Journal of Neurochemistry
Raven Press, Ltd., New York

Identification of Multiple Phosphoinositide-Linked Receptors on Human SK-N-MC Neuroepithelioma Cells

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Abstract: The biochemical and pharmacological characteristics of receptor-stimulated phosphoinositide (PPI) hydrolysis in human SK-N-MC neuroepithelioma cells have been examined. Of 11 ligands tested, the addition of four, i.e., norepinephrine, oxotremorine-M, endothelin-1, and ATP, each resulted in an increased release (three- to eightfold) of inositol phosphates from [^{3}H]inositol-prelabeled cells. Agonist-stimulated PPI turnover was sustained for at least 30 min and required the addition of Ca^{2+} for full effect. An increased release of inositol phosphates could also be elicited by the addition of the Ca^{2+} ionophore, ionomycin. All four agonists enhanced the release of radiolabeled inositol mono- and bisphosphates, inositol 1,3,4-trisphosphate, and inositol tetrakisphosphate. Increases in inositol 1,4,5-trisphosphate were smaller and only consistently observed in the presence of norepinephrine or oxotremorine-M. Norepinephrine-stimulated PPI turnover was potently inhibited by prazosin, WB-4101, and 5-methylurapidil (K_i < 2.5 n*M*), but was relatively insensitive to chloroethylclonidine pretreatment. This pharmacological profile is consistent with the involvement of an α_{1A}-receptor subtype. The presence of an M_1 muscarinic cholinergic receptor is also indicated, because pirenzepine blocked oxotremorine-M-stimulated inositol phosphate release (K_i = 35 n*M*) with a 30-fold greater potency than the M_2-selective antagonist, AF-DX 116. Of the three endothelins tested, only the addition of endothelin-1 and endothelin-2 promoted PPI hydrolysis, whereas endothelin-3 was essentially inactive. A P_2 nucleotide receptor of broad agonist specificity is also present on these cells and activates PPI turnover in the absence of a generalized increase in plasma membrane permeability. These results indicate that SK-N-MC cells express at least four PPI-linked receptors. Because the functional coupling of three of these receptors, i.e., α_{1A}-adrenergic, endothelin, and P_2 nucleotide, has not been extensively characterized previously in neural tissues, the SK-N-MC cell line may provide a useful model system for studies of these receptors and their regulation. **Key Words:** Phosphoinositide hydrolysis—Neuroepithelioma—M_1 muscarinic cholinergic receptor—α_{1A}-Adrenergic receptor—P_2 nucleotide receptor—Endothelin. **Fisher S. K. and Landon R. E.** Identification of multiple phosphoinositide-linked receptors on human SK-N-MC neuroepithelioma cells. *J. Neurochem,* **57,** 1599–1608 (1991).

The CNS and other neural-related tissues possess a large number of pharmacologically distinct receptors coupled to phosphoinositide (PPI) hydrolysis. Activation of these receptors elicits a phosphodiesteratic breakdown of phosphatidylinositol 4,5-bisphosphate with the concomitant formation of two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (for review, see Fisher and Agranoff, 1987). Due to the complexity of the nervous system, the use of homogeneous cultured neurotumor cell lines, such as neuroblastomas and astrocytomas, has proved invaluable both in the identification of individual receptor subtypes coupled to PPI turnover and in studies of their regulation. However, although the characterization of several receptor subtypes linked to PPI turnover has been facilitated by this approach, for other functionally linked receptors, few if any suitable neural cell lines are presently available. In the present study, we have investigated the presence of PPI-linked receptors on human SK-N-MC neuroepithelioma cells. Although previously designated as a neuroblastoma (Biedler et al., 1973), this cell line is more appropriately

Received January 10, 1991; revised manuscript received April 4, 1991; accepted April 8, 1991.

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Abbreviations used: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); AF-DX 116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Epi, epinephrine; ET, endothelin; fura-2/AM, pentaacetoxymethyl ester derivative of fura-2; IP_1, 1-D-*myo*-inositol monophosphate; IP_2, 1-D-*myo*-inositol bisphosphate; IP_3, 1-D-*myo*-inositol trisphosphate; IP_4, 1-D-*myo*-inositol tetrakisphosphate (isomeric positioning of phosphate groups are indicated when appropriate); 2MeSATP, 2-methylthioadenosine 5′-triphosphate; NE, norepinephrine; Oxo-M, oxotremorine-M; PPI, phosphoinositide (phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate); TCA, trichloroacetic acid.

defined as a neuroepithelioma (McKeon et al., 1988). The results indicate that SK-N-MC cells express at least four distinct receptors linked to PPI hydrolysis, one of which is the muscarinic M_1 subtype, previously identified on a number of other neurotumor cell lines. In addition, a P_2 nucleotide receptor is functionally linked, as well as two other receptor subtypes not yet established to operate through enhanced PPI turnover in neural tissues, i.e., an α_{1A}-adrenergic receptor and an endothelin (ET) receptor subtype selectively activated by ET-1 and ET-2.

MATERIALS AND METHODS

SK-N-MC cells (passage no. 42) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.), *myo*-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.) or Amersham Corp. (Arlington Heights, IL, U.S.A.). The following radiolabeled inositol phosphates [1-D-*myo*-inositol monophosphate (IP_1), 1-D-*myo*-inositol bisphosphate (IP_2), 1-D-*myo*-inositol trisphosphate (IP_3), and 1-D-*myo*-inositol tetrakisphosphate (IP_4)] were obtained from New England Nuclear (Boston, MA, U.S.A.): [2-^{3}H]I(1)P_1 (5 Ci/mmol), [2-^{3}H]I(4)P_1 (4.5 Ci/mmol), [2-^{3}H]I(1,4)P_2 (4.5 Ci/mmol), [1-^{3}H]I(1,4,5)P_3 (20 Ci/mmol), [1-^{3}H]I(1,3,4)P_3 (17 Ci/mmol), and [2-^{3}H]I(1,3,4,5)P_4 (5 Ci/mmol). (−)-Norepinephrine (NE), serotonin, histamine, Arg^8-vasopressin, substance P, bombesin, neurotensin, ATP, ADP, GTP, ITP, XTP, UTP, AMP, adenosine, phenylephrine, bethanechol, arecoline, digitonin, and (−)-isoproterenol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chloroethylclonidine, (−)-epinephrine (Epi) bitartrate, clonidine, WB-4101, 5-methylurapidil, 6-fluonorepinephrine, prazosin, yohimbine, phentolamine, oxotremorine-M (Oxo-M), adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), and 2-methylthioadenosine 5′-triphosphate (2MeSATP) were purchased from Research Biochemicals, Inc. (Natick, MA, U.S.A.). Ionomycin and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were purchased from CalBiochem (San Diego, CA, U.S.A.). ET-1, ET-2, and ET-3 were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). Phytic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and a partial hydrolysate was prepared as previously described (Heacock et al., 1990). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY, U.S.A.). Powdered Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY, U.S.A.). Dowex-1 (100–200 mesh, X8 in the formate form) was obtained from Bio-Rad (Rockville Center, NY, U.S.A.). The pentaacetoxymethyl ester derivative of fura-2 (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Pirenzepine and 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepin-6-one (AF-DX 116) were obtained from Boehringer-Ingelheim (Ridgefield, CT, U.S.A.).

Cell culture conditions

Human SK-N-MC neuroepithelioma cells (passage numbers 43–67; Biedler et al., 1973) were grown in tissue culture flasks (75 cm^2/250 ml) in 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% (by volume) fetal bovine serum (medium I). Cells were grown for 5–14 days at 37°C in an atmosphere consisting of 10% CO_2 and 90% humidified air. Subculture was achieved by aspirating medium I and isolating the cells following incubation in a modified Puck's D_1 solution containing 0.5 m*M* EDTA and 0.05% trypsin (medium II; Honegger and Richelson, 1976). (Although the use of trypsin was essential for efficient detachment of the neuroepithelioma cells, the possibility of its adverse effect on receptor-effector coupling events, while unlikely, cannot be excluded.) After centrifugation of the cell suspension for 1 min at 300 *g*, the supernatant was removed and cells resuspended in 10 ml of medium I which was then inoculated into flasks (one confluent flask into five to 10 flasks) on day 0. The culture medium was changed on day 4 and on alternate days thereafter by the addition of 10 ml of fresh medium I and removal of 10 ml of medium.

Measurement of PPI turnover

SK-N-MC cells were allowed to prelabel for 2–3 days in Dulbecco's modified Eagle's medium/10% fetal bovine serum containing 2.5–10 μCi/ml [^{3}H]inositol. In preliminary experiments, it was determined that a 24-h labeling period was sufficient for the lipids to attain an isotopic equilibrium of labeling. Cells were detached in medium II and washed twice in buffer A (142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 5.6 m*M* D-glucose, and 30 m*M* sodium HEPES buffer, pH 7.4). Cells were then resuspended in buffer A and incubated at 37°C in the presence of agonists and/or antagonists (final volume 0.5 ml). The accumulation of ^{3}H-inositol phosphates was then monitored in the presence of Li^+, as previously described (Thompson and Fisher, 1990). Unless stated otherwise, a 30-min incubation period was routinely employed. In some experiments, the individual inositol phosphate isomers were separated by HPLC and quantitated as previously described (Fisher et al., 1990). Reactions were terminated by the addition of 2 ml of ice-cold 0.9% NaCl; the cells were centrifuged for 3 min at 500 *g* and the supernatants removed by aspiration. To the cell pellets was added 0.5 ml of 5% trichloroacetic acid (TCA), and the tubes were left on ice for 20 min to allow precipitation of protein. Inclusion of 200 μg of phytate hydrolysate at this stage optimized the recoveries of IP_3 and IP_4 (Horstman et al., 1988). Inositol phosphate isomers present in neutralized TCA extracts were then separated on a Whatman Partisil 10 SAX column. Radioactivity was monitored either on-line by means of a Beckman System Gold 171 Flow Counter, with an efficiency of 20–30% with Beckman Ready Flow III scintillation fluid (ratio of scintillant/eluate, 4:1) or, alternatively, fractions were collected and radioactivity determined directly by liquid scintillation counting. Inositol phosphate isomers were identified from the elution times of authentic radiolabeled standards, i.e., I(1)P_1, I(4)P_1, I(1,4)P_2, I(1,3,4)P_3, I(1,4,5)P_3, and I(1,3,4,5)P_4.

Measurement of fura-2 efflux from SK-N-MC cells

SK-N-MC cells were loaded with 1 μ*M* fura-2/AM for 15 min at 37°C under conditions previously described for SK-N-SH cells (Fisher et al., 1989). The SK-N-MC cells were then washed twice with buffer A to remove excess fura-2/AM and resuspended in fresh buffer A. Aliquots of fura-2-loaded SK-N-MC cells were then allowed to incubate at 37°C for 30 min in the presence or absence of 1 m*M* ATP. At the end of this time interval, cells were centrifuged at 300 *g* for 5 min and supernatants collected. Fluorescence of supernatants ($\lambda_{excitation}$ = 340 nm, $\lambda_{emission}$ = 490 nm) was monitored in a Shimadzu spectrophotofluorometer. The release of fura-2 was calculated as a percentage of dye initially present (ob-

tained from supernatant fluorescence following the addition of 10 μM digitonin). The concentration of free Ca^{2+} in buffer solutions was determined directly by fura-2 (free acid) fluorescence (Fisher et al., 1989). Protein was determined by the method of Geiger and Bessman (1972).

Data analysis

Values quoted are means ± SEM for the number of separate experiments performed. Student's two-tailed t tests were used to evaluate the statistical differences of the means of unpaired sets of data. The release of inositol phosphates is expressed as a percentage of control and is calculated from the equation:

$$\text{inositol phosphate release} = \frac{A - Z}{B - Z} \times 100$$

where A and B are the values obtained for inositol phosphate release in the presence of the agonist (A) or absence of the agonist (B) after a 30-min incubation period, and Z is inositol phosphate release obtained at zero time. Calculation of antagonist inhibition constants were derived from the equation: $K_i = IC_{50}/(1 + [A]/EC_{50})$, where IC_{50} is the concentration of antagonist required for a 50% inhibition, $[A]$ is the agonist concentration, and EC_{50} is the concentration of agonist required for half-maximal stimulation of PPI turnover (Cheng and Prusoff, 1973). The K_i values so obtained are apparent in that two assumptions are made: (a) a competitive interaction exists between the agonist and antagonist, and (b) there is an absence of any substantial receptor reserve for PPI turnover. In all situations examined to date, the second assumption holds true (Fisher and Agranoff, 1987). Dose–response and dose–inhibition curves were analyzed by the GraphPad InPlot curve-fitting program.

RESULTS

Biochemical characteristics of ligand-activated PPI hydrolysis in SK-N-MC cells

Of 11 ligands tested, the addition of optimal concentrations of four, i.e., NE, Oxo-M, ET-1, and ATP, each elicited an increased release of ^{3}H-inositol phosphates from SK-N-MC cells that had been allowed to label to isotopic equilibrium with [^{3}H]inositol (Table 1). Stimulation of PPI turnover by these agonists, although variable in magnitude between experiments, was observed consistently. In contrast, the addition of serotonin, histamine, quisqualate, vasopressin, substance P, bombesin, or neurotensin had little or no effect on inositol phosphate release. Activation of PPI turnover by each of the four active ligands required the addition of Ca^{2+} for a full effect. Thus, when Ca^{2+} was omitted from the incubation medium (free Ca^{2+} ~1 μM), the stimulation of ^{3}H-inositol phosphate release elicited by the addition of NE, Oxo-M, ET-1, and ATP was inhibited by 36 ± 9%, 56 ± 5%, 57 ± 6%, and 57 ± 12%, respectively (n = 3). When 1 mM EGTA was added, stimulated PPI turnover was inhibited further (data not shown). The addition of 10 μM ionomycin also increased the release of inositol phosphates to 351 ± 83% of control (n = 4).

Both basal and agonist-stimulated inositol phosphate release proceeded approximately linearly with time over a 60-min period with no evidence of receptor desensitization (Fig. 1). Unless stated otherwise, a 30-min incubation time was chosen routinely. Although the release of a total inositol phosphate fraction was monitored routinely, in some experiments the individual inositol phosphate isomers present were also identified by HPLC (Fig. 2). All four agonists elicited an increased release of [^{3}H]I(1/3)P_1 (an enantiomeric pair), [^{3}H]I(4)P_1, [^{3}H]IP_2, [^{3}H]I(1,3,4)P_3, and [^{3}H]IP_4, the largest increases (on a fold basis) being observed for I(1/3)P_1, I(4)P_1, I(1,3,4)P_3, and IP_4 (Table 2). An increased release of labeled I(1,4,5)P_3 was observed frequently, but not consistently, at the 30-min time point selected.

TABLE 1. *Ligand-stimulated ^{3}H-inositol phosphate formation in SK-N-MC cells*

Ligand	Concentration	Release of inositol phosphates (% of control)
NE	1 mM	723 ± 49 (n = 48)
Oxo-M	1 mM	879 ± 88 (n = 30)
ET-1	0.5 μM	678 ± 61 (n = 19)
ATP	1 mM	401 ± 34 (n = 20)
Serotonin	1 mM	94 ± 13 (n = 4)
Histamine	1 mM	128 ± 21 (n = 4)
Quisqualate	0.3 mM	97 ± 10 (n = 3)
Substance P	1 μM	112 ± 20 (n = 3)
Vasopressin	1 μM	118 ± 7 (n = 3)
Bombesin	1 μM	92 ± 9 (n = 3)
Neurotensin	50 μM	114 ± 11 (n = 3)

Prelabeled SK-N-MC cells (~1 mg of protein) were incubated for 30 min at 37° with the ligands as indicated. Reactions were terminated by the addition of an equal volume of 20% TCA, and labeled inositol phosphates present in the neutralized TCA extracts were quantitated by anion-exchange chromatography. Results are expressed as release of inositol phosphates relative to control incubations for the number of separate experiments indicated.

Pharmacological identification of receptor subtypes coupled to PPI turnover

Adrenergic. The addition of either NE or Epi to SK-N-MC cells resulted in a concentration-dependent increase in ^{3}H-inositol phosphate release (Fig. 3). The EC_{50} for NE was 6.3 ± 0.7 μM (n = 3), whereas that for Epi was 2 μM (n = 2). As previously noted for brain slices (Minneman and Johnson, 1984), Epi was found consistently to be a more efficacious agonist than NE. Other adrenergic agonists, such as 6-fluonorepinephrine and phenylephrine (selective for the α_1 subtype), were partial agonists for stimulated PPI turnover. Neither the addition of the α_2-selective agonist clonidine nor the β-selective agonist isoproterenol stimulated PPI hydrolysis (Table 3). Further evidence for the involvement of an α_1-receptor subtype was obtained from antagonist studies. Addition of phentolamine, a nonselective α antagonist, blocked NE-stimulated PPI turnover with a K_i of 11 nM. In contrast, the α_2-selective antagonist yohimbine inhibited with a 38-fold lower potency (K_i = 416 nM; Fig. 4). Because at least

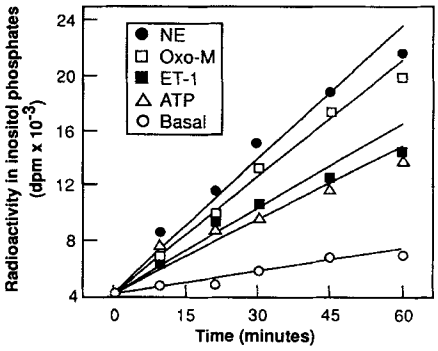

FIG. 1. Time course of basal and ligand-stimulated ^{3}H-inositol phosphate formation. Prelabeled SK-N-MC cells (~1 mg of protein) were incubated in either the absence (basal) or presence of NE (1 m*M*), Oxo-M (1 m*M*), ET-1 (0.5 μ*M*), or ATP (1 m*M*) for the times indicated at 37°C. Reactions were terminated by the addition of an equal volume of 20% TCA, and a total inositol phosphate fraction present in neutralized extracts was quantitated by anion-exchange chromatography. Values shown are means of triplicate determinations (SEM < 10% of means).

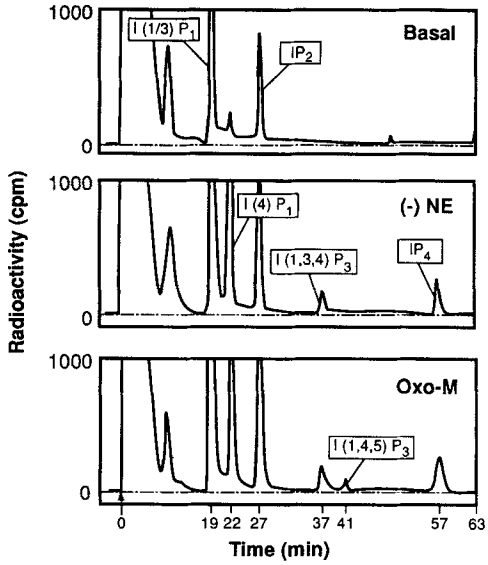

FIG. 2. HPLC separation of inositol phosphate isomers. Prelabeled SK-N-MC cells (~5 mg of protein) were incubated for 30 min at 37°C in either the absence (basal) or presence of NE (1 m*M*) or Oxo-M (1 m*M*). Reactions were terminated by the addition of ice-cold 0.9% NaCl; cells were centrifuged and supernatants removed by aspiration. Inositol phosphates were extracted from the cell pellets following the addition of 5% TCA (and 200 μg of phytate hydrolysate), and isomers present in the neutralized TCA extracts were separated by HPLC. Elution patterns shown are from one of three experiments that gave similar results. The isomer profiles obtained for ET-1 and ATP were qualitatively similar to those of NE and Oxo-M. Note that increases in I(1,4,5)P_3 were not observed consistently, whereas increases in I(1/3)P_1, I(4)P_1, IP_2, I(1,3,4)P_3, and IP_4 were always detected.

two α_1-receptor subtypes (α_{1A} and α_{1B}) can now be differentiated on the basis of antagonist potency, we evaluated the abilities of WB-4101, prazosin, and 5-methylurapidil to block NE-stimulated inositol phosphate formation. All three antagonists were potent inhibitors. 5-Methylurapidil (K_i = 2.2 n*M*), which exhibits most selectivity for the α_{1A} subtype (Hanft and Gross, 1989), was almost as effective as either prazosin (K_i = 0.6 n*M*) or WB-4101 (K_i = 0.9 n*M*) in inhibiting the PPI response (Fig. 5). The Hill coefficients for inhibition were close to 1 (0.84–0.98), indicating that these antagonists bound predominantly to a single form of the receptor. The α_{1A} and α_{1B} subtypes have also been differentiated on the basis of their susceptibilities to the alkylating agent chloroethylclonidine, the α_{1B} subtype exhibiting a greater sensitivity (Han et al., 1990). Pretreatment of SK-N-MC cells with 50 μ*M* chloroethylclonidine for 30 min resulted in a modest (36%) inhibition of NE-stimulated ^{3}H-inositol phosphate release in SK-N-MC cells (Table 4). Although neither ET-1- nor ATP-stimulated PPI turnover was influenced by chloroethylclonidine pretreatment, there was a 21% reduction of Oxo-M-stimulated inositol

TABLE 2. *HPLC separation and quantitation of inositol phosphate isomers formed in SK-N-MC cells*

	Radioactivity (cpm)				
	Basal	NE	Oxo-M	ET-1	ATP
I(1/3)P_1	6,288 ± 241	23,042 ± 689	46,715 ± 1069	21,951 ± 572	14,938 ± 444
I(4)P_1	314 ± 314	6,489 ± 506	15,898 ± 1178	5,835 ± 336	2,806 ± 212
IP_2	3,175 ± 410	6,413 ± 443	14,832 ± 1090	5,070 ± 314	4,920 ± 384
I(1,3,4)P_3	298 ± 54	1,088 ± 16	1,977 ± 79	709 ± 22	461 ± 20
I(1,4,5)P_3	517 ± 44	751 ± 42	1,120 ± 36	645 ± 62[a]	526 ± 66[a]
IP_4	693 ± 102	2,132 ± 86	3,822 ± 180	1,513 ± 49	1,247 ± 33

SK-N-MC cells were prelabeled with [^{3}H]inositol (10 μCi/ml) for 3 days. Cells (~5 mg of protein) were incubated with either NE (1 m*M*), Oxo-M (1 m*M*), ET-1 (0.5 μ*M*), or ATP (1 m*M*) for 30 min. Reactions were terminated by the addition of ice-cold 0.9% NaCl; cells were centrifuged and supernatants removed by aspiration. Inositol phosphates were extracted from the cell pellets following the addition of 5% TCA (and 200 μg of phytate hydrolysate), and isomers present in the neutralized TCA exctracts were separated by HPLC. Results shown are means ± SEM for triplicate determinations from one of three experiments that gave similar results. For all four agonists, the release of individual inositol phosphate isomers was statistically different from that obtained for control incubations ($p < 0.05$), with the exception of those instances marked with an *a*.

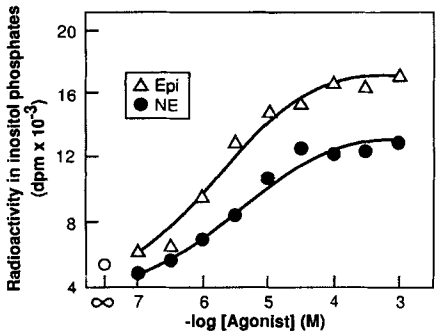

FIG. 3. Concentration dependence of NE- and Epi-stimulated inositol phosphate formation. SK-N-MC cells were incubated in either the absence (○, basal) or presence of NE or Epi at the indicated concentrations for 30 min at 37°C. Reactions were terminated and inositol phosphates quantitated as described in the legend to Fig. 1. Values shown are means of triplicate determinations (SEM < 5% of mean). The calculated EC_{50} values were 2 and 4 μM for Epi and NE, respectively. The corresponding Hill coefficients were 0.99 and 1.11, respectively.

phosphate formation, indicating that the effects of alkylation may not be restricted solely to the adrenergic receptor in this cell line.

Muscarinic cholinergic. Addition of Oxo-M resulted in a concentration-dependent increase in inositol phosphate release with an EC_{50} value of 20 ± 3 μM (n = 4). Bethanechol (10 mM) and arecoline (1 mM) were partial agonists (276 ± 80% and 202 ± 62% of control, respectively; n = 3). Pirenzepine, an M_1-selective antagonist, potently inhibited Oxo-M-stimulated PPI turnover (K_i = 35 nM). In contrast, AF-DX 116, an antagonist selective for M_2 receptors, inhibited with a 30-fold lower affinity (K_i = 1.04 μM; Fig. 6).

ET. Of the three ETs tested, ET-1 and ET-2 were markedly more effective activators of PPI hydrolysis in SK-N-MC cells than ET-3. Thus, at a concentration of 0.5 μM, addition of ET-1 increased the release of ^{3}H-inositol phosphates to 678 ± 61% of control (Table 1), whereas the corresponding values obtained for ET-2 and ET-3 were 621 ± 78% (n = 6) and 154 ± 15 (n = 4), respectively. The EC_{50} values obtained for ET-1 and ET-2 were similar (~10 nM; Fig. 7). When *both* ET-1 and ET-2 were present in the incubations, no further increase in inositol phosphate release was observed compared with that obtained in the presence of either alone, indicating that both ligands interact with a common receptor site (Fig. 8). Inclusion of 0.5 μM ET-3 did not reduce the effectiveness with which either ET-1 or ET-2 stimulated PPI hydrolysis, indicating that at this concentration ET-3 does not behave as a competitive partial agonist.

Nucleotide. The addition of ATP resulted in a dose-dependent increase in ^{3}H-inositol phosphate release with an EC_{50} value of ~100 μM. 2MeSATP, a putative P_{2y} purinergic agonist, also enhanced PPI turnover with a potency similar to that obtained for ATP (Fig. 9). ATPγS, ADP, and ADPβS were also effective (Table 5). A combination of 1 mM ATP plus 1 mM ADP did not result in a stimulation of PPI turnover greater than that elicited by ATP alone. Other purine nucleotides, such as GTP, ITP, and XTP, were able to promote PPI turnover, as was the pyrimidine UTP. AMP was only marginally effective, whereas adenosine was inactive, thereby excluding the involvement of a P_1 purinoceptor. The addition of ATP (but not other purine nucleotides) can induce a generalized increase in permeability of cell plasma membranes due to the formation of ATP^{4-} (Cockcroft and Gomperts, 1979;

TABLE 3. *Differential effects of adrenergic agonists on PPI turnover in SK-N-MC cells*

Ligand	Concentration	Release of inositol phosphates: % of control	Release of inositol phosphates: % of NE response
NE	1 mM	723 ± 49 (48)[a]	(100)
Epi	1 mM	1,113 ± 170 (6)	149 ± 7
6-Fluonorepinephrine	0.1 mM	478 ± 192 (3)	51 ± 5
6-Fluonorepinephrine	1 mM	459 ± 78 (4)	38 ± 6
Phenylephrine	0.1 mM	339 ± 76 (5)	33 ± 6
Phenylephrine	1 mM	272 ± 73 (5)	27 ± 4
Clonidine	0.1 mM	118 ± 19 (3)	4 ± 4
Clonidine	1 mM	99 ± 40 (3)	5 ± 5
Isoproterenol	10 μM	109 ± 37 (3)	6 ± 5
Isoproterenol	0.1 mM	141 ± 36 (3)	10 ± 7

SK-N-MC cells (~1 mg of protein) were incubated for 30 min at 37°C in either the absence or presence of the adrenergic ligands indicated. Reactions were terminated and inositol phosphates quantitated as described in the footnote to Table 1. Results are expressed either as release of inositol phosphates relative to control incubations or, alternatively, as the release relative to that obtained for NE in the same experiment. The number of separate experiments performed is indicated in parentheses.

[a] Data taken from Table 1.

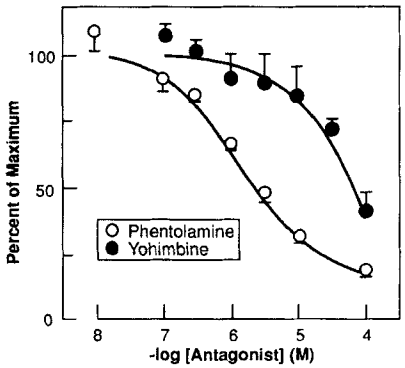

FIG. 4. Phentolamine and yohimbine inhibition of NE-stimulated PPI turnover in SK-N-MC cells. SK-N-MC cells were incubated for 30 min at 37°C in the presence of 1 m*M* NE and phentolamine or yohimbine at the concentrations indicated. Results are expressed as percentage of maximum response (obtained with NE alone) as a function of antagonist concentration. Values shown are the means ± SEM for three to four separate experiments. Neither phentolamine nor yohimbine had any effect on ^{3}H-inositol phosphate release when added alone at the highest concentration used. The calculated K_i values for phentolamine and yohimbine were 11 and 416 n*M*, respectively. The corresponding Hill coefficients were 0.85 and 0.94, respectively.

Greenberg et al., 1988). To determine whether SK-N-MC cells became leaky in the presence of ATP, the release of fura-2 (from dye-loaded cells) into extracellular fluid was monitored. Over a 30-min incubation period at 37°C, 53 ± 7% of total fura-2 initially loaded into control cells leaked into the surrounding medium. In the presence of ATP, no further increase in fura-2 leakage occurred (51 ± 7%, n = 3).

DISCUSSION

The selective ability of four groups of pharmacologically distinct ligands to enhance inositol lipid turnover indicates the presence of multiple PPI-linked receptors on SK-N-MC cells, the characteristics of which are discussed below. Although increases in I(1,4,5)P_3 production were not robust, it appears likely that the addition of each ligand promotes the Ca^{2+}-dependent hydrolysis of polyphosphoinositides, as evident from the stimulated formation of I(4)P_1, IP_2, I(1,3,4)P_3, and IP_4. The *minimum* contribution of polyphosphoinositides to inositol phosphate production (36–45%) can be calculated from the sum of radioactivities associated with these inositol phosphate isomers. However, because this calculation ignores [^{3}H]I(1/3)P_1 formed from sequential dephosphorylations of I(1,3,4)P_3 (Shears, 1989), it is likely to be an underestimate of the true contribution of the polyphosphoinositides. The formation of IP_4 and

TABLE 4. *Effect of chloroethylclonidine pretreatment on agonist-stimulated PPI turnover*

	Release of inositol phosphates (% of untreated cells)
NE	64 ± 2[a] (4)
Oxo-M	79 ± 1[a] (4)
ET-1	102 ± 6 (3)
ATP	118 ± 14 (3)

SK-N-MC cells were incubated in the presence of 50 μ*M* chloroethylclonidine or buffer A for 30 min at 37°C. Both groups of cells were then washed twice with buffer A and challenged with either NE (1 m*M*), Oxo-M (1 m*M*), ET-1 (0.5 μ*M*), or ATP (1 m*M*) for 30 min at 37°C. Reactions were terminated and inositol phosphates quantitated as described in the footnote to Table 1. Results are expressed as stimulated inositol phosphate release obtained in the chloroethylclonidine-treated cells relative to that observed for control cells. The number of separate experiments performed is indicated in parentheses.

[a] Statistically different from untreated cells ($p < 0.02$).

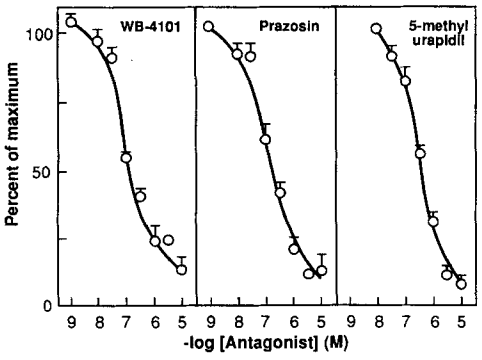

FIG. 5. WB-4101, prazosin, and 5-methylurapidil inhibition of NE-stimulated PPI turnover. SK-N-MC cells were incubated for 30 min at 37°C in the presence of 1 m*M* NE and the antagonists at the concentrations indicated. Results are expressed as percentage of maximum response (obtained with NE alone) as a function of antagonist concentration. Values shown are the means ± SEM for four or five separate experiments. Where no error bar is shown, the SEM fell within the symbol. The calculated K_i values for WB-4101, prazosin, and 5-methylurapidil were 0.6, 0.9, and 2.2 n*M*, respectively. The corresponding Hill coefficients were 0.84, 0.96, and 0.98, respectively.

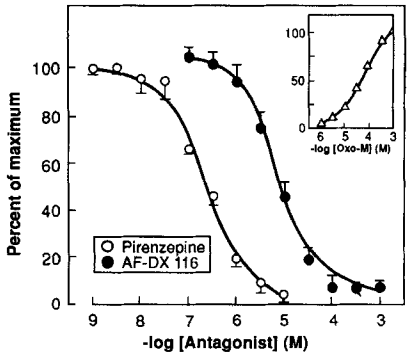

FIG. 6. Pirenzepine and AF-DX 116 inhibition of Oxo-M-stimulated inositol phosphate formation. SK-N-MC cells were incubated for 30 min at 37°C in the presence of 0.1 m*M* Oxo-M and the antagonists at the concentrations indicated. Results are expressed as percentage of maximum response (obtained with Oxo-M alone) as a function of antagonist concentration. Values shown are means ± SEM for three separate experiments. The calculated K_i values for pirenzepine and AF-DX 116 were 35 n*M* and 1.04 μ*M*, respectively. The corresponding Hill coefficients were 1.02 and 1.10, respectively. **Inset:** Dose–response curve for Oxo-M. Maximum stimulation of inositol phosphate release was obtained at an agonist concentration of 1 m*M*.

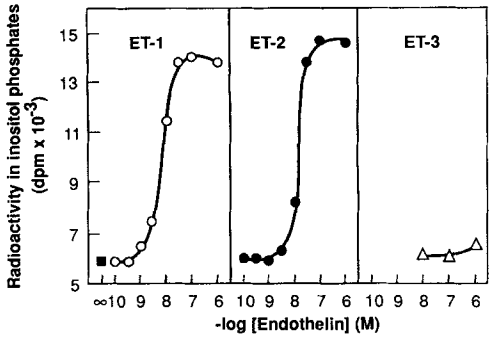

FIG. 7. Differential effects of ETs on PPI turnover. SK-N-MC cells were incubated for 30 min at 37°C in either the absence (■, basal) or presence of ET-1, ET-2, or ET-3 at the concentrations indicated. Reactions were terminated and inositol phosphates quantitated as described in the legend to Fig. 1. Values shown are means of triplicate determinations (SEM < 5% of mean) for one of two experiments that gave similar results. The calculated EC_{50} values for ET-1 and ET-2 were 7 and 15 n*M*, respectively.

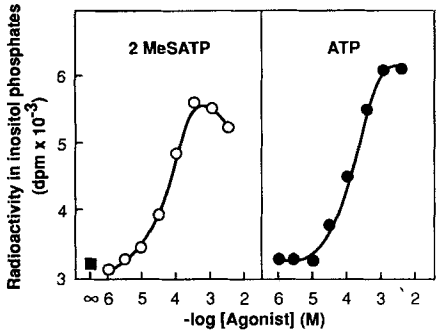

FIG. 9. Both ATP and the putative P_{2y} agonist 2MeSATP stimulate inositol phosphate formation. SK-N-MC cells were incubated for 30 min at 37°C in either the absence (■, basal) or presence of either ATP or 2MeSATP at the concentrations indicated. Reactions were terminated and inositol phosphates quantitated as described in the legend to Fig. 1. Values shown are means of triplicate replicates (SEM < 5% of mean) for one of three experiments (ATP) or two experiments (2MeSATP) that gave similar results. The calculated EC_{50} values in the experiment shown were 47 and 121 μM for 2MeSATP and ATP, respectively.

I(1,3,4)P_3 is sustained in SK-N-MC cells and, therefore, the 3'-kinase pathway of I(1,4,5)P_3 metabolism appears to operate continuously. As a consequence, IP_1s subsequently formed would be expected to accumulate steadily in the I(1/3)P_1 fraction in the presence of Li^+. Thus, although the possibility of some direct breakdown of phosphatidylinositol [and hence the formation of I(1)P_1] cannot be discounted, the polyphosphoinositides appear to be a primary source of inositol phosphates in SK-N-MC cells. A similar conclusion as to the lipid origin of stimulated inositol phosphate formation has been drawn recently for parotid glands (Hughes and Putney, 1989), brain slices (Batty and Nahorski, 1989), and SK-N-SH neuroblastoma cells (Fisher et al., 1990). The Ca^{2+} dependence of stimulated PPI turnover observed for SK-N-MC cells is similar to that previously reported for SK-N-SH neuroblastoma. In the latter cells, phospholipase C activity is regulated by changes in intracellular Ca^{2+}, and a continuous influx of Ca^{2+} is proposed to facilitate PPI hydrolysis (Fisher et al., 1989).

There is now compelling evidence for the involvement of α_1-adrenergic receptors in stimulated PPI turnover in brain (Brown et al., 1984; Minneman and Johnson, 1984; Schoepp et al., 1984). Similarly, in SK-N-MC cells, an increased PPI hydrolysis is elicited by the addition of the α_1 agonist phenylephrine, but not

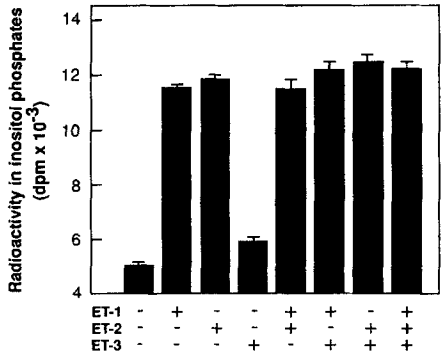

FIG. 8. Lack of additivity between ET-1, ET-2, and ET-3-stimulated inositol phosphate formation. SK-N-MC cells were incubated for 30 min at 37°C in either the absence or presence of 0.5 μM concentrations of ET-1, ET-2, and ET-3 as indicated. Reactions were terminated and inositol phosphates quantitated as described in the legend to Fig. 1. Values shown are the means ± SEM for triplicate determinations obtained in a single experiment. Similar results were obtained in a further four experiments. Note that the stimulations of PPI turnover elicited by ET-1 and ET-2 are not additive, whereas inclusion of ET-3 does not block the more efficacious ETs, ET-1 and ET-2.

TABLE 5. *Effect of purinergic nucleotides on inositol phosphate formation in SK-N-MC cells*

	Release of inositol phosphates	
	% of control	% of ATP response
ATP	401 ± 34 (20)[a]	(100)
ATPγS	256 ± 31 (3)	71 ± 3
2MeSATP	343 ± 46 (4)	86 ± 10
ADPβS	298 ± 56 (3)	68 ± 10
ADP	355 ± 46 (4)	88 ± 8
GTP	292 ± 59 (3)	68 ± 5
ITP	277 ± 45 (3)	65 ± 1
XTP	308 ± 65 (3)	74 ± 6
UTP	296 ± 57 (3)	70 ± 3
AMP	182 ± 20 (5)	30 ± 3
Adenosine	108 ± 15 (5)	6 ± 3

SK-N-MC cells (~1 mg of protein) were incubated for 30 min at 37°C in either the absence or presence of the ligands indicated (all at a 1 m*M* concentration). Reactions were terminated and inositol phosphates quantitated as described in the footnote to Table 1. Results are expressed either as release of inositol phosphates relative to control incubations or, alternatively, the release relative to that obtained for ATP in the same experiment. The number of separate experiments performed is indicated in parentheses.

[a] Data taken from Table 1.

by the α_2 agonist clonidine or the β agonist isoproterenol. However, two distinct differences can be discerned for adrenergic stimulation of inositol lipid hydrolysis in SK-N-MC cells from that in brain. First, relative to that of NE, the efficacies of the two α_1-selective agonists phenylephrine and 6-fluonorepinephrine (27–51%; Table 3) are markedly less than those observed for brain (62–94%; Johnson and Minneman, 1986; Minneman, 1988). Second, from antagonist studies, the functional coupling of an α_{1A} receptor is indicated for SK-N-MC cells, rather than the α_{1B} subtype previously implicated in cerebral cortex (Minneman, 1988; Michel et al., 1990). The two α_1 subtypes can be distinguished pharmacologically on the basis of (a) 10- and 70-fold greater affinities of the competitive antagonists WB-4101 and 5-methylurapidil, respectively, for the α_{1A} subtype (K_D $\sim$ 1 n*M*; Minneman, 1988; Hanft and Gross, 1989), and (b) a greater susceptibility of the α_{1B} subtype to irreversible blockade by chloroethylclonidine ($IC_{50} \sim 1$ μM; Minneman, 1988). In SK-N-MC cells, NE-stimulated PPI turnover is inhibited potently by both WB-4101 and 5-methylurapidil ($K_i < 2.5$ n*M*), whereas only a modest inhibition of inositol lipid hydrolysis occurs following preincubation of the cells with a high concentration of chloroethylclonidine. The antagonist profile of NE-stimulated PPI turnover in SK-N-MC cells and its susceptibility to alkylation are very similar to those recently observed for α_{1A} receptors identified on renal cells (Han et al., 1990). The present results, together with those from a recent study with cultured glial cells (Wilson and Minneman, 1990*a*), indicate that *both* α_{1A} and α_{1B} subtypes are linked to PPI hydrolysis in neural tissues. Although it has been suggested that activation of α_{1A} receptors may result in the breakdown of phosphatidylinositol rather than of the polyphosphoinositides (Wilson and Minneman, 1990*b*), such appears not to be the case for the same receptors on SK-N-MC cells.

From transfection studies, at least three muscarinic receptor subtypes (M_1, M_3, and M_5) are known to couple to PPI turnover (Bonner et al., 1988; Ashkenazi et al., 1989). Only two of these subtypes (M_1 and M_3) have been demonstrated to be functionally linked in the CNS and neural-related tissues (Fisher and Agranoff, 1987, and references therein; Ellis et al., 1990; Forray and El-Fakahany, 1990). Muscarinic acetylcholine receptors linked to PPI turnover in SK-N-MC cells appear to be predominantly of the M_1 subtype, because pirenzepine inhibited the response with a K_i of 35 n*M*, a value that is close to its affinity binding constant to M_1 receptors in neural tissues (Watson et al., 1983) and similar to that obtained for inhibition of PPI turnover in other M_1-enriched neural preparations (Gonzales and Crews, 1984; Fisher and Bartus, 1985). AF-DX 116, an antagonist selective for M_2 receptors, inhibits with a 30-fold lower potency in SK-N-MC cells. In contrast, M_3 receptors (such as those on SK-N-SH and 1321N1 cells) are inhibited relatively weakly by *both* pirenzepine and AF-DX 116 (Fisher and Heacock, 1988; Kunysz et al., 1989). The potency with which Oxo-M stimulated inositol phosphate formation in SK-N-MC cells and the relative efficacies of the partial agonists bethanechol and arecoline are similar to those previously observed for the M_1-muscarinic acetylcholine receptor linked to PPI turnover in brain and in NIE-115 cells (Fisher et al., 1984; Fisher and Snider, 1987).

High-affinity binding sites for the ETs (and their structural homologues, the sarafotoxins) have been identified recently in the CNS (Kloog et al., 1988; Jones et al., 1989). Moreover, the functional coupling of these sites to PPI turnover has been demonstrated for a number of brain regions (Kloog et al., 1989*b*; MacCumber et al., 1990). In SK-N-MC cells, the addition of ET-1 elicits an increase in PPI turnover comparable in magnitude to that observed in brain. However, a notable difference is the inability of ET-3 either to enhance PPI turnover in SK-N-MC cells by itself or to block the effects of ET-1 and ET-2. In contrast, ET-3 is a full agonist in brain, C_6 glioma, and primary neuronal cultures (Crawford et al., 1990; Lin et al., 1990; MacCumber et al., 1990). This result raises the possibility that more than one functionally linked subtype of ET receptor may exist. In keeping with this suggestion, Kloog et al. (1989*a*) have identified three subtypes of the ET receptor on the basis of radioligand binding data. Whereas both ET-1 and ET-3 readily bind to the E-Sβ and E-Sγ subtypes (found in cerebellum and caudate, respectively), only ET-1 binds to the E-Sα subtype (found in smooth muscles and aorta). Furthermore, two cDNAs encoding separate ET receptors have recently been cloned and expressed in host cells (Arai et al., 1990; Sakurai et al., 1990). One of the receptors (ET_A) shows a high specificity for ET-1, whereas the second (ET_B) recognizes ET-1 and ET-3 with equal affinity. Although further characterization is necessary, the profile of agonist selectively obtained for stimulation of PPI turnover in SK-N-MC cells is consistent with the involvement of an ET receptor subtype distinct from those previously described in the CNS.

A fourth receptor linked to PPI turnover in SK-N-MC cells is activated by the presence of ATP and other purine nucleotides. Because neither AMP nor adenosine exerted a substantial stimulatory effect on inositol lipid hydrolysis, the receptors involved appear to be of the P_2 subtype (Burnstock and Kennedy, 1985). P_2 receptors linked to PPI turnover have also been demonstrated in astrocytes (Pearce et al., 1989), pituitary cells (Davidson et al., 1990), and adrenal chromaffin cells (Sasakawa et al., 1989; Allsup and Boarder, 1990). In SK-N-MC cells, all purine nucleotide triphosphates tested were able to promote PPI turnover, indicating a broad agonist specificity. However, given the ability of the pyrimidine UTP to enhance inositol phosphate release in this cell line and in other tissues (Fine et al., 1989; Sasakawa et al., 1989), it may be more appropriate to term the receptor involved a "nucleotidoceptor" (Pfeilschifter, 1990). Of the P_2-receptor subtypes

thus far described, it is the P_{2y} receptor that has been linked to activation of PPI turnover in nonneural tissues (Martin and Harden, 1989; Allsup and Boarder, 1990), based largely upon the potency exhibited by the putative P_{2y}-selective agonist 2MeSATP for both binding to the receptor and eliciting a functional response. However, although 2MeSATP and ATP are equally effective enhancers of PPI hydrolysis in SK-N-MC cells, they are also equipotent (Fig. 9). Furthermore, 2MeSATP has been reported to be either a partial agonist (Pfeilschifter, 1990) or devoid of activity (Fine et al., 1989) at P_2 receptors linked to PPI hydrolysis. These observations raise the possibility that the current definition of P_2 subtypes is inadequate to account fully for the observed properties of this class of receptors.

In conclusion, the present results indicate that SK-N-MC cells possess multiple PPI-linked receptors, two of which (α_{1A}-adrenergic and ET) have received little previous attention. The availability of this cell line may facilitate future studies of both the characterization and regulation of these receptors.

Acknowledgment: The authors wish to thank Dr. A. M. Heacock for her helpful comments and Ms. Jo Ann Kelsch for preparation of the manuscript. This study was supported by NIMH grant no. MH 42652.

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