Differential Ca\(^{2+}\) Signaling Induced by Activation of the Epidermal Growth Factor and Nerve Growth Factor Receptors*

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Stimulation by epidermal growth factor (EGF) of NIH3T3 cells overexpressing the EGF receptor (EGFR) results in a release of Ca\(^{2+}\) from internal stores. Ca\(^{2+}\) release is followed by an influx of extracellular calcium which can be recorded by the influx of the calcium surrogate Mn\(^{2+}\). Both Ca\(^{2+}\) release and Mn\(^{2+}/Ca\(^{2+}\) influx are inhibited by expression of the dominant negative Asn17-Ha-Ras mutant and abrogated by microinjected neutralizing anti-Ras antibody Y13-259, whereas microinjection of the anti-Ras antibody Y13-238 which does not interact with the effector binding domain of Ras is without any effect on the EGF-induced Ca\(^{2+}\) transient. Neither Asn17-Ha-Ras nor the Y13-259 antibody interferes with the thapsigargin-induced Mn\(^{2+}/Ca\(^{2+}\) influx. The nerve growth factor receptor (Trk)-mediated Ca\(^{2+}\) transient was found to be unaffected by the dominant negative Ras mutant or microinjected neutralizing anti-Ras antibodies. Substitution of the phospholipase C\(\gamma\) (PLC\(\gamma\)) binding site of the EGFR by the PLC\(\gamma\) binding domain of Trk renders the EGFR-induced Ca\(^{2+}\) influx insensitive to the expression of Asn17-Ha-Ras, whereas the Ca\(^{2+}\) signal induced by Trk carrying the PLC\(\gamma\) binding site of EGFR is Ras-dependent and abrogated by the dominant negative Ras mutant. It is concluded that the Ca\(^{2+}\) transient induced by the activated EGFR, not however, the Ca\(^{2+}\) transient elicited by the activated NGFR/Trk, is a Ras-mediated phenomenon and that the role of Ras in regulating EGFR-induced Ca\(^{2+}\) influx depends on the structure of the PLC\(\gamma\) binding domain.

In previous reports, we and others have expressed that expression of transforming Ha-Ras leads to an enhanced Ca\(^{2+}\) influx (5, 7–11). It remained unclear, however, whether this effect represents a gain-of-function of the mutated Ras protein or whether cellular Ras is also involved in regulating Ca\(^{2+}\) entry mechanisms operating in nontransformed cells activated by growth factors which activate Ras-dependent signaling pathways.

In order to address this question, Ca\(^{2+}\) transients induced by activation of epidermal growth factor receptor (EGFR)\(^{1}\) and nerve growth factor receptor (NGFR/Trk) were investigated.

Both receptors are known to activate Ras and to cause an elevation of cytosolic free Ca\(^{2+}\) (1, 12–14). The studies presented here were performed with NIH3T3 cells overexpressing either EGFR or an EGFR/Trk chimeric receptor consisting of an extracellular EGFR domain and the cytosolic domain of Trk as described previously (1). This system permits the study of both receptors in the same cellular environment employing the same agonist. In PC12 cells under physiological conditions, EGFR and NGFR induce opposite effects, activation of EGFR elicits proliferation, whereas stimulation of NGFR leads to differentiation (15–19). When expressed in fibroblasts, however, activation of NGFR causes a proliferative response (20). Both receptors have been shown to activate a phosphatidylinositol-specific phospholipase C, resulting in the generation of inositol 1,4,5-trisphosphate (IP\(_3\)) and a release of Ca\(^{2+}\) from intracellular stores (1). In fibroblasts, Ca\(^{2+}\) release is followed or accompanied by an enhanced influx of Ca\(^{2+}\) through voltage-independent Ca\(^{2+}\) channels of the plasma membrane (21, 22). Although the Ca\(^{2+}\) transients elicited by activation of different receptor tyrosine kinases appear to be rather similar, the underlying mechanisms have been shown to differ in a receptor-specific fashion (23). These differences may result from variations in the contribution of Ca\(^{2+}\) influx to the total Ca\(^{2+}\) transient and the Ca\(^{2+}\) channels involved. The data presented here demonstrate that the Ca\(^{2+}\) influx following activation of EGFR is blocked by expression of a dominant negative (Asn\(^{7}\)) mutant of Ha-Ras and abrogated by microinjection of antibodies interacting with the effector binding domain of Ras whereas the Ca\(^{2+}\) influx elicited by an activation of Trk is not affected by Asn\(^{7}\)-Ha-Ras or anti-Ras antibody and appears to proceed by a Ras-independent mechanism. In an attempt to identify the mechanism responsible for the differences in signaling by the two receptor types, it was investigated whether the significantly different affinities to phospholipase C\(\gamma\) (PLC\(\gamma\)) described in a preceding publication (1) are related to the distinct signal transmission. The studies revealed that an exchange of the phospholipase C\(\gamma\) (PLC\(\gamma\)) binding sites between Trk and EGFR renders the Trk-induced Ca\(^{2+}\) influx Ras-dependent and abolishes the Ras independence of the EGFR-mediated Ca\(^{2+}\) influx.

EXPERIMENTAL PROCEDURES

Materials—Fura-2/AM was obtained from Molecular Probes; culture media and sera were from Boehringer Ingelheim Bioproducts; SK&FP96365 was from Smith Kline Beecham Pharmaceuticals; EGF and nifedipine were purchased from Sigma; pRSV-Asn\(^{7}\)-Ha-Ras was kindly provided by L. de Vries, Laboratory for Physiological Chemistry, University of Utrecht, and pEF-neo GFP-S65T by S. Geley, Institute of Pathology, University of Innsbruck.

Cells—NIH3T3 fibroblasts overexpressing EGFR (EGFR6), NIH3T3 cells expressing a chimeric EGFR/Trk (ETR2), and NIH3T3 cells expressing the mutant receptors EGF-R.X2 or ET-R.X3 were grown in

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Fig. 1. Effect of Asn17-Ha-Ras on Ca2+ release from intracellular stores and Ca2+ influx induced by the activated EGF receptor (A) and the activated NGF/Trk receptor (B). Fura-2-loaded EGFR6 cells (A), and ETR2 cells expressing a chimeric EGF/Trk receptor (B) were kept in nominally Ca2+-free HEPES buffer and stimulated with 50 ng/ml EGF. Where indicated, 1 mM Ca2+ was added. Representative single-cell recordings of control cells and Asn17-Ha-Ras transfectant cells (dashed line) are shown.

The Ca2+ Transient Elicited by the Activated EGF Receptor Is Ras-dependent—Fig. 1A shows a representative single-cell recording of an EGF-induced Ca2+ signal in EGFR6 cells. In order to discriminate between Ca2+ release and Ca2+ influx, cells were initially kept in Ca2+-free medium. Addition of EGF leads to a release of Ca2+ from internal stores. After store depletion, Ca2+ was added to the medium. This results in a second peak of intracellular Ca2+ representing Ca2+ influx and release of refilled stores. For a more sensitive determination of calcium influx, the Ca2+ surrogate Mn2+ was employed, and the quench of fura-2-loaded cells by exogenous Mn2+ was determined in a single-cell imaging system. As shown in Table I, this EGF-induced Mn2+ influx is sensitive to the Ca2+ channel blockers SK&F96365 and La3+, but insensitive to nifedipine up to concentrations of 10 μM. These results were likewise obtained by whole-cell patch clamp studies (data not shown).

In order to determine the role of Ras in the EGF-induced Ca2+ transient, cells were transiently transfected with an expression plasmid encoding the dominant negative Asn17-Ras mutant. Transfection was performed by microinjection, and the transfected cells were identified by co-transfection with a vector encoding a mutated version of the green fluorescent protein (GFP-S65T). As shown in Fig. 1 and Table II, Asn17-Ras inhibits the EGF-induced Ca2+ transient by interfering with both Ca2+ release and Ca2+ influx. Whereas Ca2+ release is strongly but incompletely suppressed (Fig. 1A), Ca2+/Mn2+ influx is completely abrogated (Table II). The results obtained with the dominant negative Ras mutant were confirmed by microinjection of anti-Ras antibodies. The monoclonal antibody Y13-259 had been shown to interact with the effecter binding domain of Ras and to inhibit the proliferative effect of serum growth factors (24). As shown in Table III, microinjection of this antibody completely inhibits the EGF-induced Ca2+/Mn2+ influx in EGFR6 cells. Microinjection of the non-neutralizing anti-Ras antibody Y13-238 (25) did not affect Ca2+/Mn2+ influx (Table III). Microinjected unspecific fluorescein isothiocyanate-labeled antibody which was used to identify the treated cells also did not interfere with the Ca2+ influx (data not shown).

Thapsigargin-induced Ca2+ Influx Is Not Ras-dependent—The absolute Ras dependence of the EGF-induced Ca2+ influx raised the question whether Ras is involved in regulating the activity of store-operated calcium channels. For this reason, intracellular Ca2+ stores were depleted with thapsigargin, and the effects of an expression of the dominant negative Ras mutant and microinjected, neutralizing anti-Ras antibody were determined. The data revealed, however, that neither Asn17-Ras nor the neutralizing anti-Ras antibody are able to interfere with thapsigargin-induced store-regulated Ca2+ influx (Tables II and III).

The Ca2+ Transient Induced by Activated NGF/Trk Is Ras Independent—The role of Ras in the NGFR/Trk-induced Ca2+ signal was studied in ETR2 cells. These cells represent NIH3T3 fibroblasts expressing a chimeric EGF/Trk consisting of the extracellular EGF binding domain of the EGFR and the cytosolic region of Trk (26). Activation of Trk by EGF results in a calcium signal which resembles the Ca2+ transient observed after activation of EGFR in EGFR6 cells (Fig. 1B and Table I). In contrast to the EGFR-induced Ca2+ signal, neither release nor influx of Ca2+ are affected by an expression of Asn17-Ras or microinjected neutralizing anti-Ras antibodies (Fig. 1B, Tables II and III).

The Ras Dependence of the Receptor-mediated Ca2+ Influx Is Determined by the Structure of the Phospholipase Cγ1 (PLCγ1) Binding Domains—A major difference between EGFR and NGFR/Trk is the affinity to PLCγ1. Compared to the EGFR, the affinity of Trk to PLCγ1 is approximately 100-fold higher (28). The high affinity of activated Trk was shown to be defined by ±5 amino acid residues flanking phosphorylated tyrosine 785. Changing the tyrosine at this position to phenylalanine causes a PLCγ binding-incompetent mutant receptor that cannot induce any IP3 or Ca2+ signal upon EGF stimulation (1). In the EGFR, a PLCγ1binding site surrounding tyrosine 992 had been identified. In addition to the EGFR domain surrounding Tyr992, which exhibits the highest affinity for PLCγ1, secondary low affinity binding sites such as Tyr1068 and Tyr1173 could be characterized (27). In order to investigate the significance of
the distinct binding properties for the Ras dependence of the Ca\(^{2+}\) signal, exchange mutants were employed. The exchange mutant EGF-RX carried the Trk residues 780–790 in place of the PLC\(^{-}\) binding domain of the EGFR rendered the Ca\(^{2+}\) binding sites and can be equally effective in competing with PTP1b for binding to the corresponding binding domain of the receptors.

According to a recent publication, the EGF-induced Ca\(^{2+}\) signal is usually mediated by inositol 1,4,5-trisphosphate (IP\(_{3}\)) generated by a phospholipase C (PLC) (30). Ligand-activated EGFR is known to bind and activate PLC\(_{Y}\) (31). Although evidence for Ras as an upstream effector or regulator of PLC\(_{Y}\) has been presented (32, 33), the detailed mechanism by which Ras could regulate PLC activity is still unclear. Recently, it has been shown that the SH2 domains of p120\(^{Ras-GAP}\) exhibit a similar affinity to a binding site of the EGFR as the SH2 domains of PLC\(_{Y}\) (34). The region around the phosphorylated tyrosine at position 992 was also described as a high affinity binding site for protein-tyrosine phosphatase 1b (PTP1b) and PLC\(_{Y}\) as well as GAP are equally effective in competing with PTP1b for binding to the

**TABLE I**

| DNA plasmid (100 µg/ml) | EGF (100 ng/ml) | Thapsigargin (1 µM) | Thapsigargin + Y13-259 (1 µM) |
|-------------------------|-----------------|---------------------|-------------------------------|
| EGFR6                   | 20.8 ± 1.1      | 19.0 ± 1.3          | 18.9 ± 1.7                    |
| ETR2                    | 20.2 ± 1.8      | 21.2 ± 2.8          | 19.8 ± 2.3                    |
| ETR2 FCS               | 19.6 ± 0.7      | 20.1 ± 2.5          | 18.8 ± 1.6                    |

**DISCUSSION**

The data presented here demonstrate that the Ca\(^{2+}\) signal induced by an activation of the epidermal growth factor receptor (EGFR) is mediated by a Ras-dependent mechanism. The complete inhibition of Ca\(^{2+}\) influx which is seen in cells expressing the dominant negative Asn\(^{17}\)-Ras mutant or observed after microinjection of neutralizing anti-Ras antibodies is probably due to the suppression of Ca\(^{2+}\) release (Fig. 1A). The attenuated depletion of internal Ca\(^{2+}\) stores may be insufficient to activate a store-operated Ca\(^{2+}\) influx. That Ras is not required for the activation of store-operated Ca\(^{2+}\) channels of the plasma membrane is supported by the insensitivity of the thapsigargin-mediated Ca\(^{2+}\) influx to Asn\(^{17}\)-Ras or microinjected neutralizing anti-Ras antibodies. The mechanism by which Ras regulates Ca\(^{2+}\) release remains to be elucidated. According to a recent publication, the EGF-induced Ca\(^{2+}\) transient is completely abolished by dominant negative Asn\(^{17}\)-Rac\(_{1}\) (23) indicating that Asn\(^{17}\)-Rac\(_{1}\) also interferes with Ca\(^{2+}\) release; otherwise, the initial rise in cytosolic free Ca\(^{2+}\) release would have been unaffected. These findings together with the data reported here suggest that the EGF-induced Ca\(^{2+}\) release involves Ras and Rac\(_{1}\). The activation of c-Jun amino-terminal kinases (JUNKs) by EGF has also been shown to require Ras and Rac1 (28, 29). Thus, the activated EGFR may employ a similar pathway for the generation of the Ca\(^{2+}\) signal and the activation of JUNK. The release of internal Ca\(^{2+}\) is usually mediated by inositol 1,4,5-trisphosphate (IP\(_{3}\)) generated by a phospholipase C (PLC) (30). Ligand-activated EGFR is known to bind and activate PLC\(_{Y}\) (31). Although evidence for Ras as an upstream effector or regulator of PLC\(_{Y}\) has been presented (32, 33), the detailed mechanism by which Ras could regulate PLC activity is still unclear. Recently, it has been shown that the SH2 domains of p120\(^{Ras-GAP}\) exhibit a similar affinity to a binding site of the EGFR as the SH2 domains of PLC\(_{Y}\) (34). The region around the phosphorylated tyrosine at position 992 was also described as a high affinity binding site for protein-tyrosine phosphatase 1b (PTP1b) and PLC\(_{Y}\) as well as GAP are equally effective in competing with PTP1b for binding to the

**TABLE II**

| DNA plasmid (100 µg/ml) | EGF (100 ng/ml) | Thapsigargin (1 µM) | Thapsigargin + Y13-259 (1 µM) |
|-------------------------|-----------------|---------------------|-------------------------------|
| EGFR6                   | 20.8 ± 1.1      | 19.0 ± 1.3          | 18.9 ± 1.7                    |
| ETR2                    | 20.2 ± 1.8      | 21.2 ± 2.8          | 19.8 ± 2.3                    |
| ETR2 FCS               | 19.6 ± 0.7      | 20.1 ± 2.5          | 18.8 ± 1.6                    |

**TABLE III**

| DNA plasmid (100 µg/ml) | EGF (100 ng/ml) | Thapsigargin (1 µM) | Thapsigargin + Y13-259 (1 µM) |
|-------------------------|-----------------|---------------------|-------------------------------|
| EGFR6                   | 20.8 ± 1.1      | 19.0 ± 1.3          | 18.9 ± 1.7                    |
| ETR2                    | 20.2 ± 1.8      | 21.2 ± 2.8          | 19.8 ± 2.3                    |
| ETR2 FCS               | 19.6 ± 0.7      | 20.1 ± 2.5          | 18.8 ± 1.6                    |

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requires a release from the receptor (37), conditions which bind to the effector domain of GTP-charged Ras (36), which same binding site of the EGF receptor. Since Ras-GAP also domain of Trk instead of the endogenous PLC
bindingsites of EGF receptor and Trk reduce the binding of PLC
could be confirmed by studies employing receptor mutants.

We had shown previously (1) that the EGF-induced IP3 response in cells overexpressing the EGF/Trk (ETR) is proportional to the affinities of PLCγ to the corresponding receptors, i.e. high for ETR cells and low for EGFR cells. Substitution of tyrosine at position 785 of the ETR by a phenylalanine eliminates the binding of PLCγ to the ETR and abrogates the EGF-induced IP3 and Ca2+ signals (1). Exchange of the PLCγ binding sites of EGFR and Trk reduces the binding of PLCγ to the mutated ETR and enhances PLCγ binding to the EGF/Trk exchange mutant. Accordingly, EGF-induced IP3 and Ca2+ signals were found to be strictly proportional to the binding affinities of PLCγ to the corresponding receptor mutants (1).

Activation of a mutant EGFR carrying the PLCγ binding domain of Trk instead of the endogenous PLCγ binding domain leads to a Ca2+ influx which is unaffected by dominant negative Ras, although the expression levels of the wild type EGFR6 and the mutant EGF-R.X2 were found to be similar. Further-
more, the Trk-induced Ca2+ influx which was found to be unaffected by dominant negative Ras, becomes Ras-dependent if the PLCγ binding site of Trk is replaced by the PLC binding domain of the EGFR. The data obtained with the exchange mutants also demonstrate that the insensitivity of the Trk-induced Ca2+ influx to dominant negative Asn17-Ras is not explained by an inefficient blockade of Ras activation. Both the ETR2 cells and the ET-R.X3 cells overexpress the corresponding chimeric EGF/Trk receptors to similar levels (1). Whereas Asn17-Ras does not affect the Ca2+ influx following activation of the ET-R receptors which contain the wild-type Trk, the Ca2+ signal observed after stimulation of the ET-RX receptor carrying the mutated PLC binding site is completely abrogated, demonstrating that Asn17-Ras is indeed active in the cells expressing the chimeric EGF/Trk receptors.

The implication of Rac1 in the EGF-induced Ca2+ signal which had been reported by others (23) may indicate an additional requirement for an increased pool of phosphatidylinositol 4,5-bisphosphate. Rac1 and RhoA have been shown to stimulate phosphatidylinositol-4'-kinase and phosphatidyl-4-phosphate-5'-kinase, respectively (40, 41). Dominant negative RhoN19 causes indeed a partial inhibition of the EGF-induced Ca2+ signal under conditions where the ATP-mediated Ca2+ transient is unaffected (23). Constitutively active V12Ras has been shown to activate Rac1 (28, 29). Although the mechanism by which Ras activates Rac1 is still unclear, evidence is accumulating that Rac1 can be activated by Ras-dependent and independent pathways (29). Evidence for an activation of RhoA by Rac1 has been presented (42). Phospholipase A2 has also been implicated in the generation of the EGF-induced Ca2+ signal (43). Depending on the cell type, activation of PLA2 appears to be mediated by either Ras or Rac1, but the role of this PLA2-dependent pathway for the EGF-induced Ca2+ signal is still obscure (43).

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