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Protein Kinase C Mediates Delayed Inhibitory Feedback Regulation of Human Neurokinin Type 1 Receptor Activation of Phospholipase C in UC11 Astrocytoma Cells

ALASTAIR J. BARR[1] and STEVE P. WATSON
Department of Pharmacology, Oxford University, Oxford, OX1 3QT, UK
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SUMMARY

Human UC11 astrocytoma cells were used to investigate the role of protein kinase C (PKC) and other kinases in neurokinin $(NK)_1$ receptor desensitization. The selective NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]-substance P stimulated a biphasic accumulation of [^{3}H]inositol phosphates ([^{3}H]IPs) in the presence of 10 mM LiCl in cells that had been prelabeled with [^{3}H]inositol. An initial rapid phase of [^{3}H]IP accumulation during the first 1 min was followed by a slower sustained phase for up to 90 min. These results demonstrate that the human NK_1 receptor desensitizes rapidly but only partially. The selective PKC inhibitor Ro31-8220 did not prevent rapid NK_1 receptor desensitization but after a longer incubation significantly potentiated human NK_1 receptor agonist-stimulated accumulation of [^{3}H]IPs. These results suggest that, although PKC does not mediate the process of rapid desensitization, it does have an inhibitory role at later times. This conclusion is supported by studies with staurosporine, phorbol dibutyrate, and the protein phosphatase inhibitor okadaic acid. Studies using AlF_4^-, an agent that can directly activate G proteins, and Ro31-8220 suggested that PKC can exert inhibitory effects 'downstream' of receptor activation, although immunoprecipitation of the G proteins α_q/α_{11} demonstrated that they do not undergo phosphorylation in UC11 cells and are unlikely to be the target of PKC-mediated inhibitory feedback. Delayed inhibitory feedback by PKC may be mediated by phosphorylation of phospholipase C, although an additional site of action on the NK_1 receptor cannot be ruled out.

It is established that NK_1 tachykinin receptors are linked via a G protein to PLC and the hydrolysis of phosphatidylinositol-4,5-bisphosphate, to generate the second messengers $(1,4,5)IP_3$ and diacylglycerol (1-3). $(1,4,5)IP_3$ is involved in mobilizing intracellular calcium, and diacylglycerol activates PKC (4, 5). These two messengers provide the link to the physiological response, e.g., contraction or secretion. A great deal of evidence indicates that NK_1 tachykinin receptor responses undergo rapid desensitization within 1 min. For example, NK_1 receptor-stimulated contraction of the guinea pig ileum desensitizes rapidly (6), as does SP-stimulated $(1,4,5)IP_3$ and calcium mobilization in the rat parotid gland (7-9). The desensitization is homologous (receptor specific) in nature, i.e. responses to other agonists activating PLC-linked receptors are not attenuated. The molecular basis underlying this process of desensitization remains unclear.

One possible route of desensitization after PLC activation involves inhibitory feedback via PKC (10-12). Much of the evidence for a role of PKC is derived from studies with activators of PKC, e.g., phorbol esters, and relatively nonselective protein kinase inhibitors, e.g., staurosporine, H7, and K252a. However, the ability to stimulate or inhibit a process with direct activators of PKC, such as phorbol esters, does not preclude the existence of alternative pathways that might be used by the physiological agonist. Putney and co-workers (13) concluded that, although PDBu inhibits $(1,4,5)IP_3$ formation after NK_1 receptor activation in rat parotid cells, this mechanism plays little if any role in regulation of the NK_1 receptor-PLC pathway. For example, H7, a relatively nonselective kinase inhibitor, reversed the effect of PDBu but did not block desensitization (14). A similar conclusion was reached after studies of SP receptor desensitization in frog sympathetic ganglion cells (15). Furthermore, activation of PLC and the generation of IPs do not seem to be requirements for desensitization (16). A number of studies on other PLC-linked receptors have also excluded a role for PKC in receptor desensitization (17, 18, 19).

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[1] Present address: Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6084.

ABBREVIATIONS: NK, neurokinin; PLC, phospholipase C; $(1,4,5)IP_3$, D-*myo*-inositol-1,4,5-trisphosphate; PKC, protein kinase C; PDBu, phorbol dibutyrate; IP, inositol phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; β-ARK, β-adrenergic receptor kinase; SP, substance P; BSA, bovine serum albumin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PVDF, polyvinylidene difluoride.

Homologous desensitization of the NK_1 receptor as well as other PLC-linked receptors may operate by a mechanism similar to that of the extensively studied β-adrenergic receptor, involving serine/threonine phosphorylation of the agonist-occupied form of the receptor by a constitutively active kinase, β-ARK, and consequent uncoupling from the G protein (20). Recent evidence suggests that receptors coupled to stimulation of the PLC pathway can be regulated in a similar manner. Kwatra *et al.* (21) demonstrated *in vitro* phosphorylation of a reconstituted rat NK_1 receptor by β-ARK-1 and β-ARK-2; Tobin and Nahorski (22) demonstrated rapid M_3 receptor phosphorylation that was independent of PKC, and studies on the cholecystokinin receptor in pancreatic acinar cells have shown agonist-dependent phosphorylation of the receptor by both PKC and a second group of kinases, which are resistant to staurosporine (23, 24).

The present study has investigated whether PKC or other kinases play a role in regulation of human NK_1 tachykinin receptor activation, using the UC11 astrocytoma cell line as a model. UC11 cells exhibit high levels of expression of the NK_1 receptor, and pharmacological studies have indicated the absence of other tachykinin receptors (25, 26). The involvement of kinases has been examined by studying the effects of a variety of agents that regulate protein kinases and protein phosphatases on [Sar^9,$Met(O_2)^{11}$]-SP (an NK_1 receptor-selective agonist)-stimulated formation of [^{3}H]IPs. The role of PKC has been investigated using a potent selective inhibitor of PKC, Ro31–8220 (27, 28), and the phorbol ester PDBu, which activates PKC. The involvement of other kinases has been investigated using the nonselective kinase inhibitor staurosporine, which inhibits several other serine/threonine-specific and tyrosine-specific kinases (29). Protein phosphatases play a key role in terminating phosphorylation induced by kinases and may be involved in recovery from desensitization (resensitization). Okadaic acid, which inhibits protein phosphatases 1 and 2A (30), can be used to elucidate the role of these two phosphatases in receptor regulation by prolonging recovery from desensitization. Agents regulating kinases and phosphatases can therefore be used to determine the role of phosphorylation in the regulation of human NK_1 receptor activation of PLC.

Experimental Procedures

Materials. Tissue culture flasks, media, antibiotics, and calf serum were from GIBCO Ltd. (Paisley, Scotland, UK). *myo*-[^{3}H]Inositol (15.4 Ci/mmol) and [^{32}P]orthophosphate (8500–9120 Ci/mmol) were from Amersham International (Bucks, UK). CP-96,345 [(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine] was a gift from Dr. J. G. Stam (Pfizer Inc., Groton, CT). [Sar^9,$Met(O_2)^{11}$]-SP was obtained from Peninsula Laboratories (Belmont, CA). Antibody QL was obtained from New England Nuclear-DuPont (Boston, MA) and NA 934 was from Amersham. Okadaic acid was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Ro31–8220 was a gift from Roche Products Ltd. (Welwyn Garden City, Herts., UK). Staurosporine, PDBu, and pertussis toxin were from Sigma Chemical Co. (Poole, UK). KT5720 was from Calbiochem (Nottingham, UK) and Dowex AG1-X8 resin was from Bio-Rad (Hemel Hempstead, UK). Other reagents were of analytical grade.

Cell culture. The UC11 human astrocytoma cells were kindly provided by Dr. C. L. Johnson (University of Cincinnati, Cincinnati, OH). The cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. Cells were incubated at 37° in a humidified incubator equilibrated with 5% CO_2.

Measurement of [^{3}H]IP formation. Cells grown in 75-cm^2 flasks were labeled for ~24 hr in medium containing 1 μCi/ml [^{3}H]inositol. The cells were washed in phosphate-buffered saline, trypsinized, and resuspended in Hanks' buffer (143 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 with NaOH at 37°, gassed with 100% O_2), at a cell density of 5×10^5 cells/ml. Aliquots (200 μl) of the cell suspension were transferred to plastic tubes containing LiCl (final concentration, 10 mM) and 30 μl of Hanks' buffer. Agonist was added in 10-μl volumes, and the tubes were gassed, capped, and incubated in a shaking water bath at 37° for the required time. Agents regulating kinases and phosphatases were preincubated with samples for 5 min before the addition of agonist.

Incubations were terminated by addition of 0.94 ml of chloroform/methanol/HCl (100:200:1, v/v/v). Total [^{3}H]IPs were extracted essentially as described by Berridge *et al.* (31). Chloroform (0.31 ml) and water (0.31 ml) were added, the samples were vortex-mixed, and the phases were separated by centrifugation at 1000 × *g* for 5 min. A portion of the upper aqueous phase (0.7 ml) was applied to a glass column containing 1 ml of an approximately 1:1 (w/v) slurry of Dowex AG1-X8 resin (100–200 mesh, formate form) and distilled water. [^{3}H]Inositol and [^{3}H]glycerophosphoinositol were removed with 5 mM disodium tetraborate/60 mM ammonium formate (2 × 8 ml). Total [^{3}H]IPs (i.e., inositol mono-, bis-, and trisphosphates) were eluted with 6 ml of 800 mM ammonium formate/0.1 M formic acid. This eluant was collected in scintillation vials and counted in the gel phase for radioactivity, after the addition of 10 ml of Liquiscint (National Diagnostics) scintillation fluid.

[^{3}H]IP studies examining the effect of pertussis toxin were carried out essentially as described by Johnson and Johnson (25). Cells plated at a density of 3×10^4 cells/ml in 24-well plates were labeled for ~24 hr in medium containing 1 μCi/ml [^{3}H]inositol. Pertussis toxin was added to wells and preincubated for the required time. The cells were washed three times in 500 μl of Hanks' buffer and then incubated at 37° with 200 μl of Hanks' buffer containing LiCl (final concentration, 10 mM) for 10 min. Agonist (50 μl) was added and the incubation was continued for the required time. The reaction was terminated by aspiration of the buffer and rapid addition of 1 ml of ice-cold methanol/0.1 M HCl (1:1). The extraction of water-soluble [^{3}H]IPs was allowed to continue for 1 hr. The extract was then removed, diluted with 6 ml of water, and assayed for [^{3}H]IPs as described above.

Data analysis. Results are expressed as means ± standard errors of at least three determinations. Where appropriate, statistical significance was assessed using Student's *t* test and was considered significant when p was <0.05.

Pertussis toxin-dependent [^{32}P]ADP-ribosylation of UC11 cell membranes. Cells were trypsinized as described above and were resuspended in 1 ml of 50 mM NaH_2PO_4 buffer, pH 7.4. Cells were homogenized with a 10-sec pulse in a probe sonicator, rapidly frozen in liquid N_2, thawed, and centrifuged at 500 × *g* for 5 min to pellet unbroken cells. The supernatant was removed and centrifuged at 14,000 × *g* for 10 min at 4°; the membrane pellet was resuspended in 100 mM KH_2PO_4 buffer, pH 7.5. Protein concentration was measured by the method of Bradford (32) with BSA as a standard, and membranes were stored at −70° until used.

Pertussis toxin (1 μg) was preactivated in 50 μl of 0.05 M Tris buffer, pH 7.5, containing 20 mM DTT and 50 mM glycine, for 45 min at 37°; the mixture was then cooled on ice for 20 min. For treatment with pertussis toxin, cell membranes (100 μg) were incubated in 20 mM Tris, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 0.1% (v/v) Lubrol PX, 1 mM ATP, 1 mM GTP, 5 mM $MgCl_2$, 10 mM thymidine, 10 μM NAD, 5 μCi of [^{32}P]NAD, 1 μg of preactivated pertussis toxin, and a regenerating system consisting of 20 mM phosphocreatine and 36.3 units/ml creatine phosphokinase, in a final volume of 200 μl. All reactions were carried out at 37° for 30 min and the incubations were terminated by the addition of 700 μl of ice-cold 20 mM Tris buffer, pH 7.5, containing 1 mM EDTA. Control samples were prepared by incubating membranes in the same medium in the absence of toxin. After termination, samples

were centrifuged at 11,000 × *g* for 20 min at 4° and were then washed and recentrifuged three times. Membrane pellets were solubilized in 20 μl of 50 mM Tris, pH 6.8, containing 10% (w/v) SDS and 0.5 mM DTT, and were then boiled for 10 min. Samples were cooled, 10 μl of 9 mM *N*-ethylmaleimide were added, and samples were then allowed to stand for 15 min. An equal volume of 2× sample solvent (Tris-HCl 50 mM, pH 6.8, SDS 4% (w/v), glycerol 20% (v/v), β-mercaptoethanol 10% (v/v), brilliant blue) was added and proteins were resolved by 12% SDS-PAGE. Gels were dried under vacuum at 80° for 2 hr and exposed to Amersham Hyperfilm to assess the extent of ADP-ribosylation.

Immunoprecipitation and immunoblotting. Human UC11 cells were trypsinized and resuspended in Hanks' buffer (gassed with 100% O_2), at a cell density of ~2 × 10^7 cells/ml, as described above. [^{32}P] Orthophosphate (1 mCi/ml) was added and cells were incubated at 37° for 1 hr in a shaking water bath. An aliquot of the cell suspension (500 μl; ~10^7 cells) was transferred to a plastic tube and challenged with various agents administered in a 50-μl volume for the required time. Immunoprecipitation was carried out essentially as described by Harlow and Lane (33). Incubations were terminated by addition of an equal volume of ice-cold 2× cell lysis buffer (2%, v/v, Triton, 20%, v/v, glycerol, 100 mM HEPES, 300 mM NaCl, 2 mM EGTA, 2 mM Na_3VO_4, 200 mM NaF, 20 mM sodium pyrophosphate, 20 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, pH 7.5), vortex-mixed, and sonicated in an ice-water bath for 30 min. Samples were centrifuged in a microfuge at 13,000 × *g* for 30 min to remove insoluble cell debris, and the supernatant was transferred to an Eppendorf tube. Samples were precleared with 50 μl of Protein A-Sepharose CL-4B (swollen gel made up in 5%, w/v, BSA) by mixing for 1 hr at 4°, followed by centrifugation at 13,000 × *g* in a microfuge for 30 min. The pellet of Protein A-Sepharose was retained from each sample as a control to define nonspecific binding of proteins. Each sample was washed and then analyzed by SDS-PAGE, immunoblotting, and autoradiography (see below).

Precleared supernatant was transferred to a new Eppendorf tube and primary antibody QL (1 μg), raised to the carboxyl-terminal decapeptide of G proteins α_q/α_{11} (34), and 50 μl of Protein A-Sepharose CL-4B were added to samples. The samples were incubated overnight at 4° with continual mixing and were then centrifuged in a microfuge at 13,000 × *g* for 30 min. The Protein A-Sepharose CL-4B pellet was washed five times with a 1:1 mixture of cell lysis buffer and Hanks' buffer, followed by an additional wash in Hanks' buffer alone. The immunoprecipitated protein was removed from Protein A-Sepaharose by addition of sample solvent and heating to 80° for 30 min. Protein was resolved by 12% SDS-PAGE and transferred to PVDF membranes for immunoblotting by semi-dry transfer (2 hr, 15 V). Blots were blocked in TBS-T (20 mM Tris, 137 mM NaCl, 0.1%, v/v, Tween-20, pH 7.6) with BSA (10%, w/v) for 1 hr at room temperature. Primary antibody QL (1/1000) diluted in TBS-T with BSA (1%, w/v) was then applied for 1 hr at room temperature. Blots were washed thoroughly with TBS-T and incubated with secondary antibody, i.e., donkey anti-rabbit IgG coupled to horseradish peroxidase (NA934; 1/1000 dilution), for 1 hr at room temperature. Blots were washed, and reactive bands were detected using the Amersham enhanced chemiluminescence system and exposed to enhanced chemiluminescence Hyperfilm. To assess the extent of [^{32}P]phosphorylation, PVDF membranes were autoradiographed by exposure to Amersham Hyperfilm at −70°.

Results

Time course of [Sar^9,Met(O$_2$)^{11}]-SP-stimulated accumulation of [^{3}H]IPs in human UC11 cells. [Sar^9,Met(O$_2$)^{11}]-SP stimulated a biphasic accumulation of [^{3}H]IPs in human UC11 cells, with an initial rapid accumulation during the first 60 sec followed by a slower sustained phase during which [^{3}H]IPs accumulated at an approximately linear rate over 90 min (Fig. 1). The selective NK_1 receptor antagonist CP-96,345 (35), added after a 15-min exposure to [Sar^9,Met(O$_2$)^{11}]-SP, completely inhibited the increase in [^{3}H]IPs (the further accumulation of [^{3}H]IPs was parallel to base-line values) (Fig. 1A, *inset*). Moreover, because no significant decrease in [^{3}H]IPs was observed during the remaining incubation, the presence of 10 mM LiCl in the assay was sufficient to fully inhibit the degradation of [^{3}H]IPs.

Effects of Ro31–8220 and okadaic acid. The selective PKC inhibitor Ro31–8220 had no significant effect on [Sar^9,Met(O$_2$)^{11}]-SP-induced accumulation of IPs during the first 5 min of stimulation (Fig. 1). However, after 15 min Ro31–8220 significantly potentiated the response to [Sar^9,Met(O$_2$)^{11}]-SP (Fig. 1A; see also Table 1 for 30-min incubation). This suggests that PKC does not mediate the initial rapid desensitization but has an inhibitory role at longer times of incubation. Incubation of cells with okadaic acid also had no effect on [Sar^9,Met(O$_2$)^{11}]-SP-induced accumulation of IPs during the first 5 min but caused a significant inhibition by 15 min (Fig. 1B; see also Table 1 for 30-min incubation). This result indicates the presence of a phosphorylation event, which is reversed by protein phosphatases 1 and/or 2A, regulating NK_1 receptor signaling at longer times of stimulation. Ro31–8220 and okadaic acid did not affect basal levels of [^{3}H]IPs in human UC11 cells during a 30-min incubation (data not shown).

Delayed involvement of PKC. The involvement of PKC-mediated regulation of the pathway was studied in more detail using Ro31–8220 and the phorbol ester PDBu. [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) stimulated a 5.0 ± 0.31-fold increase in [^{3}H]IPs over 30 min (Table 1). PDBu (1 μM) significantly reduced [Sar^9,Met(O$_2$)^{11}]-SP-stimulated accumulation of IPs, to 47 ± 2.0% ($p < 0.01$) of control levels. This effect was blocked by Ro31–8220 (1 μM), under which conditions the response to [Sar^9,Met(O$_2$)^{11}]-SP was increased to 158 ± 6.5% of control; this response is not significantly different from that to Ro31–8220 and [Sar^9,Met(O$_2$)^{11}]-SP alone, indicating that this concentration of Ro 31–8220 is sufficient to induce complete inhibition of PKC (Table 1). Ro31–8220 (1 μM) potentiated the response to [Sar^9,Met(O$_2$)^{11}]-SP over the length of the agonist concentration-response curve (Fig. 2).

Involvement of additional kinases in receptor desensitization. To investigate the possible existence of additional protein phosphorylation events involved in the regulation of PLC by NK_1 receptors, the relatively nonselective protein kinase inhibitor staurosporine was used. The effect of staurosporine on the [Sar^9,Met(O$_2$)^{11}]-SP response (Fig. 3) is complicated by a significant effect on basal levels of [^{3}H]IPs. Staurosporine (1 μM), in contrast to Ro31–8220, increased the levels of [^{3}H]IPs to 2.7 ± 0.3 times basal values during a 30-min incubation, a response equivalent to 54 ± 6% of the [Sar^9,Met(O$_2$)^{11}]-SP response (Fig. 3). The response to [Sar^9,Met(O$_2$)^{11}]-SP was increased in the presence of staurosporine to 189 ± 4.6% of basal levels, a value similar to the combined responses induced by Ro31–8220 and [Sar^9,Met(O$_2$)^{11}]-SP (158 ± 3.9%) and staurosporine alone (Fig. 3; Table 1). These results suggest that the effect of staurosporine on the response to [Sar^9,Met(O$_2$)^{11}]-SP may reflect two actions, i.e., direct stimulation of basal [^{3}H]IPs and inhibition of PKC. The effect of staurosporine on the response to [Sar^9,Met(O$_2$)^{11}]-SP was not altered in the presence of Ro31–8220, suggesting that the two kinase inhibitors have, in part, a common mechanism of action (i.e., inhibition of PKC).

The inhibitory effect of okadaic acid on the [Sar^9,Met(O$_2$)^{11}]-SP-stimulated time course of [^{3}H]IP accumulation

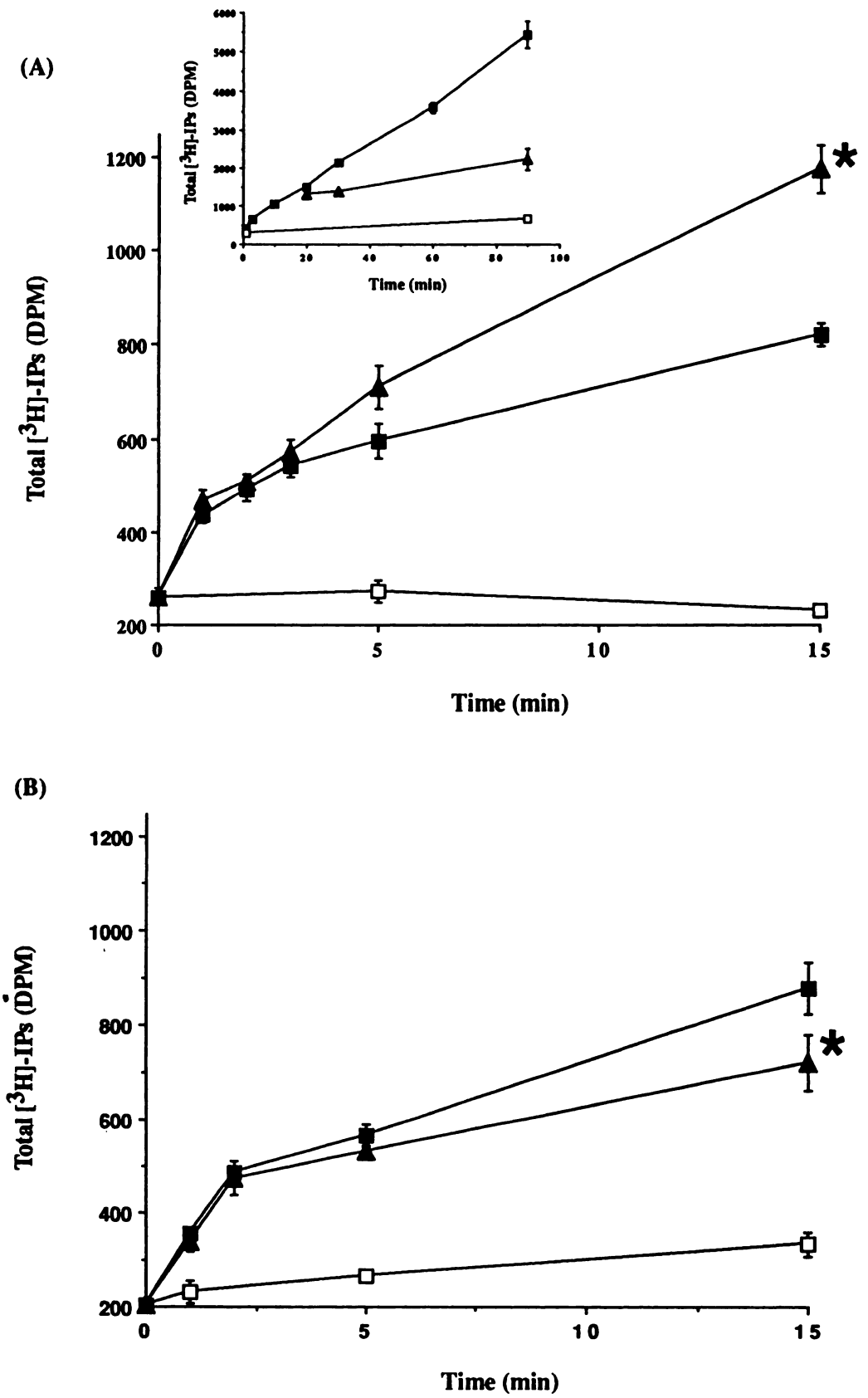

Fig. 1. Time course of [Sar^9,Met(O$_2$)^{11}]-SP-stimulated [^{3}H]IP accumulation in human UC11 cells. Human UC11 cells that had been prelabeled with [^{3}H]inositol were stimulated for the indicated times and then assayed for [^{3}H]IP accumulation as described in Experimental Procedures. A, ■, Cells stimulated with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM); □, cells treated with buffer; ▲, cells incubated with Ro31–8220 (1 μM) and then challenged with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM). *Inset*, time course over 90 min. ■, cells stimulated with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM); □, cells treated with buffer; ▲, cells stimulated with [Sar^9,Met(O$_2$)^{11}]-SP, with CP-96,345 (100 nM) added at 15 min. B, ■, Cells stimulated with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM); □, cells treated with buffer; ▲, cells incubated with okadaic acid (1 μM) and then challenged with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM). Experiments were carried out in the presence of 10 mM LiCl. Each *point* represents the mean of triplicate determinations; *error bars*, standard error (three experiments). *, $p < 0.05$.

TABLE 1

Effects of agents regulating protein kinases and protein phosphatases on [Sar^9,Met(O$_2$)^{11}]-SP-stimulated total [^{3}H]IP accumulation in human UC11 astrocytoma cells

Agents regulating kinases or phosphatases were added 5 min before [Sar^9,Met(O$_2$)^{11}]-SP. Results are expressed as a percentage of the [Sar^9,Met(O$_2$)^{11}]-SP (1 μM)-stimulated [^{3}H]IP accumulation during a 30-min incubation (mean ± standard error). [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) stimulated a mean 5.0 ± 0.3-fold increase in [^{3}H]IP (26 experiments). Compounds used were as follows: [Sar^9,Met(O$_2$)^{11}]-SP (Sar^9-SP) (1 μM), Ro31-8220 (1 μM), staurosporine (ST) (1 μM), okadaic acid (OKA) (1 μM), PDBu (1 μM), forskolin (10 μM), isobutylmethylxanthine (IBMX) (1 mM) and KT5720 (1 μM). Results are from four or more separate experiments performed in triplicate.

	Total [^{3}H]IPs	
	dpm	*% of Sar^9-SP response*
Basal	390 ± 22	
Sar^9-SP	1889 ± 18	100 ± 1
Sar^9-SP + Ro31-8220	2766 ± 68	158 ± 3.9
Sar^9-SP + PDBu	1096 ± 48	47 ± 2.0
Sar^9-SP + PDBu + Ro31-8220	2762 ± 113	158 ± 6.5
Sar^9-SP + ST	3229 ± 78	189 ± 4.6
Sar^9-SP + Ro31-8220 + ST	3216 ± 144	189 ± 8.5
Sar^9-SP + PDBu + ST	3368 ± 263	199 ± 15.5
Sar^9-SP + OKA	1285 ± 98	60 ± 4.5
Sar^9-SP + OKA + PDBu	566 ± 87	12 ± 3.9
Sar^9-SP + OKA + ST	2952 ± 231	171 ± 13.4
Sar^9-SP + OKA + Ro31-8220	1638 ± 100	83 ± 5.1
Sar^9-SP + forskolin	1949 ± 88	104 ± 4.7
Sar^9-SP + forskolin + IBMX	1994 ± 97	107 ± 5.2
Sar^9-SP + KT5720	1964 ± 75	105 ± 4.0

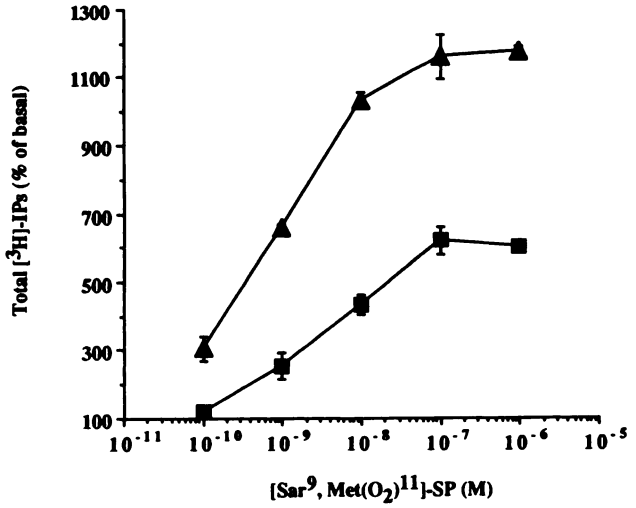

Fig. 2. Potentiation by Ro31–8220 of [Sar^9,Met(O$_2$)^{11}]-SP concentration-dependent stimulation of [^{3}H]IPs in human UC11 cells. Human UC11 cells that had been prelabeled with [^{3}H]inositol were stimulated for 30 min with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) in the presence (▲) or absence (■) of Ro31–8220 (1 μM). Cells were preincubated with Ro31–8220 for 5 min before the addition of [Sar^9,Met(O$_2$)^{11}]-SP. Results are expressed as a percentage of basal levels of [^{3}H]IPs. Experiments were carried out in the presence of 10 mM LiCl. Each *point* represents the mean of triplicate determinations; *error bars*, standard error (three experiments).

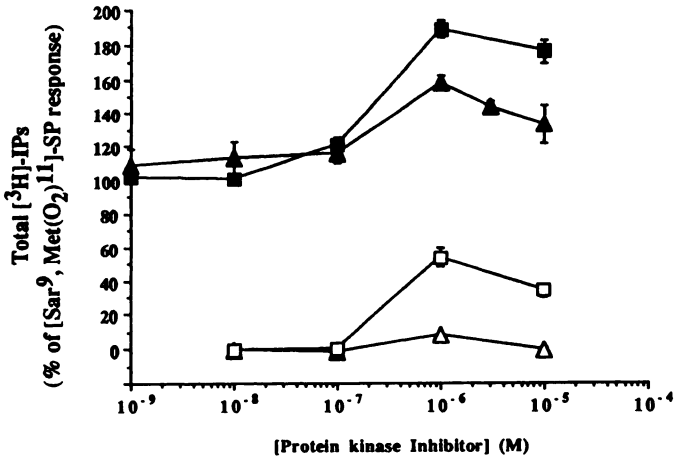

Fig. 3. Concentration-dependent effects of Ro31–8220 and staurosporine on [Sar^9,Met(O$_2$)^{11}]-SP-stimulated and basal levels of total [^{3}H]IPs in human UC11 cells. Human UC11 cells that had been prelabeled with [^{3}H]inositol were stimulated for 30 min with [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) in the presence of the kinase inhibitors staurosporine (1 μM) (■) and Ro31–8220 (1 μM) (▲). The effect of kinase inhibitors on basal [^{3}H]IP accumulation during a 30-min incubation is also shown [□, staurosporine (1 μM); △, Ro31–8220 (1 μM)]. Cells were preincubated with kinase inhibitors for 5 min before addition of [Sar^9,Met(O$_2$)^{11}]-SP. Results are expressed as a percentage of the [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) response. Experiments were carried out in the presence of 10 mM LiCl. Each *point* represents the mean of triplicate determinations; *error bars*, standard error (three experiments).

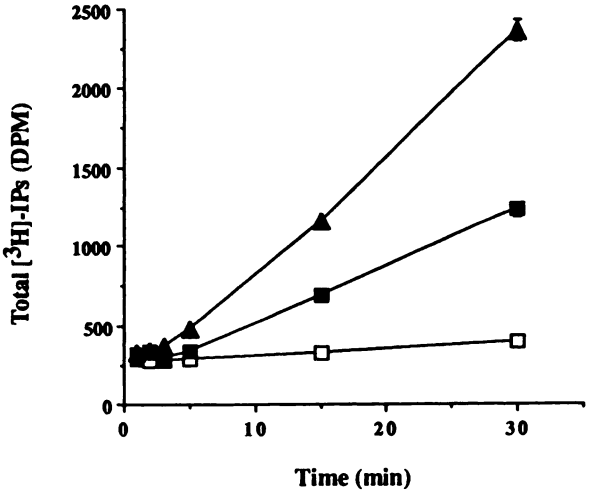

Fig. 4. Time course of AlF_4^--stimulated total [^{3}H]IP accumulation in human UC11 cells in the absence and presence of Ro31–8220. Human UC11 cells were prelabeled with [^{3}H]inositol and then stimulated for the indicated times with AlF_4^- (10 mM NaF and 10 μM $AlCl_3$) (■), buffer (□), or AlF_4^- and Ro31–8220 (1 μM) (▲). Experiments were carried out in the presence of 10 mM LiCl. Each *point* represents the mean of triplicate measurements; *error bars*, standard error. This experiments is representative of two others.

was studied in more detail after a 30-min incubation. Okadaic acid significantly reduced [Sar^9,Met(O$_2$)^{11}]-SP-stimulated accumulation of [^{3}H]IPs, to 60 ± 4.5% of control values ($p < 0.01$) (Table 1), and the effect was additive with that of PDBu, with the response to [Sar^9,Met(O$_2$)^{11}]-SP being reduced to 12 ± 3.9% of control values in the presence of the two inhibitors (Table 1). Ro31–8220 partially reversed the inhibitory effect of okadaic acid on the response to [Sar^9,Met(O$_2$)^{11}]-SP, to 83 ± 5.1%, a value that is significantly lower than that for Ro31–8220 and [Sar^9,Met(O$_2$)^{11}]-SP. In contrast, the response to [Sar^9,Met(O$_2$)^{11}]-SP in the presence of staurosporine and okadaic acid reached 171 ± 13.4% of control values, which was not significantly different from the response to [Sar^9,Met(O$_2$)^{11}]-SP and staurosporine alone ($p > 0.05$). These results suggest that the inhibitory effect of okadaic acid is mediated by PKC and an additional staurosporine-sensitive kinase.

Forskolin, which directly activates adenylyl cyclase, causing

elevation of cAMP, had no significant effect on [Sar⁹,Met$(O_2)^{11}$]-SP-stimulated accumulation of [³H]IPs in either the absence or presence of isobutylmethylxanthine (Table 1). Similarly, KT5720, an inhibitor of cAMP-dependent protein kinase (36), had no significant effect on [Sar⁹,Met$(O_2)^{11}$]-SP-stimulated accumulation of [³H]IPs (Table 1).

Site of action of PKC. To determine the site at which PKC exerts its inhibitory action, studies were carried out with AlF_4^-, which induces PLC activation by a direct effect on G proteins (37). AlF_4^- stimulated a linear accumulation of [³H]IPs, with an increase to 286 ± 15% of basal values at 30 min (Fig. 4; Table 2). Ro31-8220 significantly potentiated the response to AlF_4^- after 5 min (Fig. 4), and this reached a value of 179 ± 12% by 30 min, relative to control values. Consistent with this, PDBu reduced the response to AlF_4^- to 36 ± 6% of control values after 30 min (Table 2). These results suggest that PKC exerts an inhibitory action 'downstream' of receptor activation.

Effect of pertussis toxin. The pertussis toxin-insensitive G protein family α_q/α_{11} is thought to be involved in linking the NK_1 receptor to activation of PLC (21). Consistent with this, pretreatment of UC11 cells with pertussis toxin (3 μg/ml) for 8 hr had no significant effect on [Sar⁹,Met$(O_2)^{11}$]-SP-induced formation of [³H]IPs (accumulation of [³H]IPs in pertussis toxin-pretreated cells was 103 ± 5.1%, relative to 100 ± 2.0% in control cells), despite complete ADP-ribosylation of pertussis toxin-sensitive G proteins (Fig. 5).

Immunoprecipitation of G proteins α_q/α_{11}. To determine whether the G protein α subunits α_q/α_{11} are the site of PKC-mediated inhibition, they were immunoprecipitated (using antibody QL) from [³²P]orthophosphate-labeled UC11 cells challenged with various agents. Immunoblotting using the secondary antibody detected a band of 50–60 kDa, which is probably the IgG heavy chain fraction of the antibody used for immunoprecipitation (Fig. 6A). Using the primary antibody QL and a secondary antibody, an additional band was detected in the region of 42–43 kDa, the molecular mass of α_q/α_{11} (Fig. 6B). Immunoprecipitation was dependent on the presence of specific antiserum (QL) in the incubation.

Fig. 6C shows an autoradiograph of the PVDF membrane to which immunoprecipitated samples had been transferred. Comparison with the immunoblot (Fig. 6B) demonstrates that the G proteins α_q/α_{11} are not phosphorylated in unstimulated or [Sar⁹,Met$(O_2)^{11}$]-SP-stimulated cells at time intervals of 1 and 30 min (Fig. 6, B and C, *lanes 1-4*). Similarly, PDBu and okadaic acid had no effect on phosphorylation of α_q/α_{11} (Fig. 6, B and C, *lanes 5* and *6*).

TABLE 2

Effect of PDBu and Ro31-8220 on AlF_4^--stimulated total [³H]IP accumulation in human UC11 cells

Results are expressed as a percentage of both basal and AlF_4^--stimulated total [³H]IP accumulation during a 30-min incubation (mean ± standard error). Results are from four separate experiments performed in triplicate. Compounds used were as follows: NaF (10 mM) and $AlCl_3$ (10 μM) (AlF_4^-), PDBu (1 μM), Ro31-8220 (1 μM), and [Sar⁹,Met$(O_2)^{11}$]-(1 μM).

	Total [³H]IPs	
	% of basal	% of AlF_4^--stimulated
Basal	100 ± 4	0 ± 2
AlF_4^-	286 ± 15	100 ± 3
AlF_4^- + PDBu	160 ± 17	36 ± 6
AlF_4^- + Ro31-8220	445 ± 39	179 ± 12
[Sar⁹,Met$(O_2)^{11}$]-SP	448 ± 24	253 ± 25

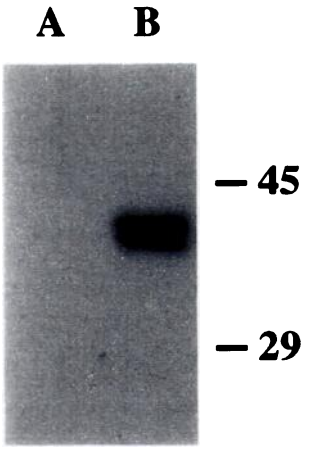

Fig. 5. [³²P]ADP-ribosylation by pertussis toxin of membrane protein from human UC11 cells. Membranes were incubated with pertussis toxin (20 μg/ml) and [³²P]NAD for 30 min at 30°. After protein precipitation, the samples were denatured and proteins were resolved by 12.5% SDS-PAGE (run for ~6 hr). The gel was stained with Coomassie blue, dried, and subjected to autoradiography for 3 hr. *Lane A*, UC11 cell membranes treated with [³²P]NAD in the absence of pertussis toxin; *lane B*, UC11 cell membranes treated with [³²P]NAD and pertussis toxin. Similar results were obtained in two other experiments. Pretreatment of UC11 cells with pertussis toxin (3 μg/ml) for 8 hr before membrane preparation completely blocked subsequent [³²P]ADP-ribosylation of substrate proteins.

Discussion

[Sar⁹,Met$(O_2)^{11}$]-SP (1 μM) stimulated a biphasic accumulation of [³H]IPs in the presence of 10 mM LiCl in human UC11 cells that had been prelabeled with [³H]inositol. The maintained level of [³H]IPs in the presence of CP-96,345 suggests that LiCl completely prevents the final step of dephosphorylation of [³H]IPs to [³H]inositol in UC11 cells; therefore, measuring [³H]IP accumulation provides a measure of PLC activity irrespective of IP metabolism. The initial rate of formation of [³H]IPs is rapid during the first 1 min, followed by a slower rate of accumulation for up to 90 min (Fig. 1). The initial rapid increase in [³H]IPs correlates with the transient peak of (1,4,5)IP_3 production described by Johnson and Johnson (25). The same authors (25) also reported that (1,4,5)IP_3 production returns to basal values within 1 min in UC11 cells, in agreement with the time course of Ca^{2+} mobilization.[2] During the sustained phase of [³H]IP accumulation, the production of (1,4,5)IP_3 may be below the threshold level detected by the assay or, alternatively, metabolism of membrane phospholipids other than phosphatidylinositol-4,5-bisphosphate may occur. These results demonstrate that the human NK_1 receptor desensitizes rapidly but only partially.

A similar biphasic time course of formation of [³H]IPs has been reported after bombesin stimulation of AR42J pancreatic acinar cells (38) and carbachol stimulation of Chinese hamster ovary cells transfected with the M_3 muscarinic receptor (39). Both reports account for this phenomenon by a rapidly developing partial desensitization of the receptor. The time course of formation of [³H]IP correlates with an initial rapid increase in (1,4,5)IP_3 followed by a plateau phase, which may account for the later phase of [³H]IP formation. The initial transient nature of the (1,4,5)IP_3 response could also reflect a receptor-regulated increase in (1,4,5)IP_3 metabolism; however, investi-

[2] A. J. Barr and S. P. Watson, unpublished observations.

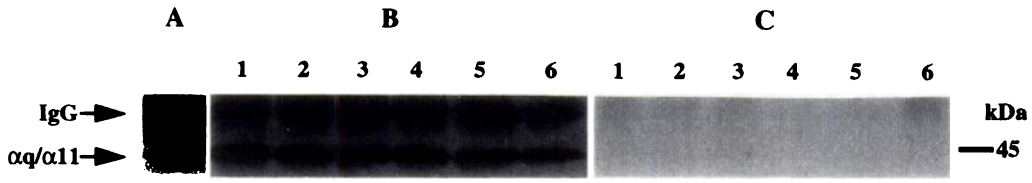

Fig. 6. Immunoprecipitation of G proteins α_q/α_{11} from [^{32}P]P_i-labeled human UC11 cells using antibody QL. A, Representative lane from immunoblot using 1/10,000 dilution (secondary antibody). B, Immunoblot of samples using primary antibody QL (1/1000) and secondary antibody (1/10,000). C, Autoradiograph of samples on PVDF membrane (5-day exposure). *Lane 1*, basal (1 min); *lane 2*, [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) (1 min); *lane 3*, basal (30 min); *lane 4*, [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) (30 min); *lane 5*, [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) plus PDBu (1 μM) (30 min); *lane 6*, [Sar^9,Met(O$_2$)^{11}]-SP (1 μM) plus okadaic acid (1 μM) (30 min). Human UC11 cells that had been labeled with [^{32}P]P_i (1 mCi/ml) for 1 hr were challenged with agents as indicated, and G proteins α_q/α_{11} were immunoprecipitated as described in Experimental Procedures.

gations into this have not uncovered convincing evidence of a general regulatory process in most cells (40).

Results with the selective PKC inhibitor Ro31–8220 indicate that PKC does not mediate rapid desensitization of the human NK_1 receptor in UC11 cells. This is in agreement with the studies of Sugiya and Putney (14) (see the introduction). During longer incubations, Ro31–8220 significantly potentiated human NK_1 receptor agonist-stimulated accumulation of [^{3}H]IPs. These results suggest that, although PKC is not involved in the process of rapid desensitization, it does have an inhibitory role at longer times. The studies with staurosporine, although complicated by a significant effect on basal levels of [^{3}H]IPs, are consistent with an inhibitory feedback action of PKC on NK_1 receptor activation of PLC.

The inhibitory effect of okadaic acid on NK_1 receptor activation of PLC activity provides additional evidence for kinase-mediated phosphorylation inhibiting NK_1 receptor activation of PLC. If the inhibitory action of okadaic acid is mediated by PKC, then Ro31–8220 should fully reverse its action. Therefore, the observation that Ro31–8220 only partially reversed the effect of okadaic acid suggests the involvement of an additional kinase whose activity is reversed by protein phosphatases 1 and 2A. In contrast, the effect of okadaic acid is fully reversed by staurosporine. The Ro31–8220-resistant inhibitory action of okadaic acid, which is sensitive to staurosporine, does not appear to be involved in NK_1 receptor-induced desensitization, because the increase in response to staurosporine is equal to the sum of that induced by Ro31–8220 and the stimulatory effect of staurosporine on basal IPs. The mechanism of stimulation of basal [^{3}H]IP accumulation observed with staurosporine indicates the presence of a constitutively active staurosporine-sensitive kinase involved in inhibitory regulation of PLC in resting cells. It is worth noting that Périanin *et al.* (41) have reported that staurosporine stimulates phospholipase D in human leukocytes.

PKC may exert its inhibitory action on NK_1 receptor activation of PLC at the level of either the receptor, the G protein, or PLC; more than one of these sites may be involved. Indirect evidence from studies with AlF_4^-, an agent that can directly activate G proteins, suggests that PKC can exert inhibitory effects downstream of receptor activation. AlF_4^--stimulated accumulation of [^{3}H]IPs was reduced in the presence of PDBu and potentiated by Ro31–8220 in UC11 cells. Immunoprecipitation studies of the G proteins (α_q/α_{11}) that are believed to link the NK_1 receptor to PLC (21) demonstrated that these proteins do not undergo phosphorylation and are therefore unlikely to be the site of inhibition. These results are in agreement with the studies of Lounsbury *et al.* (42) demonstrating that the G proteins α_q/α_{11} are not phosphorylated in permeabilized human platelets challenged with PLC-coupled receptor agonists.

PLC-β isozymes are targets for regulation by PKC in certain cells. Treatment of several cell lines with phorbol 12-myristate-13-acetate elicited a large increase in phosphorylation of PLC-β_1 but not of PLC-δ_1 or PLC-γ_1 (43). Phosphorylation of PLC-β_1 *in vitro* results in incorporation of phosphate at a single serine residue but has no effect on the catalytic activity of PLC-β_1 *in vitro* (43). It has been proposed that, rather than having an effect on enzyme activity, phosphorylation by PKC of PLC-β_1 may alter its interaction with α_q. PKC inhibitory feedback of NK_1 receptor stimulation of PLC in human UC11 cells may be mediated partly by phosphorylation of PLC-β_1, leading to its uncoupling from α_q/α_{11}.

The human NK_1 receptor, which has a number of potential phosphorylation sites for PKC, may also be a target for inhibitory feedback. Evidence suggests that PLC-coupled receptors, e.g., the α_1-adrenoceptor (44) and the cholecystokinin receptor (23, 24), can be regulated directly by PKC-mediated phosphorylation. Clearly, additional studies are required to establish the molecular target of PKC inhibitory regulation. The possible involvement of a receptor-specific kinase in the initial phase of NK_1 receptor desensitization, as suggested for the cholecystokinin receptor (23, 24), is also worthy of investigation.

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Send reprint requests to: Alastair J. Barr, Department of Pharmacology, University of Pennsylvania School of Medicine, 36th St. and Hamilton Walk, Philadelphia, PA 19104–6084.