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Agonist-Specific Calcium Signaling and Phosphoinositide Hydrolysis in Human SK-N-MCIXC Neuroepithelioma Cells

*‡R. Kyle Palmer, †David I. Yule, *Edward L. McEwen, †John A. Williams, and *‡Stephen K. Fisher

**Neuroscience Laboratory, Mental Health Research Institute, and Departments of †Physiology and ‡Pharmacology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

Abstract: Fura-2 digital imaging microfluorimetry was used to evaluate the Ca^{2+} signals generated in single clonal human neuroepithelioma cells (SK-N-MCIXC) in response to agonists that stimulate phosphoinositide hydrolysis. Addition of optimal concentrations of either endothelin-1 (ET-1), ATP, oxotremorine-M (Oxo-M), or norepinephrine (NE) all resulted in an increase in the concentration of cytosolic calcium (Ca^{2+}_i) but of different magnitudes (ET-1 = ATP > Oxo-M > NE). The Ca^{2+} signals elicited by the individual agonists also differed from each other in terms of their latency of onset, rate of rise and decay, and prevalence of a sustained phase of Ca^{2+} influx. The Ca^{2+} signals that occurred in response to ATP had a shorter latency and more rapid rates of rise and decay than those observed for the other three agonists. Furthermore, a sustained plateau phase of the Ca^{2+} signal, which was characteristic of the response to Oxo-M, was observed in <40% of cells stimulated with ET-1 and absent from Ca^{2+} signals elicited after NE addition. Removal of extracellular Ca^{2+} enhanced the rate of decay of Ca^{2+} signals generated by ATP, ET-1, or Oxo-M and, when evident, abolished the sustained phase of Ca^{2+} influx. In the absence of extracellular Ca^{2+}, NE elicited asynchronous multiple Ca^{2+} transients. In either the absence or presence of extracellular Ca^{2+}, >94% of cells responded to ET-1 or ATP, whereas corresponding values for Oxo-M and NE were ~74 and ~48%. Sequential addition of agonists to cells maintained in a Ca^{2+}-free buffer indicated that each ligand mobilized Ca^{2+} from a common intracellular pool. When monitored as a release of a total inositol phosphate fraction, all four agonists elicited similar (four- to sixfold) increases in phosphoinositide hydrolysis. However, the addition of ET-1 or ATP resulted in larger increases in the net formation of inositol 1,4,5-trisphosphate than did either Oxo-M or NE. These results indicate that, in SK-N-MCIXC cells, the characteristics of both Ca^{2+} signaling and inositol phosphate production are agonist specific. **Key Words:** Calcium signals—Agonist specificity—Phosphoinositide hydrolysis—Inositol 1,4,5-trisphosphate—Neuroepithelioma—SK-N-MCIXC.
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Agonist occupancy of a diverse range of pharmacologically distinct receptors in the CNS results in the activation of phosphoinositidase C, the hydrolysis of phosphatidylinositol 4,5-bisphosphate, and the attendant formation of two intracellular second messengers, namely, inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$] and diacylglycerol (for review, see Fisher et al., 1992*a*). The latter serve to mobilize intracellular Ca^{2+} (Ca^{2+}_i) and to activate protein kinase C, respectively (Berridge and Irvine, 1989; Hug and Sarre, 1993). The probability that multiple receptors linked to phosphoinositide (PPI) hydrolysis are present on individual neural cells raises the question of how specificity of signaling is attained. One possibility, not yet extensively evaluated in neural tissues, is that qualitative and quantitative differences in the Ca^{2+} signals generated upon receptor activation may confer the required degree of specificity. In this context, evidence for the generation of distinctive Ca^{2+} signaling patterns has previously been obtained for nonneural preparations challenged with agonists that promote PPI turnover, either when monitored as Ca^{2+} oscillations (Woods et al., 1987*a*; Rooney et al., 1989; Osipchuk et al., 1990) or as sustained increases in $[Ca^{2+}]_i$ (Lynch et al., 1992). Conceivably, these differences might reflect agonist-specific differences in the rate of production and/or metabolism of inositol phosphates. Alternatively, other factors such as the cellular distribution of Ca^{2+} channels and pumps, or the distribution of Ca^{2+} binding proteins, could explain the diverse range of Ca^{2+} signals observed.

Previously, we demonstrated that the human neuroepithelioma cell line SK-N-MC and its subclone SK-N-MCIXC possess at least four receptors (endothelin,

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Address correspondence and reprint requests to Dr. S. K. Fisher at Neuroscience Laboratory, University of Michigan, 1103 E. Huron St., Ann Arbor, MI 48104-1687, U.S.A.

Abbreviations used: Ca^{2+}_i, cytosolic Ca^{2+}; ET, endothelin; fura-2/AM, fura-2 acetoxymethyl ester; $I(1,4,5)P_3$, inositol 1,4,5-trisphosphate; NE, norepinephrine; Oxo-M, oxotremorine-M; PPI, phosphoinositide; SK-N-MCIXC, subclone of human neuroepithelioma cell line SK-N-MC.

muscarinic cholinergic, nucleotide, and α_1-adrenergic) coupled to PPI hydrolysis (Fisher and Landon, 1991; Fisher et al., 1992*b*). In the present study, we have used fura-2 digital imaging microfluorimetry to evaluate the Ca^{2+} signals generated in single SK-N-MCIXC cells after activation of these receptors. We demonstrate that in these cells, the Ca^{2+} signals that accompany enhanced PPI hydrolysis can be differentiated on the basis of their magnitude, latency of onset, kinetics of rise and decay, and the prevalence of a sustained phase of Ca^{2+} influx. Agonist-specific differences in the net formation of $I(1,4,5)P_3$ were also observed for SK-N-MCIXC cells. The possibility that the intracellular concentration of $I(1,4,5)P_3$ attained after receptor activation might explain, in part at least, some of the diversity of Ca^{2+} responses is discussed. A preliminary account of part of this work has appeared elsewhere (Palmer et al., 1993).

MATERIALS AND METHODS

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [^{3}H]$I(1,4,5)P_3$ (15 Ci/mmol) was purchased from NEN/Du Pont (Boston, MA, U.S.A.). ATP, atropine, (−)-isoproterenol, (−)-norepinephrine (NE), and UTP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endothelin-1 (ET-1) and endothelin-3 (ET-3) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). Oxotremorine-M (Oxo-M), 2-methylthio ATP, WB-4101, clonidine, and prazosin were obtained from Research Biochemicals, Inc. (Natick, MA, U.S.A.). Fura-2 acetoxymethyl ester (fura-2/AM) and fluo-3 (free acid) were from Molecular Probes, Inc. (Eugene, OR, U.S.A.). $I(1,4,5)P_3$ was obtained from Calbiochem (La Jolla, CA, U.S.A.). Endothelin receptor antagonists (PD 147953 and PD 142893) were gifts from Parke-Davis (Ann Arbor, MI, U.S.A.). Pirenzepine was a gift from Boehringer-Ingelheim (Ridgefield, CT, U.S.A.). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY, U.S.A.). Powdered Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GIBCO (Grand Island, NY, U.S.A.). SK-N-MCIXC neuroepithelioma cells were obtained from Dr. June Biedler, Sloan Kettering Institute (New York, NY, U.S.A.).

Cell culture conditions

Human SK-N-MCIXC cells (passages 28–46, subcloned twice from the parent SK-N-MC line; see 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 calf serum. Cells were grown for 7–30 days at 37°C in an atmosphere consisting of 10% CO_2 and 90% humidified air. Subculture and maintenance of the cells were as previously described (Fisher and Landon, 1991).

Measurement of $[Ca^{2+}]_i$ in cell suspensions

SK-N-MCIXC cells were resuspended in 10 ml of 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* Na^+-HEPES buffer, pH 7.4) at a concentration of ~1 mg of protein/ml. Cells were then incubated with 2 μ*M* fura-2/AM for 15 min at 37°C and $[Ca^{2+}]_i$ measured by monitoring fura-2 fluorescence in a Shimadzu RF-5000 spectrofluorophotometer using the dual wavelength method, as described previously (Thompson and Fisher, 1991). Concentrations of free Ca^{2+} in buffers were measured directly after the addition of fluo-3 (free acid).

Measurement of $[Ca^{2+}]_i$ in single cells

SK-N-MCIXC cells were seeded on glass coverslips (22 mm diameter) and allowed to grow in Dulbecco's modified Eagle's medium/fetal calf serum for 1–3 days, during which time 30–50% confluency was achieved. Culture medium was then aspirated and cells washed gently with 10 ml of buffer A. Cells were then incubated for 45–60 min at room temperature with 5 ml of buffer A, which contained 1 mg/ml bovine serum albumin and 4 μ*M* fura 2/AM. At the end of the loading period, cells were washed with buffer A and the coverslips transferred to a closed chamber (which was mounted on the stage of a Zeiss Axiovert inverted microscope) and the cells constantly superfused with buffer A (1 ml/min at 37°C). For most experiments, solution changes were accomplished by means of a valve attached to a gravity driven eight-chambered superfusion reservoir. For determination of latencies, an electrically powered perfusion pump was used. Measurement of $[Ca^{2+}]_i$ was performed using an Attofluor digital imaging system (Rockville, MD, U.S.A.), as described previously (Yule et al., 1993*a*). In brief, excitation of cells at 334 and 380 (10 nm band pass) was accomplished by a computer selectable filter and shutter system. Resultant emission at 510 nm was monitored by an intensified CCD camera and subsequently digitized. A ratio was obtained every 0.5–1.0 s. Calibration of $[Ca^{2+}]_i$ signals was determined by applying the equation of Grynkiewicz et al. (1985). The fluorescent emission of external Ca^{2+} standards containing 1 μ*M* fura-2 either in the absence (1 m*M* EGTA) or presence of Ca^{2+} (1 m*M*) was monitored allowing the determination of R_{max}, R_{min}, and β. The calibration was then applied to use defined areas of interest, where mean gray level values were computed to give a measurement of $[Ca^{2+}]_i$ as described previously (Brooker et al., 1990).

Measurement of PPI turnover

SK-N-MCIXC cells were allowed to prelabel for 2 days in Dulbecco's modified Eagle's medium/fetal calf serum containing 10 μCi/ml [^{3}H]inositol. Cells were then detached after the addition of a modified Puck's D_1 solution (Honneger and Richelson, 1976) containing 0.5 m*M* EDTA and 0.05% trypsin, washed with buffer A, and incubated in the presence or absence of agonists for 15 min at 37°C. The accumulation of ^{3}H-inositol phosphates was monitored during a 15-min incubation period in the presence of Li^+, as described previously (Fisher et al., 1990). For each agonist, the release of labeled inositol phosphates was linear over this time interval.

Measurement of $I(1,4,5)P_3$ mass

Aliquots of SK-N-MCIXC cells (~1 mg of protein) were incubated in 0.5 ml of buffer A at 37°C in the presence or absence of agonist. Reactions were terminated by the addition of 0.5 ml of ice-cold 20% (wt/vol) trichloroacetic acid. The trichloroacetic acid extract was washed with water-saturated diethyl ether and neutralized as described previously (Fisher et al., 1990). Aliquots of the neutralized water-soluble extract were diluted in buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1 m*M* β-mercaptoethanol, pH 8.8) and incubated (in the same buffer) for 15 min at 4°C with 400 μg of a rat

cerebellar membrane preparation (Bredt et al., 1989) in the presence of 0.01 μCi [^{3}H]1(1,4,5)P_3. Bound and free radioligand were separated by rapid vacuum filtration (at 4°) using Whatman GF/B glass–fiber filters. Preliminary studies indicated that, when maintained at 4°C, the rapid filtration method gave results comparable with the more frequently used centrifugation assay. Protein was measured by the method of Geiger and Bessman (1972).

Data analysis

Values are means ± standard errors for the number (n) of individual cells examined or separate experiments performed. Statistical analyses were performed with the InStat program (GraphPad Software, San Diego, CA, U.S.A.). Due to significant differences among the standard deviations (detected by Bartlett's test for homogeneity of variance), nonparametric methods of statistical analysis were used. Thus, the Kruskal–Wallis ANOVA (with Dunn's multiple comparisons posttest) was used to evaluate differences among three or more groups, and the Mann–Whitney *U* test was used for comparisons between two groups.

RESULTS

Agonists elicit specific Ca^{2+} "signatures" in SK-N-MCIXC cells

The addition of maximally effective concentrations of either ET-1, ATP, Oxo-M, or NE to single SK-N-MCIXC cells resulted in a rapid rise in $[Ca^{2+}]_i$. Although some degree of heterogeneity of response was observed, the magnitude, shape, and duration of the Ca^{2+} signals obtained for the majority of cells were characteristic for each agonist. The addition of 1 μM ET-1 resulted in an increase in $[Ca^{2+}]_i$ at a rate of 20 nM/s to ~200 nM above basal. After the peak $[Ca^{2+}]_i$ had been achieved, the $[Ca^{2+}]_i$ decayed with a $t_{1/2}$ of 41 s (Table 1, A). In the majority of cells tested, no sustained level of Ca^{2+} (plateau phase) was evident after this initial peak (Fig. 1a). This pattern of Ca^{2+} signaling was observed at all concentrations of ET-1 tested (30–1,000 nM). The increase in $[Ca^{2+}]_i$ elicited by 1 μM ET-1 could be blocked by 100 nM concentrations of either PD 147953 (a selective ET_A receptor antagonist) or PD 142893 (a nonselective ET receptor antagonist). The addition of ET-3 (1 μM) had little effect on $[Ca^{2+}]_i$, further indicating the involvement of an ET_A receptor in the generation of Ca^{2+} signals (Arai et al., 1990). The addition of 1 mM ATP resulted in an increase in $[Ca^{2+}]_i$ of similar magnitude to that observed for ET-1 (Fig. 1 and Table 1, A). However, the Ca^{2+} signals generated by ATP (concentration range, 10–1,000 μM) differed from those elicited by ET-1 in two respects. First, the rate of increase in $[Ca^{2+}]_i$ observed in the presence of 1 mM ATP was more rapid than that observed for ET-1 (59 nM/s) and the decay phase of the Ca^{2+} signal was also more short lived ($t_{1/2}$ = 11 s for ATP, see Table 1, A). Second, in contrast to ET-1, the majority (>70%) of cells exhibited a marked plateau phase of the Ca^{2+} signal in response to ATP (Fig. 1c). The addition of 100 μM concentrations of the pyrimidine, UTP, or 2-methylthio ATP also resulted in Ca^{2+} signals that were similar in size, shape, and duration to those elicited by ATP. This pharmacological profile for Ca^{2+} signaling is similar to that previously obtained for PPI hydrolysis in SK-N-MC cells (Fisher and Landon, 1991). Smaller increases in $[Ca^{2+}]_i$, achieved at relatively slower rates (8–11 nM/s) than those monitored for ET-1 and ATP, were observed after the addition of 1 mM concentrations of either Oxo-M or NE (Table 1, A). A characteristic of the response to Oxo-M observed in >70% of cells was that of a sustained plateau phase of the Ca^{2+} signal (Fig. 1e), which was maintained in the continued presence of agonist. This pattern was observed for Oxo-M in the concentration range of 10–1,000 μM. Inclusion of 10 μM concentrations of either atropine or pirenzepine blocked the increase in $[Ca^{2+}]_i$ that resulted from the addition of 500 μM Oxo-M, indicating the involvement of a muscarinic cholinergic receptor. In contrast to the sustained Ca^{2+} signals observed in response to Oxo-M, the addition of NE (10–1,000 μM) resulted in a transient increase in $[Ca^{2+}]_i$, which returned to baseline in all cells (Fig. 1g, h). In ~20% of the cells, repeated transients were observed (Fig. 1h). The response to NE (100 μM) appeared to be mediated by an α_1-adrenergic receptor because it was blocked by inclusion of 1 μM concentrations of either prazosin or WB-4101 and was not mimicked by addition of either 10 μM (−)-isoproterenol or 1 mM clonidine.

Agonist-mediated increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

To determine the role, if any, played by extracellular Ca^{2+} in the generation of agonist-specific Ca^{2+} signals, the ability of ET-1, ATP, Oxo-M, and NE to increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was determined. Chelation of extracellular Ca^{2+} had little or no effect on the Ca^{2+} signals elicited by the addition of ET-1 in terms of either the magnitude of response, or on kinetics of rise and decay of the Ca^{2+} signals (Table 1, B; Fig. 2a, b). Apart from a small decrease in $t_{1/2}$ for decay of the Ca^{2+} signal, the only marked difference observed was the complete absence of the plateau phase in all cells tested. The latter was also absent from ATP-generated Ca^{2+} signals (Fig. 2c, d) (Table 1, B). However, as observed for ET-1, the peak $[Ca^{2+}]_i$ elicited by ATP was also unaltered by chelation of extracellular Ca^{2+} (Table 1, B). Moreover, the characteristic rapidity of the rise and decay of the ATP-mediated Ca^{2+} signal was maintained in the absence of extracellular Ca^{2+} (Table 1, B; Fig. 2c). In ~25% of cells, a transient series of oscillations was observed after the addition of ATP (Fig. 2d). For Oxo-M, the pronounced plateau phase of the Ca^{2+} signal elicited after agonist addition was also abolished in Ca^{2+}-free medium, and $[Ca^{2+}]_i$ returned to baseline in the majority of cells with a $t_{1/2}$ of 46 s (Table 1, B; Fig. 2e). In the remaining cells, Oxo-M addition resulted in the generation of heterogeneous Ca^{2+} signals, some of

TABLE 1. *Magnitude and kinetics of rise and decay of agonist-induced Ca^{2+} signals*

	Number of cells	ΔPeak $[Ca^{2+}]_i$ (n*M*)	Rate of rise (n*M*/s)	$t_{1/2}$ decay (s)	ΔPlateau $[Ca^{2+}]_i$ (n*M*)
A. $[Ca^{2+}]$ = 2.2 m*M*					
ATP	257 (8)	227 ± 4[a]	59 ± 2[b]	11 ± 1[b]	30 ± 3[c]
ET-1	179 (7)	213 ± 5[a]	20 ± 1[d]	41 ± 2[d]	28 ± 3[c]
Oxo-M	119 (6)	127 ± 5[c]	11 ± 1	ND	52 ± 3[c,e]
NE	73 (5)	87 ± 6	8 ± 1	23 ± 2	2 ± 1
B. $[Ca^{2+}]$ < 10 n*M*					
ATP	102 (3)	215 ± 7	54 ± 3	8 ± 1[f]	1 ± 0[f]
ET-1	145 (4)	214 ± 6	21 ± 1	33 ± 1	2 ± 4[f]
Oxo-M	143 (4)	125 ± 4	14 ± 1	46 ± 4	0 ± 0[f]
NE	78 (4)	101 ± 6	11 ± 2	13 ± 2[f]	0 ± 1

SK-N-MCIXC cell grown on coverslips were loaded with fura-2 and superfused with either ATP (1 m*M*), ET-1 (1 μ*M*), Oxo-M (1 m*M*), or NE (1 m*M*) in the presence (A) or absence (B) of extracellular Ca^{2+}, as detailed in the legends to Figs. 1 and 2. ΔPeak and Δplateau $[Ca^{2+}]_i$ values are the maximal and plateau increases above basal $[Ca^{2+}]_i$, respectively. The average rate of increase of the Ca^{2+} signal was calculated as Δpeak $[Ca^{2+}]_i$/time between onset and peak $[Ca^{2+}]_i$. The $t_{1/2}$ decay was calculated as time required for Δpeak $[Ca^{2+}]_i$ to decline by 50%. Values shown are mean ± SEM for the number of cells analyzed. The number of individual coverslips examined is indicated in parentheses. Values for basal $[Ca^{2+}]_i$ were ~60–90 n*M*. ND, could not be determined ($t_{1/2}$ > 120 s).

[a] Different from Oxo-M or NE, $p < 0.001$.
[b] Different from ET-1, Oxo-M, or NE, $p < 0.001$.
[c] Different from NE, $p < 0.001$.
[d] Different from ATP, Oxo-M, or NE, $p < 0.001$.
[e] Different from ATP or ET-1, $p < 0.001$.
[f] Different from $[Ca^{2+}]$ = 2.2 m*M*, $p < 0.001$.
[a–e] Kruskal–Wallis ANOVA; [f] Mann–Whitney *U* test.

which exhibited repeated Ca^{2+} transients (Fig. 2f). Removal of extracellular Ca^{2+} also resulted in the appearance of asynchronous repeated Ca^{2+} transients in response to NE addition (Fig. 2g, h). The latter did not diminish in amplitude or increase in periodicity in the majority of cells during the 10-min exposure to agonist, indicating little reliance on extracellular Ca^{2+} for maintenance of the NE response. Although not extensively evaluated, Ca^{2+} signals generated in a nominally Ca^{2+}-free buffer A (EGTA omitted) displayed characteristics similar to those obtained in the presence of EGTA.

Fraction of SK-N-MCIXC cell population responsive to each of the four agonists

Not all cells responded to maximally effective concentration of ET-1, ATP, Oxo-M, or NE. Thus, whereas the addition of either ATP or ET-1 resulted, on average, in an increase in $[Ca^{2+}]_i$ in >94% of cells examined, the corresponding values obtained for Oxo-M and NE were ~74 and ~48%, respectively. In addition, more interexperimental variability in terms of the number of responsive cells per coverslip were observed for Oxo-M and NE (ranges, 43–96 and 16–93% of total, respectively), than for either ET-1 or ATP

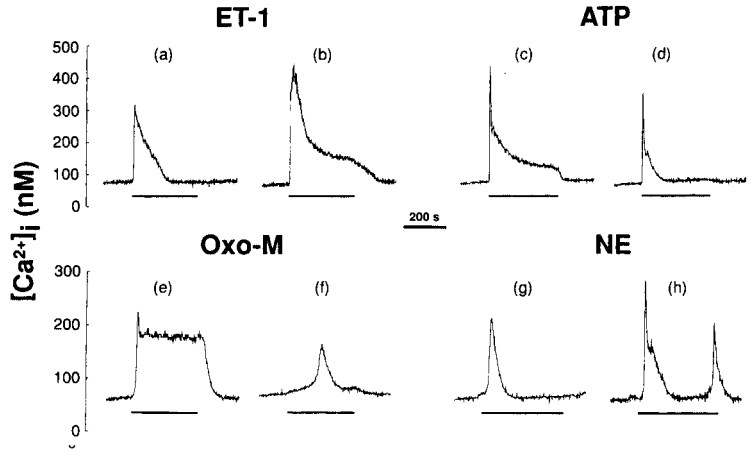

FIG. 1. Agonist-specific Ca^{2+} signaling in Ca^{2+}-containing buffer. SK-N-MCIXC cells were grown on coverslips, loaded with fura-2, and then superfused with buffer A for 3–5 min at 37°C before the addition of either ET-1 (1 μ*M*) or 1 m*M* concentrations of ATP, Oxo-M, or NE, for the time indicated by the solid bar. The two most frequently observed patterns of Ca^{2+} signaling generated by each agonist are shown, with the initial traces, i.e., **a, c, e,** and **g,** representing the predominant signaling pattern observed (i.e., that observed in >60–70% of cells examined). Results shown are from one of seven to eight (for ET-1 and ATP) or five to six separate experiments (for Oxo-M and NE) that gave essentially similar results.

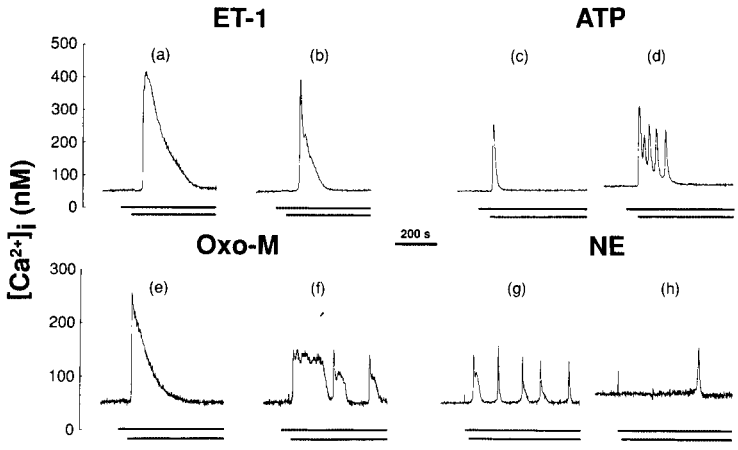

FIG. 2. Agonist-specific Ca^{2+} signals generated in Ca^{2+}-free buffer. SK-N-MCIXC cells were grown on coverslips, loaded with fura-2, and then superfused with buffer A for 3–5 min at 37°C. Approximately 30–60 s before the addition of either ET-1 (1 μM) or 1 mM concentrations of ATP, Oxo-M, or NE, and thereafter, cells were perfused with a nominally Ca^{2+}-free buffer A to which EGTA (2 mM) had been added (free $[Ca^{2+}] < 10$ nM). The brief exposure of cells to EGTA minimized the possibility of a redistribution of cellular Ca^{2+}. The two most frequently observed patterns of Ca^{2+} signal generated by each agonist are shown, with the initial traces, i.e., **a, c, e,** and **g,** representing the predominant signaling pattern observed (i.e., that observed in >70% of cells examined). Results shown are from one of three to four experiments that gave essentially similar results. The time periods of exposure of the cells to the Ca^{2+}-free medium (upper bar) and agonist (lower bar) are indicated beneath the traces.

(ranges, 86–100 and 80–100%, respectively). Similar results were also obtained when agonist-mediated Ca^{2+} signals were monitored in the absence of extracellular Ca^{2+} (Fig. 3).

Latency of agonist-mediated increased $[Ca^{2+}]_i$

The latency of onset of agonist-induced increases in $[Ca^{2+}]_i$ was also monitored. ATP exhibited the most rapid response, with >90% of cells responding within <5 s (median, 1 s; see Fig. 4). For those cells that responded to ET-1, Oxo-M, and NE (90, 81, and 50% of total, respectively), the median values for latency were 13, 5, and 8 s, respectively. Whereas >95% of cells responded to ATP, ET-1, and Oxo-M within 40 s of agonist exposure, a fraction (23% of responsive population) responded to NE with much longer latencies (41–500 s, see Fig. 4).

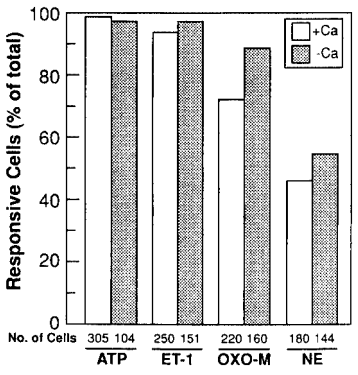

FIG. 3. Fraction of cells responsive to agonist addition. SK-N-MCIXC cells that had been grown on coverslips were loaded with fura-2 and then challenged with either ET-1 (1 μM), ATP (1 mM), Oxo-M (1 mM), or NE (1 mM) in the absence or presence of extracellular Ca^{2+}, as detailed in the legends to Figs. 1 and 2. Results are expressed as the fraction of cells (percentage of total) that responded to each agonist within a time period of 4 min. The total number of cells analyzed [obtained from either six to nine coverslips ($+Ca^{2+}$) or three to four coverslips ($-Ca^{2+}$)] is indicated. A response was defined as an increase in $[Ca^{2+}]_i$ of >20 nM.

All four agonists mobilize Ca^{2+} from a common intracellular pool

Because not all cells responded to each agonist, cell suspensions were used to determine whether ATP, ET-1, Oxo-M, and NE mobilized Ca^{2+} from common or discrete intracellular Ca^{2+} pools. When suspensions of SK-N-MCIXC cells were incubated in buffer A that contained 10 mM EGTA (free $[Ca^{2+}]$ ~60 nM, a value that approximates that observed in the cytoplasm of quiescent cells), the initial addition of ET-1 essentially abolished the subsequent ability of other agonists to elicit an increase in $[Ca^{2+}]_i$. When the order of addition was reversed such that NE, a less efficacious agonist, was added first, the ability of either ATP or Oxo-M to elicit an increase in $[Ca^{2+}]_i$ was severely impaired. The subsequent addition of ET-1 resulted in little or no further increase in $[Ca^{2+}]_i$ (Fig. 5).

Agonist-stimulated PPI hydrolysis

The addition of ET-1, ATP, Oxo-M, or NE elicited relatively similar increases in the release of a total ^{3}H-inositol phosphate fraction (Fig. 6). When assayed in nominally Ca^{2+}-free buffer to which 2 mM EGTA had been added, ET-1-, ATP-, Oxo-M-, or NE-stimulated ^{3}H-inositol phosphate production was reduced by 84 ± 3, 90 ± 2, 78 ± 2, and 80 ± 4%, respectively (n = 4), a result that may indicate that a continuous influx of Ca^{2+} facilitates the phosphoinositidase C activity in these cells. Because the results obtained for the accumulation of a total ^{3}H-inositol phosphate fraction appeared to be inconsistent with the differential ability of the agonists to increase $[Ca^{2+}]_i$ (see Table 1), I(1,4,5)P_3 mass was also determined. For all four agonists, the maximal increase in the net formation of I(1,4,5)P_3 was observed after 10 s of incubation. Under these conditions, larger increases in I(1,4,5)P_3 mass were observed in the presence of either ET-1 or ATP than after the addition of either Oxo-M or NE (Fig. 6).

DISCUSSION

Relatively few studies have addressed the possibility that activation of receptors coupled to PPI hydrolysis

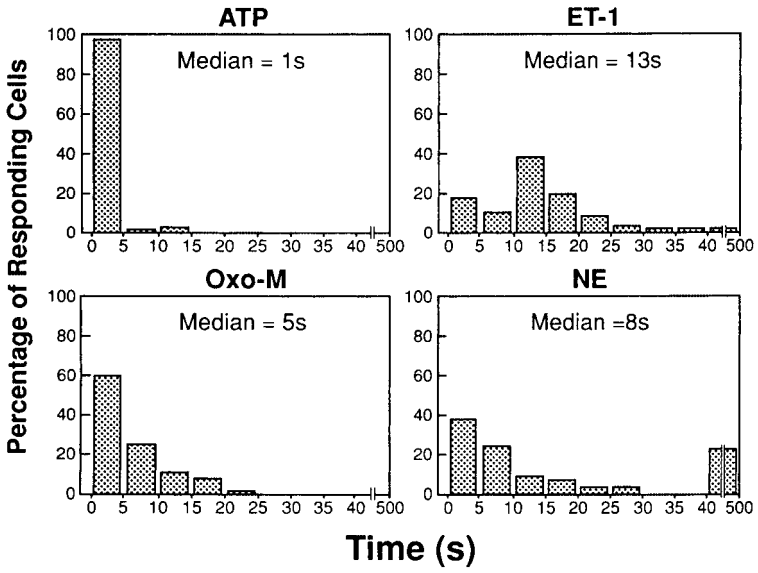

FIG. 4. Frequency histogram of latencies of agonist-induced increases in $[Ca^{2+}]_i$. SK-N-MCIXC cells that had been grown on coverslips were loaded with fura-2 and challenged (in the presence of buffer A containing 2.2 m*M* Ca^{2+}) with either ATP (1 m*M*), ET-1 (1 μM), Oxo-M (1 m*M*), or NE (1 m*M*), which were administered via an electrically powered perfusion pump. Latencies of responses were calculated after subtraction of time required for agonist to be delivered to cells (~18 s, determined before each experiment). A response is defined as an increase in $[Ca^{2+}]_i$ of >20 n*M*. Results are plotted in terms of the percentage of cells that responded to each agonist within 5 s time intervals. The number of cells analyzed (number of coverslips in parentheses) were as follows: ATP, 145 (4); ET-1, 148 (5); Oxo-M, 75 (4); NE, 56 (4).

in neural tissues is accompanied by the generation of agonist-specific Ca^{2+} signals. Although distinct patterns of Ca^{2+} signaling have been reported after the addition of individual agonists to N1E-115 neuroblastoma cells (Iredale et al., 1992), chromaffin cells (Stauderman and Pruss, 1990), and PC-12 cells (Fasolato et al., 1990), the possibility that cell heterogeneity explains the diversity of responses recorded cannot be excluded due to the use of cell suspensions in these studies. In this context, it should be noted that when analyzed at the single cell level, at least part of the heterogeneity of Ca^{2+} signals observed after addition of either bradykinin, carbamylcholine, or ATP to PC-12 cells could be attributed to the presence of multiple clones (Grohovaz et al., 1991). In the present study, we demonstrate that the addition of ATP, ET-1, Oxo-M, or NE to SK-N-MCIXC cells results in the genera-

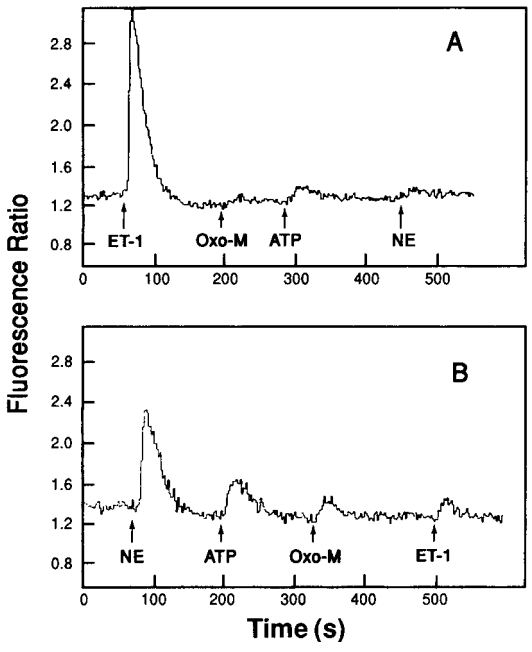

FIG. 5. All four agonists mobilize Ca^{2+} from a common intracellular pool. Suspensions of SK-N-MCIXC cells were loaded with fura-2 and incubated for 2 min at 37°C in buffer A that contained 10 m*M* EGTA (free $[Ca^{2+}]$ = ~60 n*M*). **A:** ET-1 (1 μM) or 1 m*M* concentrations of either Oxo-M, ATP, or NE were then added sequentially, at the times indicated by arrows. **B:** The order of agonist addition was reversed. Values expressed are fluorescence ratios (F_{340}/F_{380}) as a function of time. Results are from one of three experiments that gave similar results.

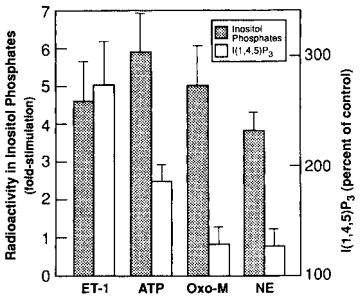

FIG. 6. Agonist-stimulated formation of a total inositol phosphate fraction and $I(1,4,5)P_3$ mass in SK-N-MCIXC neuroepithelioma. Cells (~1–2 mg of protein) were incubated in the presence of buffer A (2.2 m*M* Ca^{2+}) for either 15 min (for measurement of a total inositol phosphate fraction) or 10 s [for measurement of $I(1,4,5)P_3$ mass] with 1 m*M* concentrations of ATP, Oxo-M, or NE, or 1 μM ET-1. Reactions were terminated by the addition of trichloroacetic acid and labeled inositol phosphates present in neutralized extracts were quantitated by anion-exchange chromatography. $I(1,4,5)P_3$ present in neutralized extracts was assayed using a cerebellar membrane preparation as the source of $I(1,4,5)P_3$ receptors. Values shown are mean ± SEM for either seven (total inositol phosphate fraction) or five to six [$I(1,4,5)P_3$ mass] separate experiments. $I(1,4,5)P_3$ concentrations at zero time were 31 ± 4 pmol/mg of protein (n = 6).

tion of Ca^{2+} signals that exhibit a pharmacological profile similar to that previously observed for PPI hydrolysis. Moreover, when analyzed at the single cell level, the Ca^{2+} signals generated in the majority of cells tested are characteristic for each agonist over a range of concentrations. Two considerations lend support to the notion that these distinctive Ca^{2+} signals can be generated within single neuroepithelioma cells. First, the SK-N-MCIXC cell line is clonal and has been subcloned three times from the parent SK-N-MC cell (Biedler et al., 1973), thus reducing (but not eliminating) the possibility of cell heterogeneity. Second, whereas not all cells responded to NE or Oxo-M (48 and 74%, respectively), very distinctive Ca^{2+} signals were observed after the addition of either ATP or ET-1, both of which elicited responses from >94% of the cells tested. Furthermore, individual SK-N-MCIXC cells that responded to all four agonists were routinely encountered. Thus, the probability that agonist specificity of Ca^{2+} signaling is due to cell heterogeneity is minimal. Taken collectively, these results indicate that distinct agonist-specific Ca^{2+} signals can be elicited within an individual SK-N-MCIXC cell after activation of pharmacologically distinct receptors coupled to PPI hydrolysis. Some cell-to-cell variation in the characteristics of Ca^{2+} signals elicited by the same agonist was also observed (Figs. 1 and 2). Similar observations have been made in other nonneural clonal cell lines (Ambler et al., 1988; Prentki et al., 1988). Although the reason for this intercellular variation remains unknown, it appears to be unrelated to either the degree of fura-2 loading (Rooney et al., 1989) or to the specific stages of the cell cycle (Ambler et al., 1988).

The Ca^{2+} signals generated in SK-N-MCIXC cells by the four agonists can be differentiated quantitatively based upon five parameters, namely, the latency of onset, magnitude of increase in $[Ca^{2+}]_i$, rate of rise and decay of the Ca^{2+} transient, and magnitude of the plateau phase of the Ca^{2+} signal. Although the largest increases in $[Ca^{2+}]_i$ were observed in response to either ATP or ET-1, other characteristics of these Ca^{2+} signals were very different. Thus, in response to ATP, the onset of the signal was rapid (median latency, 1 s) as were the rate of rise (59 n*M*/s) and decay ($t_{1/2}$ = 11 s) phases of the Ca^{2+} transients. This type of Ca^{2+} signaling in response to ATP is reminiscent of that observed for type I astroglia (Kastritsis et al., 1992). In contrast, the Ca^{2+} signals elicited after ET-1 addition to SK-N-MCIXC cells were initiated slowly (median latency, 13 s) and were also slower to develop (rate of rise, 20 n*M*/s) and decay ($t_{1/2}$ = 40 s) than those observed for ATP. Because the kinetic differences between Ca^{2+} signals generated by ATP and ET-1 persisted in the absence of extracellular Ca^{2+}, this indicates agonist specificity in terms of the efficacy of mobilization of intracellular Ca^{2+}, and the latter's subsequent removal from the cytosol by means of either sequestration into intracellular organelles or extrusion from the cell. In the latter context, it should be noted that agonist modulation of the plasma membrane Ca^{2+} pump has recently been demonstrated (Zhang et al., 1992). A further difference between ATP- and ET-1-induced Ca^{2+} signals was the frequent absence of a plateau phase from the Ca^{2+} signal elicited after ET-1 addition, as has been previously observed for NG 108-15 cells (Reiser and Donié, 1990; Chau et al., 1993).

The addition of Oxo-M or NE resulted in Ca^{2+} signals that were not only of smaller magnitude than those observed for either ET-1 or ATP, but also developed less rapidly (8–11 n*M*/s). However, their response latencies (median, 5–8 s) were shorter than those obtained for ET-1 but distinctly slower than that monitored for ATP. The muscarinic receptor response exhibited a very pronounced plateau phase (~40% of peak $[Ca^{2+}]_i$), which indicates that a marked influx of extracellular Ca^{2+} accompanies receptor activation, as has previously been observed for other neural cells (Fisher et al., 1989; Lambert and Nahorski, 1990; Iredale et al., 1992). Consistent with this conclusion was the observation that chelation of extracellular Ca^{2+} abolished this phase of the Ca^{2+} signal, under which conditions Ca^{2+} was cleared from the cytosol at a rate ($t_{1/2}$ = 46 s) similar to that observed for ET-1 ($t_{1/2}$ = 33 s). Ca^{2+} signals generated by NE were also distinctive in that they consisted of a transient increase in $[Ca^{2+}]_i$ (which on occasions occurred repetitively), followed by a return to basal $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+}, neither the magnitude nor rate of increase of NE-mediated Ca^{2+} transients was altered. However, an increase in the frequency of Ca^{2+} transients was observed, along with a significant reduction in the time required to clear cytosolic Ca^{2+} ($t_{1/2}$ = 13 s). The latter result indicates that despite a rapid return to basal $[Ca^{2+}]_i$ (Fig. 1g, h), a component of the NE-mediated Ca^{2+} signal does involve the influx of extracellular Ca^{2+}. Although all four agonists differ in terms of the rates at which they mobilize and clear $Ca^{2+}{}_i$, each agonist appears to interact with a common intracellular pool of Ca^{2+}, as evident from the lack of additive effects of agonist-mediated increases in $[Ca^{2+}]_i$ observed in the absence of extracellular Ca^{2+} (Fig. 5).

The results obtained in the present study also raise the possibility that, in addition to the generation of Ca^{2+} signals, the metabolism of inositol phosphates may be agonist specific in SK-N-MCIXC cells. When measured as the release of a total ^{3}H-inositol phosphate fraction, all four agonists resulted in similar increases in PPI turnover (four- to sixfold). In contrast, marked differences in the net increases in the mass of I(1,4,5)P_3 attained for the individual agonists were observed. Thus, whereas a 10-s exposure of cells to either ET-1 or ATP resulted in a consistent two- to threefold increase in I(1,4,5)P_3 mass, only small and variable increases were observed for either Oxo-M or NE. Furthermore, I(1,4,5)P_3 concentrations declined

more rapidly in the presence of ATP than when ET-1 had been added (data not shown). It appears unlikely that the discrepancy between the total ^{3}H-inositol phosphate and I(1,4,5)P_3 measurements is due to the coupling of muscarinic and α_1-adrenergic receptors on SK-N-MCIXC cells to the hydrolysis of lipids other than phosphatidylinositol 4,5-bisphosphate (e.g., phosphatidylinositol), given previous results obtained for the four agonists in the parent SK-N-MC cell line (Fisher and Landon, 1991). More plausible is the possibility that the metabolism of I(1,4,5)P_3 is compartmentalized in SK-N-MCIXC cells such that the intracellular concentrations of the second messenger attained in the presence of either ET-1 or ATP are significantly greater than for Oxo-M or NE (see also Zhao et al., 1990).

Regardless of the mechanism involved, that the maximum increases in I(1,4,5)P_3 differ for each agonist raises the issue, posed previously by Stauderman and Pruss (1990), of whether such differences can be related to the characteristics of the Ca^{2+} signals generated. Although a direct comparison is complicated by differences in the technical procedures used to obtain these measures (i.e., cell suspensions vs. single cells), the possibility that some facets of the Ca^{2+} signals generated in SK-N-MCIXC cells are linked to the concentrations of I(1,4,5)P_3 achieved can be considered. First, recent studies in nonneural cells have indicated that the I(1,4,5)P_3 concentration attained may be important in determining the percentage of cells that respond to an agonist (Rénard-Rooney et al., 1993). Our studies would support that conclusion because, on average, fewer SK-N-MCIXC cells responded to Oxo-M or NE than to either ATP or ET-1 and that the attainment of a threshold concentration of I(1,4,5)P_3, which is consistently met by both ATP and ET-1, appears to be necessary for the initiation of a Ca^{2+} response. Second, although not proportionally related, the magnitude of increase in $[Ca^{2+}]_i$ is greater for those agonists (ET-1 or ATP) that induce the largest increases in I(1,4,5)P_3 mass. This result is in agreement with a previous study of NG 108-15 cells in which the addition of bradykinin was found to result in a five- to sixfold greater increase in I(1,4,5)P_3 mass but only a 70% increase in the magnitude of the rise in $[Ca^{2+}]_i$, when compared with the responses observed for either ET-1 or ATP (Chau et al., 1993). Thus, in both NG 108-15 and SK-N-MCIXC cells, it appears likely that increases in I(1,4,5)P_3 mass beyond a certain level result in little or no further increase in $[Ca^{2+}]_i$. Third, the frequency with which repetitive Ca^{2+} transients are observed in SK-N-MCIXC cells for NE and Oxo-M may also be related to I(1,4,5)P_3 generation, because at all concentrations, weak partial agonists for PPI hydrolysis elicit Ca^{2+} oscillations rather than sustained Ca^{2+} signals (Yule et al., 1993*b*). In contrast, however, other characteristics of Ca^{2+} signals (such as the prevalence of the plateau phase and rate of decline of initial peak $[Ca^{2+}]_i$) appear to be unrelated to I(1,4,5)P_3 production and, thus, additional factors undoubtedly contribute to the shape, size, and duration of agonist-mediated Ca^{2+} transients. For example in hepatocytes, Ca^{2+} signals generated after α_1-adrenergic, vasopressin, angiotensin II, and ATP addition are differentially sensitive to the activation of protein kinase C (Woods et al., 1987*b*; Sanchez-Bueno et al., 1990). Furthermore, in addition to PPI hydrolysis, an agonist may activate other signaling pathways (e.g., adenylyl cyclase), which in turn can serve to modify the characteristics of the Ca^{2+} signals generated (Schöfl et al., 1991).

In summary, the present results obtained using digital imaging microfluorimetry indicate that agonist-specific Ca^{2+} signals can be generated in SK-N-MCIXC cells. We conclude that one means by which specificity of signal transduction involving PPI-linked receptors is attained in these cells is at the level of the Ca^{2+} signal.

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