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EFFECTS OF BRADYKININ, GTPγS, R59022 AND N-ETHYLMALEIMIDE ON INOSITOL PHOSPHATE PRODUCTION IN NG108-15 CELLS

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Summary: Accumulation of inositol phosphates in NG108-15 neuroblastoma x glioma hybrid cells, pre-labeled for 24h to equilibrium, was stimulated by bradykinin, guanosine 5'-0-(3-thiotriphosphate) and the diacylglycerol kinase inhibitor R59022. Only the stimulation by bradykinin was inhibited by the bradykinin receptor antagonist [D-Arg^{0}, Hyp^{3}, Phe^{7}, Thi^{5,8}] bradykinin. Neither bradykinin nor R059022 increased the labeling of the inositol phospholipids. The sulfhydryl-alkylating reagent N-ethylmaleimide at 100 μM essentially abolished the stimulation caused by all three agents, possibly by preventing the binding of GTP to a guanine nucleotide-binding regulatory protein of as yet unknown size.

Ligand binding to specific receptors on the surface of target cells is the first step in the signal transduction induced by peptide hormones and neurotransmitters (1). Bradykinin (BK) is one of these substances that can bind to specific receptors (2) and cause increased phosphoinositide turnover (3-6). The precise mechanism for this BK-induced hydrolysis of inositol phospholipids is not well understood, however by analogy the most likely mechanism is the following sequence: activation of the receptor through BK binding, followed by interaction with a guanine nucleotide-binding regulatory protein (G-protein) (7), resulting in a direct effect on the activity of a phosphoinositide-specific phospholipase C (8).

What reaction exists between the receptor and the effector G-protein is still unknown. Evidence has accumulated recently indicating that receptors for calcium-mobilizing hormones are linked through a G-protein to stimulate phospholipase C (9,10). In addition to ligands that activate receptors a number of other compounds, such as Ca^{2+} (11,12), guanine nucleotide (10,11), cyclic AMP (13), chlorpromazine (14), ATP (15), lithium (6,16), the diacylglycerol

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Abbreviations used: BK, bradykinin; DMEM, Dulbecco's modified Eagle's medium; G-protein, guanine nucleotide-binding regulatory protein; GTPγs, guanosine 5'-0-(3-thiotriphosphate); IP, inositol phosphate; IP_1, IP_2, IP_3, inositolmono-, -bis-, -trisphosphate; NEM, N-ethylmaleimide; , TCA, trichloroacetic acid.

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kinase inhibitor R59022 (17), NaF plus $AlCl_3$ (18) and vanadate (18), can stimulate inositol phospholipid hydrolysis in many types of cells.

The finding in certain systems that BK (3-6), the GTP analog GTPγS (10,11) and R59022 (17) stimulate phospholipase C, that BK-induced accumulation of IPs is inhibited by the BK antagonist B4881(6) and that N-ethylmaleimide (NEM) inhibits the binding of GTPγS to G-protein (19) prompted us to use NG108-15 cells as a model to study the possible mechanism by which R59022 activates phospholipase C.

We report here that BK (10 μM), R59022 (33-100 μM) and GTPγS (100 μM) cause inositol phosphate (IP) accumulation in this cell line. The effect of BK was partially blocked by the BK antagonist PBRA 88 whereas the effect of R59022 and GTPγS were not inhibited. All of the IP effects were uncoupled by NEM. From these results, it is suggested (1) that an essential sulfhydryl group is involved in the coupling between the BK receptor and a GTP-binding protein and (2) that the site of action of R59022 is not directly on phospholipase C but may be indirect either on the GTP-binding protein or on a step involved in the activation of this protein.

MATERIALS AND METHODS

Materials: R59022 is a Janssen Life Science product (Beerse, Belgium); BK, guanosine 5'-0-(3-thiotriphosphate) (GTPγs) and N-ethylmaleimide (NEM) were from Sigma Chemical Co. (St. Louis, MO); 2-[^{3}H]myo-inositol (15 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY). Dowex-1x8 resin (100-200 mesh, formate form) was from Bio-Rad (Richmond, CA). Aquasol-2 was from Du Pont Co.(Wilmington, DE). Silica gel HL was from Analtech (Wilmington, DE), the BK antagonist [D-Arg^0, Hyp^3,D-Phe^7,Thi^{5,8}]-BK (PBRA 88) was from Bachem Inc. (Torrance, CA). and fetal calf serum was from HyClone Laboratories (Logan, UT).

Cell culture conditions: NG108-15 cells were grown in six-well cluster dishes (Costar, Cambridge, MA) containing DMEM supplemented with 10% fetal calf serum, penicillin (100 u/ml), streptomycin (100 μg/ml), hypoxanthine (2.5 x 10^{-5}M), aminopterin (1x10^{-7}M), thymidine 14x10^{-5}M) and L-glutamine (2 mM). The cells were grown in a humidified atmosphere of 10% CO_2 and 90% air at 37°C.

Equilibrium labeling of IPs with 2-[^{3}H]myo-inositol: To determine the time necessary for labeling NG108-15 cells with 2-[^{3}H]myo-inositol to equilibrium, the medium was changed to DMEM containing 95 μM carrier myo-inositol 36 hours before cells became confluent. Final concentration of myo-inositol in the labeling medium was approximately 190 μM. Cells were prelabeled with tritiated myo-inositol (10 μCi/1.5 ml) for 12, 24 or 36 hours. After removal of the medium and a rapid rinse with ice-cold balanced salt solution, 15% ice-cold trichloroacetic acid (TCA) was added. After 15 minutes on ice, the TCA extract was transferred into tubes, washed three times with an equal volume of diethylether, neutralized with NH_4OH and used for the assay of IPs. Cells were harvested in 2 ml of ice-cold methanol by scraping with a rubber policeman and used for protein determination.

Lipid extraction and analysis: Two volumes of chloroform were added to the cell suspension in methanol and lipids were extracted and washed according to the procedure of Folch et al. (20). The extracted cell residues were recovered from the chloroform-methanol solution by filtration through small wads of cotton inserted into Pasteur pipettes and incubated in chloroform-methanol containing 0.25% of 12 M HCl at 37°C for 1 hour. The acid extracts were washed as described previously (21) and phospholipids were separated on silica gel HL plates with chloroform/methanol/concentrated ammonia/water (45:45:11:5.5 by volume) as the mobile phase (22). Lipids were visualized by radioautography using X-Omat RP film (Eastman Kodak Co., Rochester, NY). Tritiated lipids were revealed by fluorography after spraying the plates with 0.4% 2,5-diphenyloxazole in 2-methylnaphthalene containing 10% toluene and exposing the radioautograms at -70°C for 1-3 weeks (23).

Assay of ^{3}H-labeled IPs: The method of Berridge et al. (24) was used for the assay of IPs. Samples were added to columns containing 1 ml of Dowex-1x8 resin (formate form) and the phosphate esters were eluated by stepwise addition of formate solutions of increasing strength, after the column was washed with 8 ml of 5 mM inositol. All samples of eluate were counted for radioactivity in 10 ml of Aquasol-2.

Protein determination: Proteins were determined according to the method of Lowry et al. (25) with bovine serum albumin as standard.

Statistics: One way analysis of variance was used to analyze all the data, followed by Student-Newman-Keuls test between groups to determine significance.

RESULTS

Equilibrium labeling of IPs

The cells prelabeled with tritiated myo-inositol form three products of phospholipase C-catalyzed phosphoinositide hydrolysis, namely IP_1, IP_2, and IP_3 respectively. The accumulation of IP_1 with time, plotted in Fig.1, demonstrated that the equilibrium labeling time is 24 hours, since there is no significant difference in IP_1 accumulation between 24 and 36 hours ($p<0.05$). Prelabeling in subsequent experiments was for 24 hours followed by replacement of the medium with medium containing the agents to be tested.

Bradykinin-, R59022- and GTPγS-induced accumulation of IPs and phosphoinositide labeling

There was no effect of ethanol (2.5×10^{-3}M), used as solvent, on the accumulation of any of the IP classes when compared with control (Table 1). BK (10 μM), R59022 (100 μM) and GTPγS (100 μM) significantly stimulated ($p< 0.01$) the accumulation of IPs. The GTP analog, GTPγS (100 μM), a potent activator of G-protein (26), caused a 200% increase in the accumulation of IP_1. Since at a lower concentration of GTPγS (10 μM) the increased accumulation of IP_1 was 31% (data not shown), 100 μM ofGTPγS was used in all following experiments. The accumulation effect of R59022 on IPs was concentration-dependent between 10 and 100 μM.

In contrast to the findings with IPs BK and R59022 had no effect on labeling of inositol phospholipids (Table 2). In NG108-15 cells, PI, PIP and PIP_2 comprise 90%, 2% and 8% of total inositol phospholipids respectively.

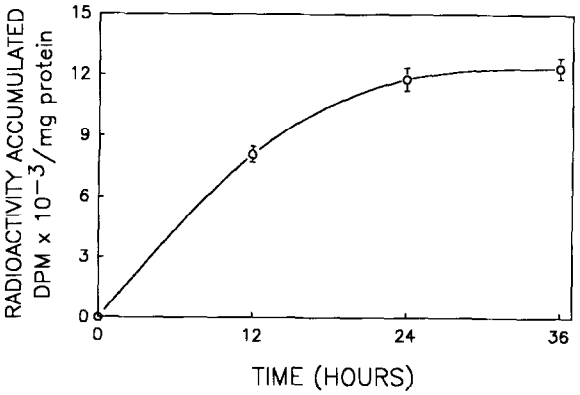

Fig. 1 Incorporation of 2-[^{3}H]myo-inositol into IPs. NG108-105 cells were incubated in media containing radioactive inositol for the times indicated as described in Materials and Methods. Total IPs were isolated by column chromatography from the TCA extract. The data points are means ± SEM from triplicate samples in each of two experiments.

TABLE 1

EFFECT OF BRADYKININ, R59022 AND GTPγS ON THE ACCUMULATION OF INOSITOL PHOSPHATES

Incubation	IP_1	IP_2	IP_3
	(DPM/10 min/mg protein)		
Control	13498 ± 678	2170 ± 151	1128 ± 91
Ethanol	13543 ± 417	1842 ± 158	1024 ± 108
Bradykinin (10 μM)	44928 ± 4458[b]	3669 ± 353[a]	2248 ± 125[a]
GTPγS (100 μM)	39545 ± 2704[b]	5126 ± 859[b]	3301 ± 549[b]
R59022 (10 μM)	16044 ± 3295	2210 ± 246	1153 ± 80
R59022 (33.3 μM)	26349 ± 5430[a]	2879 ± 310[a]	1355 ± 102
R59022 (100. μM)	31756 ± 4466[b]	4280 ± 250[b]	2147 ± 157[a]

Cells were prelabeled with tritiated myo-inositol (6.6 μCi/ml) in DMEM containing 190 μM of unlabeled myo-inositol for 24 hours. At the end of prelabeling the cells were washed and preincubated in balanced salt solution, pH 7.4, containing 25 mM LiCl and 2.5 x 10^{-3}M ethanol for 30 min. After removal of the preincubation medium, 1 ml of balanced salt solution containing 25 mM LiCl and drug at the desired concentration was added. R59022 was dissolved in ethanol and 0.3 mM HCl. This compound was present at the same concentration during both incubation periods. The BK concentration giving maximum response was used (4). GTPγS was dissolved in balanced salt solution. Incubations were terminated after 10 min with 1 ml of ice-cold TCA and IPs were separated and quantitated as described in Materials and Methods. Data are means ± SEM from two experiments, each with triplicate samples. [a]$p<0.01$, [b]$p<0.001$ as compared with control.

The effect of the bradykinin antagonist PBRA 88 and NEM on bradykinin-, R59022- and GTPγS-induced accumulation of IPs

The BK antagonist PBRA 88 (27) at a concentration of 1 μM acted as a partial agonist elevating the appearance of all radioactive IPs (Table 3). However it also partially blocked the

TABLE 2

EFFECT OF BRADYKININ AND R59022 ON THE LABELING OF INOSITOL PHOSPHOLIPIDS

Incubation	Phosphatidyl-inositol	Phosphatidyl-inositol-4-phosphate	Phosphatidyl-inositol-4,5-bisphosphate
	(CMP/10 min/mg protein)		
Control	29334 ± 2521	943 ± 60	2577 ± 414
Bradykinin (10 μM)	30196 ± 3599	1260 ± 99	3871 ± 1035
R59022 (10 μM)	32562 ± 3131	1369 ± 104	2857 ± 313
R59022 (33.3 μM)	38283 ± 5345	1148 ± 159	2905 ± 838
R59022 (100 μM)	28898 ± 3551	880 ± 129	2323 ± 400

Inositol phospholipid samples were from the same incubations as the IPs in Table 1. After prelabeling and stimulation of cells the inositol phospholipids were extracted, separated and counted as described in Materials and Methods. Data are means ± SEM from two experiments with triplicate samples each. No statistically significant differences were found.

TABLE 3

THE EFFECTS OF THE BRADYKININ ANTAGONIST PBRA 88 AND N-ETHYLMALEIMIDE ON BRADYKININ, R59022 - AND GTPγS-INDUCED ACCUMULATION OF INOSITOL PHOSPHATES

Incubation	IP_1	IP_2	IP_3
	(DPM/10 min/mg protein)		
Control	14374 ± 781	2427 ± 618	1415 ± 178
PBRA 88 (1 μM)	21073 ± 4373	4283 ± 1070	2213 ± 455
NEM (100 μM)	20332 ± 4977	1545 ± 383	1250 ± 359
BK (10 μM)	56342 ± 5676	6110 ± 560	3872 ± 282
BK + PBRA 88 (10 μM + 1 μM))	41730 ± 5738[a]	5081 ± 788	3184 ± 245
BK + NEM (10 μM + 100 μM)	18004 ± 2018[b]	1261 ± 79[b]	1007 ± 58[b]
GTPγS (100 μM)	47535 ± 4218	6699 ± 303	3900 ± 241
GTPγS + PBRA 88 (100 μM + 1 μM)	54055 ± 6110	6426 ± 262	3799 ± 255
GTPγS + NEM (100 μM + 100 μM)	22771 ± 3517[b]	2455 ± 457[b]	1460 ± 301[b]
R59022 (100 μM)	27621 ± 3085	4228 ± 716	2892 ± 277
R59022 + PBRA 88 (100 μM + 1 μM)	33078 ± 4664	4709 ± 874	2681 ± 208
R59022 + NEM (100 μM + 100 μM)	14653 ± 1460[b]	1373 ± 40[b]	1861 ± 67[b]

Conditions for prelabeling and incubation of cells and isolation of IPs were as given in the legend to Table 1. PBRA 88 (1 μM) was included in both incubations. NEM was included only in the second incubation. Data are means ± SEM from five samples. [a]$p<0.01$; [b]$p<0.001$ as compared with identical incubations without inhibitor. Stimulation by BK, GTPγS and R59022 was similar to that shown in Table 1.

accumulation of IP_1 induced by 10 μM BK but had no effect on the accumulation of IP_2 and IP_3. The R59022- and GTPγS-induced accumulation of IPs was unaffected by this compound. The failure of PBRA 88 to block the accumulation of IPs induced by GTPγS and R59022 indicates that the action of GTPγS and R59022 does not occur through activation of BK receptors.

In the presence of NEM (100 μM), the BK-, R59022- and GTPγS-induced accumulation of IP_1 was essentially completely abolished when compared with the drug-stimulated group. NEM also inhibited the accumulation of IP_2 and IP_3.

DISCUSSION

For the experiments reported here it was important to label inositol-containing phospholipids to equilibrium. In PC12 cells this was accomplished in 24 hours when 80 μM unlabeled myo-inositol was added to the culture media and incorporation of [^{3}H]myo-inositol into phosphatidylinositol was measured (28). In our experiments the production of IP_1 wasused as indicator with the final concentration of myo-inositol adjusted to 190 μM and 24 hours was adequate for equilibrium to be established.

When the accumulation of IPs induced by BK, R59022 or GTPγS was to be measured, LiCl was included in the incubation because it has been demonstrated that LiCl prolonged carbachol-induced accumulation of IP_1, as well as that of IP_2 and IP_3, in cultured cerebellar granule cells (29) and BK-induced formation of IPs in NCB-20 cells (6). Lithium has been

shown both to inhibit the enzyme that hydrolyzes IP_1 (30) and to stimulate phospholipase C (6, 16), thus augmenting the accumulation of IP_1 during stimulation.

In this report, we have shown that in the presence of BK the accumulation of IP_1 is increased when compared with control which confirms the result of Chuang and Ora (6). In our experiments, the BK antagonist PBRA 88 partially inhibited the effect of BK but not that of GTPγs or R59022 on IP_1 accumulation. This result indicated that GTPγS and R59022 bypass the receptor binding step in the stimulation of phospholipase C. The inhibitory effect of PBRA 88 on BK-induced IP_1 accumulation has also been demonstrated in neurohybrid NCB-20 cells (6) and the F-11 hybrid line (31) in the nanomolar to micromolar range. The accumulation of IPs in the presence of R59022 was concentration-dependent (Table 1), leading to similar conclusions as the report (17) on human neutrophils, stimulated with fMet-Leu-Phe. However, R59022 at a lower concentration (10 μM) exhibited no accumulation effect.

NEM prevented the R59022-induced accumulation of IP_1. The effect of R59022 on phospholipase C is not by direct stimulation since its effect was blocked by NEM, a compound which inhibits the binding of GTP to GTP-binding proteins in human platelets (18). NEM is a sulfhydryl-alkylating reagent which has been reported to uncouple the interactions between opioid (32) and α_2 adrenergic receptors (33) and the GTP-binding regulatory protein and to inhibit calcium influx induced by collagen in rabbit platelets (34). The concentration of NEM used for most experiments is from 10 μM to 1 mM (32-35). In general, to study the GTPγS effect, permeabilized cells and low concentrations of GTPγS have been used, for example, 51 μM in permeabilized vascular smooth muscle (36). In our experiments, we were able to demonstrate that phospholipase C is activated by the GTP analog GTPγS in nonpermeabilized NG108-15 cells with higher GTPγS concentration (100 μM). It has been reported that ATP concentrations higher than 50 μM are able to permeabilize the plasma membranes of rat mast cells to some extent (37) and a similar effect could have occurred in our NG108-15 cells.

Drugs that interfere with GTPγS binding to G-protein are possible inhibitors of IP accumulation, if a G-protein is involved in signal transduction between the receptor and phospholipase C. It has been suggested that at least one member of the heterotrimeric G-protein family is involved in phospholipase C activation, but the exact type of G-protein responsible for this regulation has not been definitively determined. A G-protein sensitive to ADP-ribosylation by pertussis toxin has been suggested (38-40). NEM not only blocked the GTPγS-induced IP accumulation but also the BK- and R59022-induced IP effect. This result suggest that all three agents stimulating phospholipase C were uncoupled from a G-protein by NEM which prevented the binding of GTP in this system. Since NEM specifically blocked binding of GTPγS to a small molecular weight (22 kDa) G-protein in human platelet membranes (19), our result on NEM inhibition of IP accumulation induced by GTPγS may indicate that a small molecular weight G-protein is the putative G-protein between the receptor and phospholipase C that is activated by GTPγS and R59022 in NG108-15 cells. Further evidence to support this hypothesis will be required. Since GTPγS activates phospholipase C in our experimental system, it is reasonable to speculate that factors which can affect the GTP level should be considered as possible regulators of phospholipase C. Thus, membrane-associated nucleoside diphosphate kinase

may play a role in influencing BK- and R59022-induced accumulation of IP by providing GTP for G-protein activation, a possiblility that still needs to be examined.

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