Rapid Stimulation of Ser/Thr Protein Kinases following Treatment of Swiss 3T3 Cells with Bombesin

IN涉及MENT OF CASEIN KINASE-2 IN THE SIGNALING PATHWAY OF BOMBESIN*

Patrizia Agostiniet, Johan Van Lint$, Stefania Sarno3, Peter De Witte4, Jackie R. Vandenheede**$$, and Wilfried Merlevede

From the Afdeling Biochemie, Faculteit Geneeskunde, and |Departement Farmaceutische Wetenschappen, Katholieke Universiteit te Leuven, Belgium

*This work was supported by grants from the "National Fonds voor Geneeskundig Wetenschappelijk Onderzoek" and from the "Belgisch Werk tegen Kanker". The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The amphibian tetradecapeptide bombesin, as well as the bombesin-related mammalian peptides including gastrin-releasing peptide and the neuromedins (for a review see Ref. 6), are potent mitogens for Swiss 3T3 cells (6), human small cell lung cancer cells (7), and bronchial epithelial cells (8). The bombesin receptor has been cloned from Swiss 3T3 fibroblasts: it is a member of the family of seven hydrophobic-region, G-protein-linked neuropeptide receptors (9) and its sequence does not reveal a protein kinase domain. Upon binding to its receptor, bombesin evokes a complex cascade of early biochemical events including inositol 1,4,5-trisphosphate-induced mobilization of intracellular Ca$^{2+}$, Na$^+$, and K$^+$ fluxes, protein kinase C activation, transmodulation of the EGF-receptor, and accumulation and expression of the proto-oncogenes c-Fos and c-Myc and cAMP production (6). Although this complex signaling pathway is still largely unknown, it promises to have some striking features.

First of all, bombesin by itself, just like platelet-derived growth factor or fibroblast growth factor, can act as a sole mitogen for Swiss 3T3 cells (10). The fact that many hormones and growth factors which have similar receptors with seven membrane spanning domains, are by themselves not mitogenic for Swiss 3T3 cells, suggests that bombesin is acting...
plasmic tyrosine kinase domain, bombesin stimulates the tyrosine phosphorylation of multiple substrates in quiescent through a rather special signaling pathway.

Second, although its receptor does not contain a cytoplasmic tyrosine kinase domain, bombesin stimulates the tyrosine phosphorylation of multiple substrates in quiescent Swiss 3T3 fibroblasts (11). However, as recently reported by Zachary et al. (11) none of these substrates corresponds by molecular weight or by immunological or biochemical criteria to known tyrosine kinase substrates suggesting that the tyrosine phosphorylation events initiated by this neuropeptide may be different from the classical receptor tyrosine kinase induced phosphorylations.

We were therefore interested to know whether the bombesin-induced mitogenesis in Swiss 3T3 cells encompasses any of the signaling mechanisms common of receptor tyrosine kinases. In particular, we examined the effect of bombesin on the activity of already known growth factor-activated kinases (recently reviewed in Refs. 12-14). The present study shows that, in quiescent Swiss 3T3 fibroblasts, bombesin rapidly stimulates the activity of several S6 peptide kinases as well as MBP kinases. Furthermore, our results implicate casein kinase 2 (CK-2) in the early signal transduction pathway of this mitogen.

EXPERIMENTAL PROCEDURES

RESULTS

Time-dependent Effect of Bombesin on Soluble Kinase Activities

Cytosolic extracts of either control cells or cells treated for various lengths of time with bombesin (50 nM) were assayed for their ability to phosphorylate a number of peptide and protein substrates such as the S6 peptide, the G peptide, MBP, and the RRREEESEE peptide (Fig. 1, Miniprint).

Fig. 1 shows that kinase activities toward the S6 peptide, MBP, and the G peptide peaked after 5-10 min of bombesin treatment. The rate of phosphorylation of the S6 peptide and the G peptide declined by about 40% at the 40-min time point (Fig. 1, A and B), whereas the 7-fold maximal stimulation of the MBP kinase, seen at 5-min incubation with the growth factor, was more drastically reduced at the later time points (Fig. 1C). Kinase activities toward the RRASVA peptide and the Kemptide followed the pattern of the S6 peptide phosphorylation in the time-course experiment (not shown).

The rate of phosphorylation of the RRREEESEE peptide (CK-2 activity) did not change very dramatically (Fig. 1D): a 30% increase in CK-2 activity was observed at the very most. Similar results were also obtained using casein as a substrate for CK-2 (not shown).

Dose Dependence of Protein Kinase Activation by Bombesin

In order to characterize the dose dependence of the protein kinase stimulations, quiescent Swiss 3T3 cells were treated for 5 min with various concentrations of bombesin. As shown in Fig. 2, a maximal response was observed with concentrations of bombesin around 10^-8 to 10^-9 M, which corresponds to the concentration range which elicits the mitogenic response in Swiss 3T3 fibroblasts (6). The small CK-2 activation measured with the RRREEESEE peptide as illustrated in Fig. 1 was not always evident. The higher level of activation observed in these experiments as compared to the ones reported in the time courses of Fig. 1 are possibly due to the stabilizing effect of 100 nM microcinulin which was included in the extraction buffer of the dose-dependence experiments.

Separation of Bombesin-activated Protein Kinase Activities by Mono Q FPLC Column Chromatography

The kinase activities in extracts from Swiss 3T3 control cells and from cells treated for either 5 or 40 min with bombesin, were resolved by Mono Q FPLC chromatography. Early Responses (5 Min of Bombesin Treatment)—Fig. 3A shows that the S6 peptide kinase activities in the cells treated for 5 min with bombesin were separated into several peaks: a major part of the activity came in the breakthrough of the column and four overlapping activity peaks eluted early in the gradient: at 0.12, 0.14, 0.17, and 0.2 M NaCl, respectively. All the S6 peptide kinase peaks could phosphorylate the Kemptide and, with the exception of the 0.2 M NaCl peak, also the RRASVA peptide (not shown).

The breakthrough, the 0.12 M, and the 0.17 M eluate peaks revealed comparable kinase activities toward the G peptide as substrate (Fig. 3B), suggesting that the activated kinases may phosphorylate both peptides at very similar rates. The stimulated G peptide kinase peak eluting at 0.09 M NaCl was occasionally also seen using the S6 peptide as substrate. However, the major stimulated G peptide kinase eluted later in the gradient at 0.35 M NaCl where no S6 peptide kinase activity was measurable (Fig. 3A). Usually more G peptide kinase activity (200%) was recovered after Mono Q chromatography than originally applied to the column, suggesting that a large fraction of kinase activity toward this peptide is masked in whole cytosolic extracts.

The MBP kinase activity (Fig. 3C) was resolved by the gradient in two peaks eluting at 0.11 M (MBP kinase I) and at 0.17 M NaCl (MBP kinase II): the 8-fold stimulation by bombesin measured in the cytosols (Fig. 2C) was quantitively

![FIG. 3. Elution profile of bombesin-stimulated kinase activities on Mono Q FPLC anion exchange chromatography. Cytosol from control cells (○) or from cells incubated for 5 min with 50 nM bombesin (●) were applied to a Mono Q anion exchange column. The column was developed with a linear gradient of NaCl from 0 to 0.5 M (dashed line), and aliquots of the fractions were assayed for kinase activities as in Fig. 1. The elution pattern depicted is representative of results obtained in four independent experiments with different cell preparations.](http://example.com/fig3.png)
recovered in the gradient fractions.

Surprisingly, when the RRREEESEEE peptide was used to specifically detect the CK-2 activity in the Mono Q eluted fractions, a many-fold stimulation was observed, coeluting exactly with the G peptide kinase activity at 0.35 \( \text{m NaCl} \) (Fig. 3D). This observation together with the results depicted in Fig. 1C and Fig. 2D strongly suggests that the Mono Q chromatography step may separate substances that interfere in the assay: recovery of the bombesin-stimulated CK-2 activity was several-fold greater in the Mono Q-eluted fractions. The degree of CK-2 activation, measured in the gradient fractions, varied between 2-6-fold in a series of four totally independent stimulation experiments. Differences in the level of activation of CK-2 are for instance seen in the experiments illustrated (see Figs. 3D and 5). In general it was observed that when cells of later passages were used in similar experiments or when the subculture schedule was not strictly followed, basal levels of CK-2 activity were enhanced several-fold and stimulation by bombesin greatly reduced. This would suggest that partially transformed Swiss 3T3 cells already have the CK-2 enzyme in an activated form.

Late Responses (40 Min of Bombesin Treatment)—As illustrated in Fig. 4, prolonged incubation (40 min) of Swiss 3T3 cells with bombesin resulted in a decrease in the activation level of all kinases. The activities measured in the Mono Q profiles reflected very much the respective kinase levels measured in the cytosolic extracts at the 40-min time points in Fig. 1 (except for the CK-2 activation discrepancy). The most important decrement in \( S_\alpha \) peptide kinase activities (Fig. 4A) occurred in the breakthrough fraction (80\%) as well as in those peaks eluting very early in the gradient (between 0.12 and 0.17 \( \text{m NaCl} \)), whereas the last \( S_\alpha \) peptide kinase peak was only slightly affected (about 25\%). The MBP kinase activations (Fig. 4C) decreased by 80\% after 40 min of incubation of cells with bombesin (Fig. 3C) similarly to what was observed in the extracts (Fig. 1C). The coeluting G peptide and RRREEESEEE peptide kinase activities were also reduced after prolonged bombesin treatment to about 45 and 30\%, respectively (Fig. 4D).

Implication of CK-2 in the Signaling Pathway of Bombesin

In order to further characterize the RRREEESEEE peptide kinase eluting in the 0.35 \( \text{m NaCl} \) peak on the basis of its site specificity, the GS-1 peptide (34), YRRAAVPPS\(^\text{a}\)PSLS\(^\text{a}\)RH-SS\(^\text{a}\)PHQS\(^\text{a}\)EDEE, which contains the specific CK-2 phosphorylation site (Ser\(^\text{a}\)) of glycogen synthase, was used to assay kinase activity under similar experimental conditions as in Fig. 3. As shown in Fig. 5C, the GS-1 peptide served as a substrate for three kinase activities corresponding to the stimulated MBP kinases I and II (Fig. 5B) and the RRREEESEEE peptide kinase (Fig. 5A).

Phosphorylation of the GS-1 peptide by kinase F\(_\alpha\)/GSK\(_3\), which occurs on sites Ser\(_{3b}, -3c, \) and -4, is absolutely dependent upon prior phosphorylation of site 5 by CK-2 (27, 28). Moreover, kinase F\(_\alpha\)/GSK\(_3\) activity measured with the GS-1 peptide previously phosphorylated by CK-2 eluted in the breakthrough fraction of the Mono Q column, and its activity was not changed upon bombesin treatment. The GS-1 peptide does not contain phosphorylation sites for the \( S_\alpha \) peptide kinases, so that its increased phosphorylation at the 0.11 and 0.17 \( \text{m NaCl} \) elution peaks is either due to the activated MBP...
kinases themselves or to still unknown coeluting activities.
The rate of phosphorylation of the GS-1 peptide by the two
possible to obtain a reliable measurement of the residual
peptide SEEEEE, reminiscent of the glycogen synthase Ser'
reference substrate (MBP), indicating that both substrates
were completely abolished by type 2A phosphatase action,
peak fractions, were completely abolished by the addition of
heparin in the phosphorylation assay (not shown).
In conclusion, the overlapping activities toward three differ-
ent peptide substrates (GS-1, G-, and RRREEEEEEE peptides)
containing consensus sequences for CK-2, together with the coinciding elution at 0.35 M NaCl in the Mono Q
gradient 19) and the inhibition by heparin, are three strong
arguments indicating that these phosphorylations are due to
CK-2 itself.

Phosphatase Treatment of the Bombesin-activated Kinases

The addition of microcystin, a powerful inhibitor of ATP,
Mg-dependent (type 1)- and PCS (type 2A) phosphatases
34), to the column fractions was absolutely required to main-
tain the activation of the protein kinases. This strongly sug-
gests that phosphorylations are involved in the mechanism of
the kinase activations triggered by bombesin. Therefore, frac-
tions containing activated kinases were subjected to the PCSi
(type 2A) phosphatase before addition of microcystin (Fig.
6). After 30 min of phosphatase treatment the MBP I kinase
was almost completely inactivated (Fig. 6B), whereas the
MBP II kinase activity was reduced by 60% (Fig. 6C). Since
microcystin could prevent MBP kinase inactivation (Fig. 6,
B and C) it can be concluded that the activation of both
enzymes depends upon a Ser/Thr phosphorylation reaction.
On the contrary, the stimulated CK-2 activity toward the
RRREEESEEED peptide was totally unaffected by PCSi
(PP2A i) phosphatase action (Fig. 6A). Treatment of the activated CK-2 fractions with alkaline phosphatase bound to
agarose did not inactivate the enzyme neither (not shown).
Pooled CK-2 fractions from control cytosols were also insen-
sitive to PCSi (PP2A i) phosphatase action (not shown).
This indicates either that the activation of CK-2 is not directly or
solely controlled by Ser/Thr phosphorylation or that the phos-
phorylation sites involved are not accessible to the phos-
phatases used.

Due to the high instability of the activated S6 peptide
kinases in the absence of microcystin, it was not always
possible to obtain a reliable measurement of the residual
activities after preincubation of the pooled fractions at 30 °C.
Nevertheless, the S6 peptide kinase activities that were sepa-
rated by the Mono Q gradient between 0.12 and 0.17 M NaCl
were completely abolished by type 2A phosphatase action,
whereas the more retarded S6 peptide kinase peak (eluting at
0.2 M NaCl) did not seem to be affected (not shown).

DISCUSSION

The present study unambiguously shows that treatment of
Swiss 3T3 cells with the neuromodulator bombesin results in
a rapid increase in the activity of several cytosolic Ser/Thr
kinases toward a number of protein- and peptide substrates.
In particular, incubation of cells with bombesin (5 min) pro-
duces a several-fold activation of multiple S6 peptide kinases
and of two MBP kinases. Interestingly, this mitogen also
raises a kinase activity toward the peptide RRREEESEEED
which is only revealed after chromatography of bombesin-
stimulated cytosols on a Mono Q FPLC column. This acti-
uated kinase was furthermore identified as CK-2.

Quiescent Swiss 3T3 fibroblasts can be induced to prolif-
erate by a variety of peptide growth factors and hormones,
seemingly involving different early signal transduction events.
However, the activation of certain key enzymes appears to be
common to several signaling pathways. This is the case for
the S6 kinases and MBP kinases which are established
regrowth-regulated enzymes (12-14). When comparing our re-
results with published reports it is clear that the signaling
pathways of bombesin and EGF involve the activation of
similar sets of kinases, both in the early and in the late phases
of the cellular response.

A group of five bombesin-stimulated kinases phosphorylate
the S6 peptide, and their time course of activation, substrate
specificity, chromatographic behavior, and their sensitivity to
phosphatase action relates them to the EGF-activated S6
peptide kinases described in Ref. (19).

Two distinct peaks of activated MBP kinases are observed in
the Mono Q column elution of bombesin-stimulated cyto-
sols. Their chromatographic properties as well as their sen-
sitivity to inactivation by the PCSi (PP2A i) phosphatase,
make them comparable to the EGF-stimulated MBP kinases
1 and 2 which also recognize microtubule-associated protein
2 (MAP2) as a substrate (19, 35). The MBP/MAP2 kinases
have been reported to be growth-stimulated enzymes in var-
ious systems (13, 14, 36), and both kinases activate a S6
peptide kinase from control Swiss 3T3 cells (37). While this
work was in progress, Takuya et al. (38) investigated the role of
Ca2+ influx on the mitogenic response induced by bombesin
in Swiss 3T3 cells and reported that bombesin-stimulated cyto-
sols incorporated more [32P]phosphate into exogenously
added MBP and S6 protein than control cytosols. This in-
crease in phosphorylation was found to be independent of the
extracellular Ca2+ concentration, but no attempt was made to
characterize the enzymes involved.

Probably the most intriguing finding of the present report
is the rapid stimulation of CK-2 following treatment of quies-
cent Swiss 3T3 fibroblasts with bombesin. This ubiquitous
kinase is a very well characterized enzyme as far as its subunit
structure, substrate specificity, cellular localization, and sen-
sitivity to inhibitors and activators is concerned, but a full
comprehension of its physiological regulation is still missing
(for a review see Ref. 39). An interesting but puzzling obser-
vation in our study is that a chromatography step is necessary
to reveal the bombesin-mediated activation of CK-2. The CK-
2 activity of control cytosols is quantitatively recovered in the
Mono Q column fractions, indicating that the chromatogra-
phy step by itself does not produce an artificial increment in
the CK-2 activity. Therefore, the increased CK-2 activity
measured after the ion exchange chromatography of bomb-
esin-treated cells is likely due to the separation of a factor
present in stimulated cytosols, which specifically masks the
activated CK-2 enzyme. Since a similar level of CK-2 activity
is measured in both control and bombesin-stimulated cytosols
it is conceivable that the bombesin-generated CK-2 activity
is mobilized from a latent enzyme pool. We have currently no
evidence for a direct correlation between the bombesin-in-
duced CK-2 activation and a phosphorylation of the enzyme
although clearly more information is required to clarify this
point.

As mentioned before, Swiss 3T3 cells of later passages tend
to undergo spontaneous transformation, and this is reflected

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in an altered cell morphology and an increase in the basal level of CK-2 activity. It is well documented that the CK-2 activity is increased in transformed cells and proliferating tissues (40–44) as well as during cell differentiation (45) and in most cases this correlates with an elevated expression of the enzyme. In addition to this, a transient rise in CK-2 activity has been observed upon stimulation of cells by different growth factors: in WI38 human lung fibroblasts by serum (46), in 3T3-L1 adipocytes by insulin or EGF (47), in BALB/c 3T3 fibroblasts by IGF-1 or insulin (48) and in A431 human carcinoma cells by EGF (49). The latter observations have inferred that some short-term modulation of this kinase should also exist, and it has been suggested that a covalent modification of CK-2 underlies its mechanism of regulation (47, 49, 50). The magnitude of CK-2 activity is increased in transformed cells and proliferating or 3T3 fibroblasts by IGF-1 and in A431 human lung fibroblasts by serum (48) and in proliferating cells (51). It is therefore conceivable that the activation of oncogene products Myc, Myb, Fos, p53, E1A, the SV40 T antigen (51), and the serum response factor ~67'6 H 8 (52) are substrates for CK-2 tends to confirm this.

Acknowledgments—We thank H. De Wulf, I. Vanden Bosch, and S. Vander Perre for expert technical assistance and V. Feytons for the synthesis of the peptides. The contribution of S. Debrock in the cell culture experiments is greatly appreciated.

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Supplementary Material to "Rapid stimulation of ser/thr protein kinases following treatment of wells 3T3 cells with bombesin. Involvement of G-protein(s) in the coupling pathway of bombesin receptors".

Appendix D. Assay for PKA and PKC activity

Figure 1. Time dependence of the activation by bombesin of Ser/Thr kinases in Swiss 3T3 cells. Cells were incubated for the indicated lengths of time with medium plus (D) or minus (C) bombesin (10 nM) or with buffer alone (A). The cells were prepared and kinase activities measured toward (A) G peptide (B) G peptide. The values are the means of three independent experiments.

Figure 2. Concentration dependence of bombesin in Swiss 3T3 cells. Cells were treated with the indicated concentrations of bombesin for 5 min and assayed as described in Experimental Procedures. Protein kinase activities in the extracts were measured as in Fig. 1. Values are the means of the independent experiments with different cell preparations.