essentially integrates the activity of phospholipase C over time. In the experiment depicted in Fig. 5, AR4-2J cells were pretreated with 10 mM LiCl for 10 min and then exposed to 0.2 μM bombesin. Initially, there was a rapid accumulation of [^{3}H]inositol phosphates (22.1% of IL/min). This ended abruptly after 20 sec, and accumulation thereafter proceeded at a slow but constant rate (3.5% of IL/min) for the remainder of this

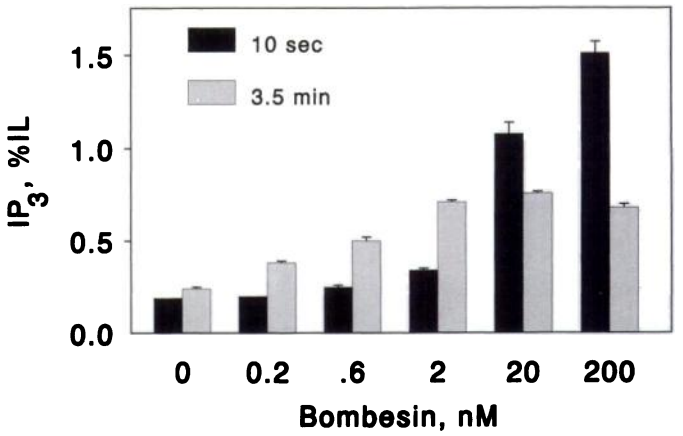

Fig. 6. Effect of bombesin concentration on the peak and sustained increases in [^{3}H]IP_3 induced by this agonist in AR4-2J cells. AR4-2J cells were exposed to different concentrations of bombesin for 10 sec or 3.5 min. The accumulation of [^{3}H]IP_3 was determined using anion exchange columns. Each value is the mean ± standard error of triplicate dishes from one experiment, representative of three similar experiments.

180-sec incubation. This slower rate of [^{3}H]inositol phosphate accumulation was constant for at least 20 min. In LiCl-treated cells not stimulated with agonist, there was no [^{3}H]inositol phosphate accumulation over basal level (data not shown). Quantitatively similar phases of [^{3}H]inositol phosphate accumulation were also observed after stimulation of LiCl-treated AR4-2J cells with 1 μM cholecystokinin (rapid phase of 23.9% of IL/min; slow phase of 4.2% of IL/min).

We next examined the concentration dependence for the initial peak and plateau accumulations of IP_3 accumulation in response to bombesin. In the experiment depicted in Fig. 6, cells were exposed to increasing concentrations of bombesin

for either 10 sec, to measure the initial peak IP_3 accumulation, or 3.5 min, to measure the steady state IP_3 accumulation (steady state IP_3 levels were attained within 30 sec at each agonist concentration). It was found that, whereas maximal steady state levels of IP_3 were attained at bombesin concentrations between 0.6 and 2 nM, maximal peak increases in IP_3 were only attained at 200 nM bombesin. Thus, there was a 100-fold or greater difference in the concentration required to activate the two phases of the IP_3 response to bombesin.

To examine the desensitization of the initial phase of inositol phosphate accumulation in response to bombesin, AR4-2J cells were incubated for 10 min with 0–200 nM bombesin and then challenged for 10 sec with a maximal concentration of bombesin (200 nM) or cholecystokinin (1 μM). As indicated above, the steady state [^{3}H]IP_3 level at the end of the 10-min incubation with bombesin was increased with increasing bombesin concentration (Fig. 7). Addition of cholecystokinin induced a further rapid increase in [^{3}H]IP_3; the final peak increase was largely independent of the bombesin concentration and was similar to that attained when cholecystokinin was administered to naive cells. However, the ability of a maximal concentration of bombesin to stimulate a further increase in the levels of IP_3 was progressively diminished with increasing concentrations of bombesin pretreatment. Thus, in cells incubated for 10 min with 20 nM bombesin, the further addition of a 10-fold higher bombesin concentration was without effect. This occurred despite the fact that these bombesin concentrations elicited clearly different responses in naive cells (Fig. 6). Thus, the initial burst of IP_3 formation induced by bombesin, but not by cholecystokinin, was inhibited by bombesin pretreatment. These data also indicate that, in the AR4-2J cells, the activation of phospholipase C by different agonists is not additive. That is, the increase in IP_3 induced by cholecystokinin in bombesin-pretreated cells reached only the level achieved by this agonist in naive cells (Fig. 7). Furthermore, in cells treated for 10 min with 0.2 μM bombesin plus 1 μM cholecystokinin, the steady state level of [^{3}H]IP_3 attained was not additive but was similar to that induced by either agonist alone (as percentage of IL: bombesin, 1.6 ± 0.1; cholecystokinin, 1.8 ± 0.1; and bombesin plus cholecystokinin, 2.1 ± 0.1; means ± standard errors for triplicate determinations).

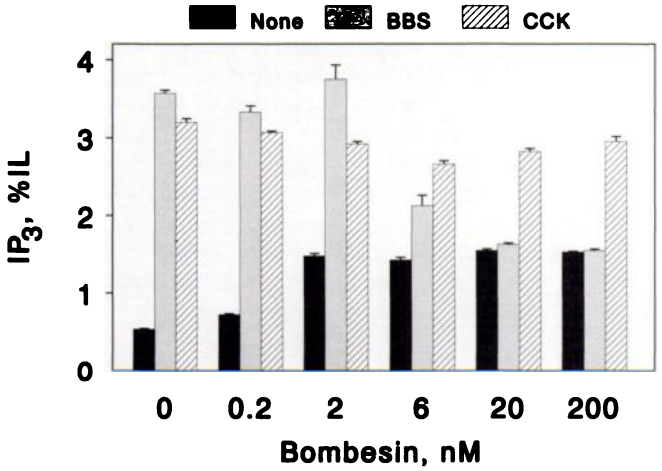

Fig. 7. Effect of preexposure to different bombesin concentrations on the subsequent [^{3}H]inositol phosphate response to maximally effective concentrations of bombesin or cholecystokinin. [^{3}H]Inositol-labeled AR4-2J cells were exposed to different concentrations of bombesin for 10 min. The incubation buffer was then rapidly aspirated, and fresh KRH was added, containing no agonist (■), 0.2 μM bombesin (□), or 1 μM cholecystokinin (▨). After 10 sec, reactions were stopped and [^{3}H]IP_3 was determined using anion exchange columns. Each value is the mean ± standard error of triplicate dishes from one of two similar experiments.

Discussion

An important locus for the regulation of intracellular messenger generation by phospholipase C appears to be at the level of the cell surface receptor. This can be seen in the results of the present study of the rat pancreatoma cell line AR4-2J. These cells express at least four types of phospholipase C-linked receptors that, when activated, give rise to distinct patterns of inositol phosphate formation (Fig. 1). There are several factors that may contribute to this phenomenon. There may be different levels of receptor expression. The receptor types may also differ with regard to the efficiency of coupling (presumably through an intermediary GTP-binding protein) to phospholipase C. This latter factor may be particularly relevant to the observed differences in initial rate of (1,4,5)IP_3 accumulation for the peptide receptors and the muscarinic cholinergic receptor. We note that, in contrast to the different IP_3 responses, there is considerable similarity between the maximally stimulated Ca^{2+} responses induced by each receptor type in the AR4-2J cells. The initial large, very rapid, increase in intracellular Ca^{2+} is of similar magnitude upon activation of each of the receptor types (23).[2] This is followed by a much smaller, sustained, Ca^{2+} increase with bombesin, cholecystokinin, or muscarinic receptor stimulation but not substance P stimulation. Thus, it seems unlikely that agonist-induced changes in intracellular Ca^{2+}, through effects on the metabolism of (1,4,5)IP_3, play a significant role in shaping the different IP_3 responses in the AR4-2J cells. Instead, the results of the present study indicate that receptor desensitization is an important mechanism for receptor-specific regulation of this signaling cascade. We have characterized receptor desensitization for three of the receptors of the AR4-2J cells, i.e., for substance P, muscarinic cholinergic, and bombesin receptors. Our results indicate that each of these receptors undergoes a pattern of desensitization that is distinct with respect to extent and time courses for onset and recovery.

The present results indicate that the substance P receptor expressed by AR4-2J cells undergoes rapid and complete desensitization. The fact that substance P methyl ester also promotes the complete desensitization of the substance P response indicates that this receptor is of the NK_1 type, as has been previously suggested (12). Prior exposure of the AR4-2J cells to substance P does not, however, alter the inositol phosphate response to other agonists, indicating that desensitization is homologous. After the onset of desensitization, the AR4-2J cells remain completely refractory to substance P for 10–15 min, after which responsiveness recovers with a half-time of approximately 1 hr. The rapid desensitization of the substance P receptor accounts for the transient accumulation of inositol phosphates induced by substance P in the AR4-2J cells, as previously noted (18–20), which, in turn, accounts for the fact that substance P induces only transient Ca^{2+} release and influx in these cells (18).

The muscarinic cholinergic receptor of the AR4-2J cells also undergoes complete desensitization. However, the onset of de-

[2] H. Takemura, F. S. Menniti, and J. W. Putney, Jr., unpublished observations.

sensitization is very much slower than that for the substance P receptor, and sustained Ca^{2+} influx is observed after muscarinic receptor stimulation.[2] Furthermore, recovery of the muscarinic response also appears to be comparatively slow, in that after complete desensitization the cells remain refractory to muscarinic stimulation for at least 45 min. As for the substance P receptor, however, muscarinic receptor desensitization is homologous.

Perhaps the most interesting pattern of desensitization occurs for the bombesin receptor of the AR4-2J cells. Radioligand-binding studies indicate a single class of bombesin binding sites on the AR4-2J cells (34), as well as on other freshly isolated pancreatic tissues (35). Furthermore, the bombesin receptor of the AR4-2J cells has been isolated, and the functional unit is believed to be a monomeric protein (34). Nonetheless, the inositol phosphate response to bombesin appears to be composed of two phases in these cells. Maximal activation of the bombesin receptor initially results in a vigorous stimulation of phospholipase C, during which the turnover rate of the IL increases to >20%/min. Within 20 sec, this gives way to a second, slower, phase of phospholipase C activity. This phase of the response does not apparently desensitize, accounting for a prolonged elevation of (1,4,5)IP_3, lasting for up to 4 hr (19), and a sustained Ca^{2+} influx evoked by bombesin.[2] The partial desensitization of the bombesin response is homologous. However, we were unable to assess accurately the time course for the recovery of this response, because we were unable to sufficiently wash the cells of the peptide to terminate its actions.

The two phases of the bombesin response can be further differentiated by their bombesin concentration dependencies. Specifically, it was observed that a higher concentration of bombesin (200 nM) was required to attain a maximal stimulation of the early desensitizing phase of the response than was necessary to maintain the slower, nondesensitizing, response phase (2 nM) (Fig. 6). However, the concentration of bombesin required to cause complete desensitization of the initial response (20 nM) (Fig. 7) appeared to be intermediate between those required to maximally stimulate the early and late responses. Similar results have been reported by Merritt and Rink (36) for the substance P receptor in parotid acinar cells. Although not studied in as great detail, desensitization of the cholecystokinin receptor appears to bear at least some of the characteristics of bombesin receptor desensitization. This is suggested by the similarity of these two agonists in their patterns of [^{3}H](1,4,5)IP_3 formation (Fig. 1) and phospholipase C activity in lithium-treated cells.

As noted above, the desensitization of each of the phospholipase C-linked receptors in the AR4-2J cells is homologous, indicating that the locus of regulation is at the level of the receptor. However, the mechanism(s) underlying these processes remain to be determined. This may be a particularly interesting avenue of research, because, in general, mechanisms for phospholipase C-linked receptor desensitization are not well understood. The most thoroughly studied mechanism for receptor desensitization is that for the β-adrenergic receptor linked to adenylate cyclase. Lefkowitz and co-workers (37, 38) have demonstrated that phosphorylation of the β_2-adrenergic receptor is the initiating signal for its homologous desensitization. Phosphorylation is catalyzed by a β-adrenergic receptor kinase; this reaction is completely dependent on agonist occupancy of the receptor. By analogy with the β-adrenergic receptor, research has focused on the role of receptor phosphorylation as the signal for desensitization of phospholipase C-linked receptors. Of particular interest has been the involvement of protein kinase C, because this kinase is known to be activated by diacylglycerol formed during the phospholipase C-catalyzed breakdown of PIP_2. Experimental support for an involvement of protein kinase C in desensitization of phospholipase C-linked receptors has come from several studies in which phorbol esters were found to inhibit responses to phospholipase C-linked agonists (11, 39–43). However, other studies indicate that the physiological activation of protein kinase C, during agonist stimulation of phospholipase C, does not play a role in homologous receptor desensitization (44, 45). Thus, other mediators of desensitization, in addition to protein kinase C, must be sought.

An intriguing possibility for one such mediator of phospholipase C-linked receptor desensitization comes from the work of Kwatra and co-workers (46, 47). This group has shown that the muscarinic receptor of chick heart is phosphorylated, in an agonist-dependent fashion, by a kinase distinct from the cyclic nucleotide-dependent or Ca^{2+}/calmodulin-dependent protein kinases or protein kinase C. Furthermore, the concentration of agonist required to induce muscarinic receptor phosphorylation correlates closely with the agonist concentration required to induce desensitization of muscarinic receptor-stimulated inositol phosphate formation in this preparation. Thus, it is not unreasonable to speculate that a kinase(s) specific for phospholipase C-linked receptors participates in their desensitization, in a manner analogous to that for the β-adrenergic receptor. If this is the case, it remains to be determined how distinct patterns of desensitization, such as reported here for the AR4-2J cells, can be accommodated within this paradigm; i.e., are different kinases indicated for each receptor, or is the pattern of desensitization encoded within the structure of the individual receptors? It also must be noted that mechanisms that do not depend on phosphorylation may also be involved in desensitization of phospholipase C-linked receptors. For example, the desensitization of the rat parotid substance P receptor (48) and the CCL39 cell thrombin receptor (49) occur under conditions that would not likely support receptor phosphorylation. The mechanisms underlying desensitization in these cases also await identification. The diverse patterns of desensitization for phospholipase C-linked receptors in the AR4-2J cells suggest that these cells may provide a useful model for the further investigation of these issues. The present study provides a body of information on which to base such studies, i.e., that the underlying mechanism(s) must account for the facts that desensitization is homologous for each receptor and occurs with a different temporal pattern.

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Send reprint requests to: Frank S. Menniti, Ph.D., NIEHS/NIH, P. O. Box 12233, Research Triangle Park, NC 27709.