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Protein phosphatase-protein kinase interplay modulates α_{1b}-adrenoceptor phosphorylation: effects of okadaic acid

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1 In the present work we studied the effect of protein phosphatase inhibitors on the phosphorylation state and function of α_{1b}-adrenoceptors.

2 Okadaic acid increased receptor phosphorylation in a time- and concentration-dependent fashion (maximum at 30 min, EC_{50} of 30 nM). Other inhibitors of protein phosphatases (calyculin A, tautomycin and cypermethrin) mimicked this effect.

3 Staurosporine and Ro 31-8220, inhibitors of protein kinase C, blocked the effect of okadaic acid on receptor phosphorylation. Neither genistein nor wortmannin altered the effect of okadaic acid.

4 The intense adrenoceptor phosphorylation induced by okadaic acid altered the adrenoceptor-G protein coupling, as evidenced by a small decreased noradrenaline-stimulated [^{35}S]GTPγS binding. Okadaic acid did not alter the noradrenaline-stimulated increases in intracellular calcium or the production of inositol trisphosphate.

5 Our data indicate that inhibition of protein phosphatases increases the phosphorylation state of α_{1b}-adrenoceptors; this effect seems to involve protein kinase C. In spite of inducing an intense receptor phosphorylation, okadaic acid alters α_{1b}-adrenergic actions to a much lesser extent than the direct activation of protein kinase C by phorbol myristate acetate.
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Abbreviations: α_1-AR, α_1-adrenoceptor; $[Ca^{2+}]_i$, intracellular calcium; DMEM, Dulbecco's modified Eagle's medium; GRK, G-protein receptor kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

Introduction

The α_1-adrenoceptors (α_1-ARs) are a heterogeneous subgroup of receptors, members of the seven transmembrane domains/G protein-coupled family of receptors that mediate important physiological actions of adrenaline and noradrenaline. Three α_1-ARs have been cloned and expressed, i.e. the α_{1a}-, α_{1b}- and α_{1d}-ARs (Hieble *et al*., 1995). The hamster α_{1b} subtype was the first to be cloned (Cotecchia *et al*., 1988), it is the best characterized and, therefore, considered prototypic of this subgroup. These receptors are mainly coupled to the phosphoinositide/calcium mobilization signal transduction pathway, although they can also activate other signalling processes (Graham *et al*., 1996).

Modulation of G protein coupled receptor function (desensitization/resensitization) is a key event in the adaptation of cells to the changes in the internal milieu of an organism and to the overall homeostasis. Different cellular processes are involved (receptor uncoupling from G proteins, internalization, degradation, regulation of receptor gene expression, etc.) with different time frames (Lefkowitz, 1998). An initial event seems to be receptor phosphorylation. Three groups of protein kinases are the major modulators of G protein-coupled receptors: (a) second messenger-activated kinases, such as protein kinase A and protein kinase C (PKC) (Clark *et al*., 1988; Houslay, 1991, (b) members of the G-protein receptor kinase (GRK) family (Benovic *et al*., 1990) and (c) some receptors with tyrosine kinase activity (Hadcock *et al*., 1992).

Regarding α_{1b} -ARs, it is known that activation of PKC blocks the actions of these receptors and that such effect is associated to receptor phosphorylation (Corvera & García-Sáinz, 1984; Corvera *et al*., 1986; Diviani *et al*., 1996; 1997; Lattion *et al*., 1994; Leeb-Lundberg *et al*., 1985; Vázquez-Prado & García-Sáinz, 1996; Vázquez-Prado *et al*., 1997). Similarly, there is also evidence indicating that GRKs phosphorylate these receptors and participate in their homologous desensitization (Diviani *et al*., 1996). The PKC- and GRK-phosphorylation sites were recently identified at the α_{1b}-AR carboxyl terminus (Diviani *et al*., 1996; 1997).

The phosphorylation state of a receptor results from the balance of activities of protein kinases and protein phosphatases. In contrast to what is known about the roles of protein kinases on G protein coupled receptor phosphorylation, little is known about the role of protein phosphatases. In this regard, Pitcher *et al*. (1995) have shown that a latent oligomeric form of protein phosphatase 2A actively dephosphorylate the β_2-AR *in vitro* and Shih *et al*. (1999) reported that protein phosphatase 2A and 2B are associated with β_2-ARs. It has also been observed that okadaic acid, an inhibitor of protein phosphatases, induces both augmentation and inhibition of β_2-AR-mediated stimulation of cyclic AMP accumulation (Clark *et al*., 1993). Inhibition of the serine/threonine protein phosphatase, calcineurin, enhances desensitization and phosphorylation of adipocyte β_1-AR (Bahouth *et al*., 1996).

In the case of the α_{1b} -ARs there is no information available. Regulation of protein phosphatase activity is a potentially important point of regulation of receptor function. Low molecular weight protein phosphatase inhibitors that are able to penetrate living cells are important tools in the study of receptor function (Holmes & Boland, 1993). Okadaic acid, tautomycin and calyculin-A are selective inhibitors, *in vitro*, for the catalytic subunits of protein phosphatase 1 and protein

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phosphatase 2A (Holmes & Boland, 1993) whereas cypermethrin is specific, *in vitro*, for protein phosphatase 2B (Wang & Stelzer, 1994). In the present work we explore the role(s) of protein phosphatases on receptor phosphorylation and function by the use of inhibitors.

Methods

Cell line and culture

Rat-1 fibroblasts transfected with the hamster α_{1b}-AR (Cotecchia *et al*., 1988), generously provided to us by Drs R.J. Lefkowitz, M.G. Caron and L. Allen (Duke University), were cultured as described previously (Vázquez-Prado & García-Sáinz, 1996; Vázquez-Prado *et al*., 1997). In the present experiments, cells at confluence were serum-deprived in unsupplemented Dulbecco's modified Eagle's medium (DMEM) for 18–24 h.

Receptor phosphorylation

Experiments were performed as described in detail (Vázquez-Prado *et al*., 1997). In brief, cells were maintained in phosphate-free DMEM during 1 h and then incubated in 3 ml of the same medium containing [^{32}P]Pi (50 μCi ml^{-1}) for 3 h at 37°C. Labelled cells were stimulated as indicated, then they were washed and solubilized with 1 ml of ice-cold solubilization buffer, containing 1% Triton X-100 and 0.05% sodium dodecyl sulphate (Vázquez-Prado *et al*., 1997). The extracts were centrifuged and the supernatants transferred to tubes containing a rabbit antiserum generated against the carboxyl terminus decapeptide of the hamster α_{1b}-AR and sepharose-coupled protein A and immunoprecipitated (Vázquez-Prado *et al*., 1997). The immunoprecipitates were subjected to electrophoresis and phosphorylated receptor was determined by PhosphorImager analysis.

Intracellular calcium concentration ($[Ca^{2+}]_i$)

Determinations were as reported previously (Vázquez-Prado *et al*., 1997). Briefly, cells were incubated overnight in G418-free DMEM without serum, loaded with 5 μM Fura 2/AM at 37°C for 1 h, detached and washed. Cells were resuspended at a concentration of approximately 10^6 cells ml^{-1} in Krebs-Ringer-HEPES, pH 7.4, containing 0.05% bovine serum albumin. When okadaic acid or phorbol 12-myristate 13-acetate (PMA) were used, the cells were in contact with these agents for 15 min and remained present during the assay. Fluorescence measurements were performed in an AMINCO-Bowman spectrofluorometer, with excitation monochromators set at 340 and 380 nm, with a chopper interval of 0.5 s, and the emission monochromator set at 510 nm. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al*. (1985) using the software provided by AMINCO-Bowman; traces were directly exported to the graphs.

[^{3}H]inositol trisphosphate production

Determinations were as previously reported (Vázquez-Prado & García-Sáinz, 1996) with minor modifications. In brief, cells were labelled with [^{3}H]inositol (6 μCi ml^{-1}) for 18–24 h in inositol-free DMEM. On the day of the experiment, cells were washed twice with Krebs-Ringer-HEPES buffer containing 1.3 mM $CaCl_2$ and preincubated for 30 min in 2 ml of the same buffer containing 10 mM LiCl. When okadaic acid or PMA were used they were added during this preincubation and remained present during the assay. Incubations continued a further 15 min (in the absence or presence of noradrenaline) and were terminated by the addition of ice-cold perchloric acid. Supernatants were neutralized and [^{3}H]inositol trisphosphate was separated by Dowex AG1-X8 chromatography (Berridge *et al*., 1983).

Membrane preparation and [^{35}S]GTPγS binding

Confluent cells were incubated in the absence (control) or presence of noradrenaline (10 μM) for 5 min or with PMA (1 μM), okadaic acid (1 μM) or okadaic acid (1 μM) plus PMA (1 μM) for 15 min at 37°C. Washing with ice-cold phosphate buffered saline terminated the reaction and cells were scraped with 1 ml of ice-cold buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 5 mM EDTA, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, plus protease inhibitors) (Vázquez-Prado *et al*., 1997). Membranes were prepared according to the method of Mattingly *et al*. (1992). [^{35}S]GTPγS binding was performed as described by Wieland & Jakobs (1994) with minor modifications. Briefly, membranes were resuspended in binding buffer (in mM): Tris 50, $MgCl_2$ 10, EDTA 1, NaCl 100, DTT 1, pH 7.4, 1 μM GDP, 0.1% bovine serum albumin. Binding was performed at 25°C for 30 min in a volume of 250 μl of binding buffer containing 0.2 nM [^{35}S]GTPγS. Membranes were stimulated with 10 μM noradrenaline. The reaction was initiated by the addition of membranes (25 μg of protein/tube) and terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filters with ice-cold buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.5). The filters were dried and the radioactivity was quantified by liquid scintillation. Nonspecific binding was determined in the presence of unlabelled GTPγS (30 μM) and represented 10% of total binding.

Statistical analysis between comparable groups was performed using analysis of variance with Newman-Keuls post test.

Materials

(−)-Noradrenaline, PMA, dl-propranolol, staurosporine, bovine serum albumin, wortmannin and protease inhibitors were obtained from Sigma Chemical Co. Genistein were from Research Biochemicals Inc. DMEM, foetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Gibco BRL. Okadaic acid, cypermethrin, tautomycin, calyculin A and Ro 31-8220 were from Calbiochem. [2,3-^{3}H]myo-inositol (22.9 Ci mmol^{-1}), [^{35}S]GTPγS (1250 Ci mmol^{-1}) and [^{32}P]Pi (8500-9120 Ci mmol^{-1}) were from New England Nuclear. Sepharose-coupled protein A was from Upstate Biotechnology. Fura-2/AM was from Molecular Probes.

Results

The effect of okadaic acid on α_{1b}-AR phosphorylation was studied. As shown in Figure 1 (left panel), okadaic acid (1 μM) increased the phosphorylation of α_{1b}-ARs. A clear increase was evident as early as 5 min after the addition of the protein phosphatase inhibitor and the effect reached its maximum ($\sim$2.5 fold) at 30 min; this effect remained constant up to 60 min. The effect of okadaic acid was concentration-dependent with an EC_{50} of 30 nM (Figure 1, right panel). The effect of okadaic acid on α_{1b}-AR phosphorylation ($P<0.001$ vs

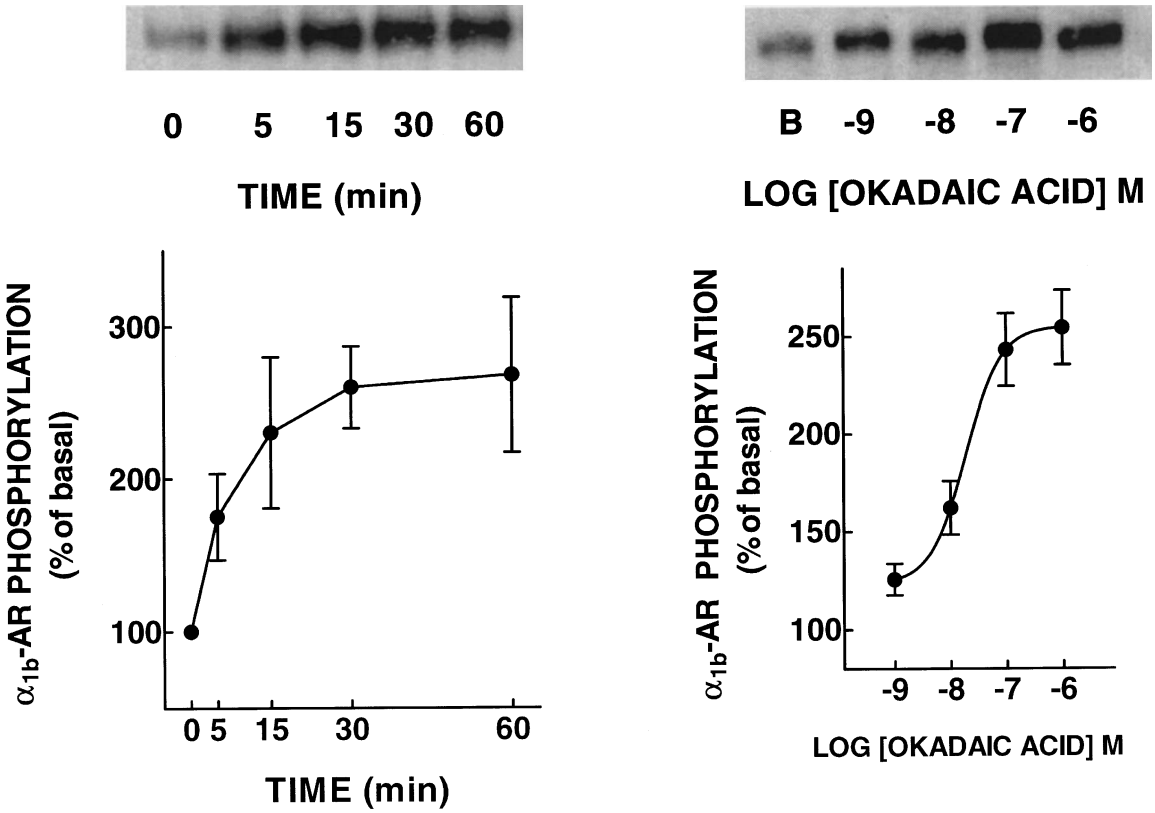

Figure 1 Effects of okadaic acid on α_{1b}-adrenoceptor phosphorylation. Left panel: cells were incubated with 1 μM okadaic acid for the times indicated. Right panel: cells were incubated with the indicated concentrations of okadaic acid for 30 min. Plotted are the means and vertical lines represent the s.e.mean of three determinations using different cell preparations. Representative autoradiographs are shown. B, basal phosphorypatium.

control) was comparable in magnitude to that induced by 1 μM PMA ($280 \pm 40\%$ of basal, $n=17$). Other protein phosphatase inhibitors were tested at a concentration of 1 μM and the effect is shown in Figure 2. Tautomycin ($P<0.001$ vs control), calyculin A ($P<0.001$ vs control) and to a lesser extent cypermethrin ($P<0.05$ vs control) increased α_{1b}-AR phosphorylation.

The data indicated that when protein phosphatases were inhibited the phosphorylated receptor accumulated. In order to gain insight into the protein kinase(s) involved in this effect, the effect of protein kinase inhibitors was tested. As shown in Figure 3, staurosporine (10 μM), an inhibitor of protein serine/threonine kinases with relative selectivity for PKC, decreased basal receptor labelling and blocked the effect of okadaic acid ($P<0.001$ vs okadaic acid alone). In contrast genistein (10 μM), a protein tyrosine kinase inhibitor, blocked neither the basal labelling nor the effect of okadaic acid on the state of phosphorylation of the receptor. The effect of wortmannin (10 μM), an inhibitor of phosphatidyl inositol 3-kinase, was also tested; this inhibitor altered neither the basal phosphorylation of the α_{1b}-AR nor the effect of okadaic acid. In order to define more clearly the role of PKC the effects of staurosporine and of the selective PKC inhibitor, Ro 31-8220, were studied in more detail. The inhibitors decreased the basal phosphorylation of the receptor at concentrations of 1 μM and higher and blocked, in a concentration-dependent fashion, the effect of okadaic acid (Figure 4). As shown, staurosporine was slightly, but consistently, more potent than Ro 31-8220.

It has been previously shown that PMA, noradrenaline and endothelin induce α_{1b}-AR phosphorylation (Diviani *et al.*, 1996; 1997; Lattion *et al.*, 1994; Leeb-Lundberg *et al.*, 1985; Vázquez-Prado *et al.*, 1997). The possibility that the action of okadaic acid could be additive to that of the previously mentioned agents was examined. However, we were unable to observe any clear additivity (data not shown).

We next examined the functional relevance of the effect of okadaic acid on the α_{1b}-AR phosphorylation state. Two parameters were examined: $[Ca^{2+}]_i$ and [^{3}H]inositol trisphosphate production. In these experiments okadaic acid and/or PMA were preincubated with the cells for 15 min ($[Ca^{2+}]_i$) or 30 min ([^{3}H]inositol trisphosphate production) before noradrenaline (in the presence of 10 μM propranolol to block any β-adrenergic action) was added. It can be observed in Figure 5 (upper panel) that noradrenaline induced a concentration dependent increase in $[Ca^{2+}]_i$. Okadaic acid, by itself, did not alter basal $[Ca^{2+}]_i$ and it did not alter in any significant way the effect of different concentrations of noradrenaline. In contrast, preincubation with PMA markedly decreased the effect of noradrenaline ($P<0.001$ vs control at noradrenaline concentrations of 100 mM and above). When the cells were preincubated with okadaic acid and PMA no effect of noradrenaline on $[Ca^{2+}]_i$ was observed at all (Figure 5, upper panel) ($P<0.05$ vs PMA alone at noradrenaline concentrations of 1 and 10 μM; $P<0.001$ vs control at noradrenaline concentrations of 100 nM and above).

Similarly, noradrenaline (in the presence of 10 μM propranolol) induced a marked increase in the production of [^{3}H]inositol trisphosphate from [^{3}H]inositol-labelled cells (Figure 5, lower panel) ($P<0.001$ vs control). Okadaic acid (Figure 5, lower panel) and PMA (not shown) by themselves did not alter the basal production of this second messenger. PMA ($P<0.01$ vs control), but not okadaic acid, markedly decreased the effect of noradrenaline (Figure 5, lower panel). When the cells were incubated with PMA, the addition of

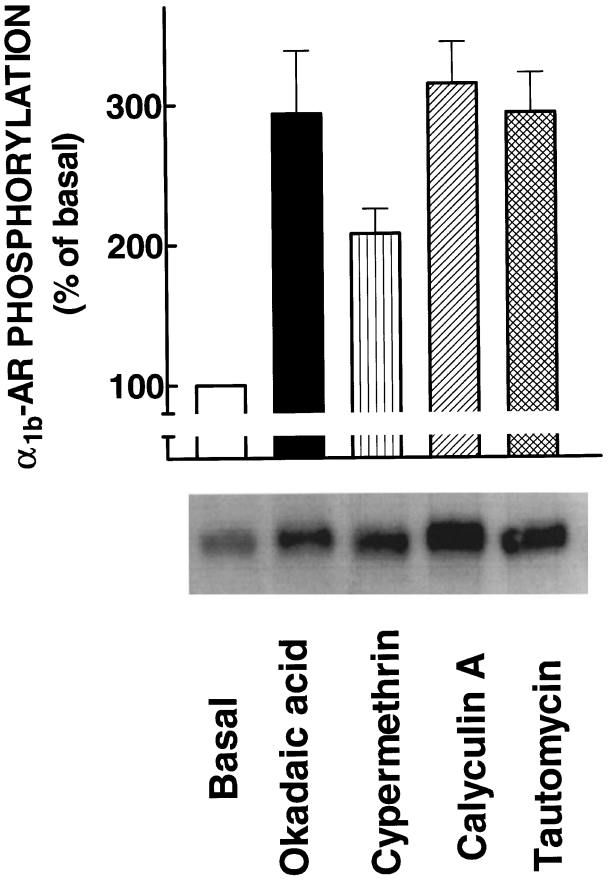

Figure 2 Effects of protein phosphatase inhibitors on α_{1b}-adrenoceptor phosphorylation. Cells were incubated in the absence of any inhibitor (Basal) or in the presence of 1 μM of the indicated inhibitors for 30 min. Plotted are the means and vertical lines represent the s.e.mean of four determinations using different cell preparations. A representative autoradiograph is shown.

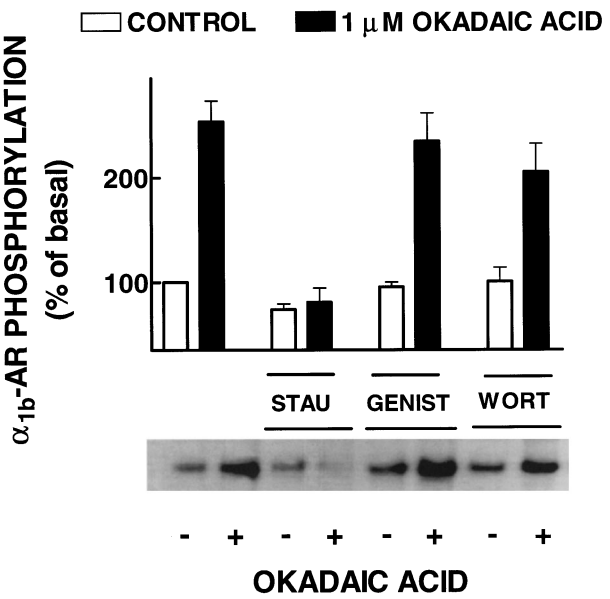

Figure 3 Effects of kinase inhibitors on the effect of okadaic acid on α_{1b}-adrenoceptor phosphorylation. Cells were incubated in the absence or presence of 1 μM okadaic acid and the inhibitors: 10 μM staurosporine (STAU), 10 μM genistein (GENIST) or 10 μM wortmannin (WORT). Plotted are the means and vertical lines represent the s.e.mean of 4–5 determinations using different cell preparations. A representative autoradiograph is shown.

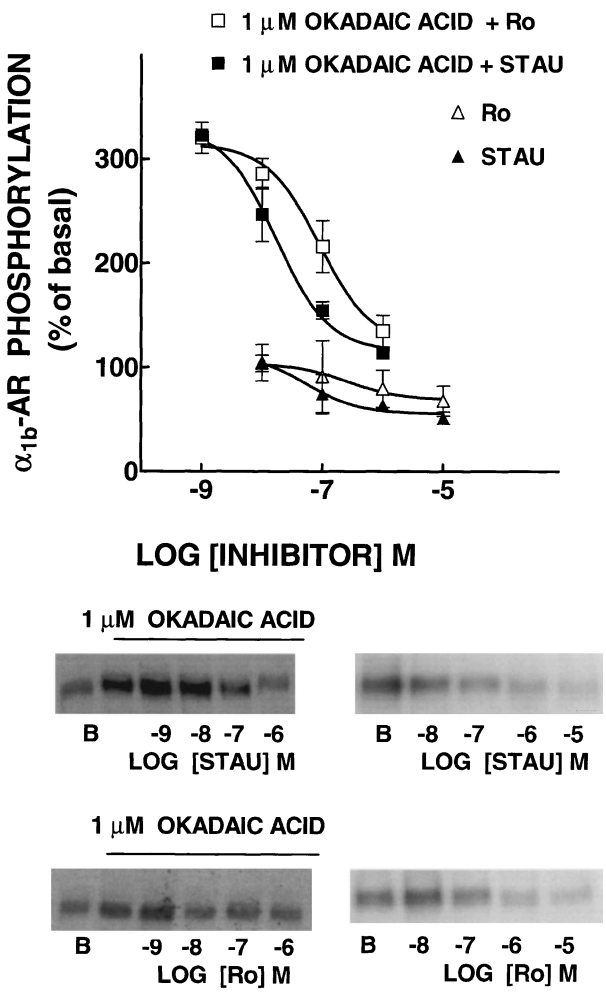

Figure 4 Effects of protein kinase C inhibitors on basal and okadaic acid-induced α_{1b}-adrenoceptor (α_{1b}-AR) phosphorylation. Cells were incubated in the absence or presence of 1 μM okadaic acid and different concentrations of staurosporine (STAU) or Ro-31-8220 (Ro). B, basal phosphorylation. Plotted are the means and vertical lines represent the s.e.mean of 3–4 determinations using different cell preparations. Representative autoradiographs are shown.

okadaic acid only slightly further decreased the α_{1b}-adrenergic effect, but did not block it; this further decrease was not statistically significant (Figure 5, lower panel).

In order to study more directly the effect of the different treatments on α_{1b}-AR-G protein coupling, noradrenaline-stimulated [^{35}S]GTPγS binding was studied. It can be observed in Figure 6 that noradrenaline increased the guanine nucleotide binding to membranes of control cells; such increase was of smaller magnitude in membranes obtained from cells incubated with 10 μM noradrenaline ($P<0.001$ vs control), or 1 μM PMA ($P<0.001$ vs control). Okadaic acid induced a small but statistically significant ($P<0.05$ vs control) decrease in agonist-stimulated binding. In membranes from cells incubated in the presence of PMA and okadaic acid, the decrease in agonist-stimulated guanine nucleotide binding was bigger than that observed with okadaic acid alone ($P<0.001$) but similar to that observed with PMA alone (Figure 6).

Discussion

Our present data clearly indicate that inhibition of serine/threonine protein phosphatases markedly increases the

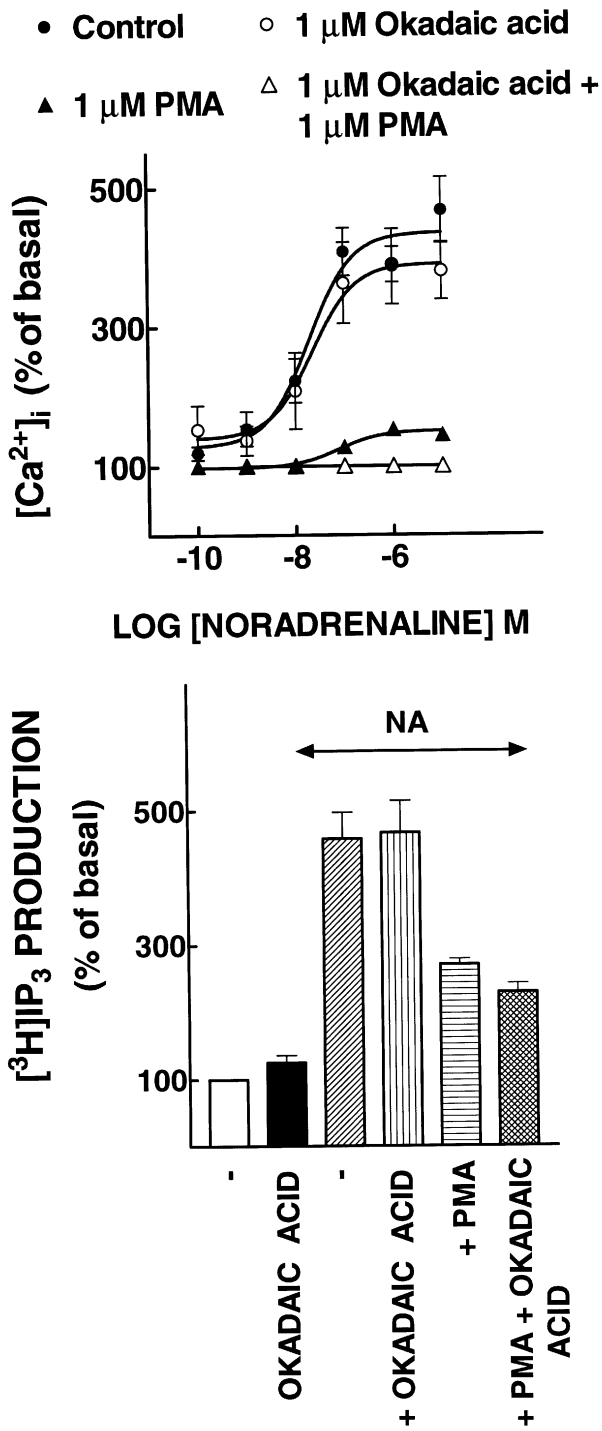

Figure 5 Functional consequences of the effects of okadaic acid and phorbol 12-myristate 13-acetate (PMA) on α_{1b}-adrenergic action. Upper panel, $[Ca^{2+}]_i$; concentration-response curves to noradrenaline of cells preincubated in the absence of any agent, 1 μM okadaic acid, 1 μM PMA or 1 μM PMA plus 1 μM okadaic acid. Lower panel, $[^3H]IP_3$. Cells were preincubated without any agent or with 1 μM of the agents indicated under the bars. After this preincubation the cells were further incubated for 15 min in the absence or presence of 10 μM noradrenaline plus 10 μM propranolol (NA). Plotted are the means and vertical lines represent the s.e.mean of 6–7 determinations using different cell preparations.

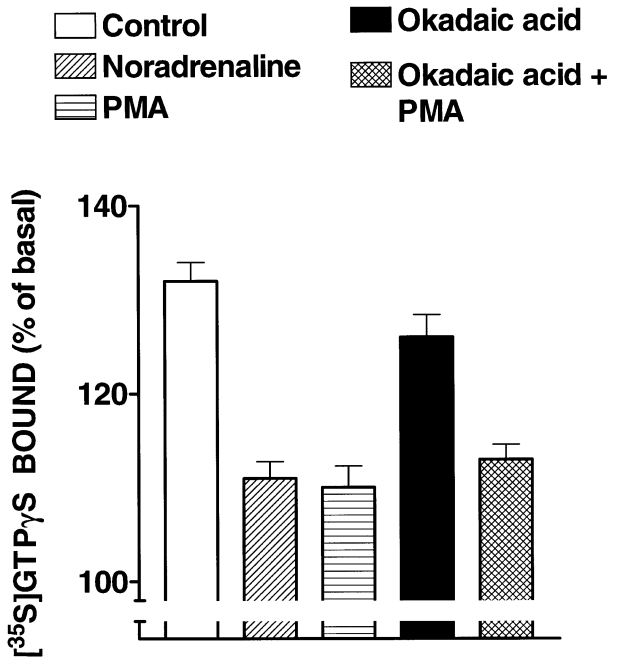

Figure 6 Effect of cell treatment with noradrenaline, okadaic acid and phorbol 12-myristate 13-acetate (PMA) on noradrenaline-stimulated $[^{35}S]GTP\gamma S$ binding. Cells were incubated in the absence of any agent (Control) or presence of 10 μM noradrenaline, 1 μM PMA, 1 μM okadaic acid or 1 μM okadaic acid plus 1 μM PMA. Membranes were stimulated with 10 μM noradrenaline. Plotted are the means and vertical lines represent the s.e.mean of 18–25 determinations using six different membrane preparations, for each condition.

phosphorylation state of the α_{1b}-AR. To the best of our knowledge this is the first demonstration of such an effect for an α_1-AR.

As indicated in the introduction, considerable insight on the roles that different protein kinases play in the modulation/phosphorylation of G protein coupled receptors has been gained, but there is very little information on the role(s) of protein phosphatases. Nevertheless, it has been observed that peroxovanadate enhances desensitization and phosphorylation of adipocyte β_1-AR by inhibiting the activity of the serine/threonine protein phosphatase, calcineurin (Bahouth *et al.*, 1996). A latent oligomeric form of protein phosphatase 2A has been shown to actively dephosphorylate the β_2-AR *in vitro* (Pitcher *et al.*, 1995).

Current evidence suggest that scaffold, anchoring and adapter proteins contribute to the specificity of signal transduction by recruiting active enzymes into signalling complexes (Pawson & Scott, 1997). This has been extensively observed in tyrosine kinase receptor signalling (Pawson & Scott, 1997) and was very elegantly extended for G protein coupled receptors by Shih *et al.* (1999). Very recently, these authors reported that protein phosphatase 2A and 2B are associated with β_2-ARs in the basal state and that the latter protein phosphatase and PKA display a robust association with these receptors following challenge with the agonists. The anchoring protein gravin participates in the formation of these dynamic complexes with protein kinases, protein phosphatases and the β_2-AR (Shih *et al.*, 1999).

A complex interplay seems to exist between PKC and protein phosphatases to modulate α_{1b}-AR phosphorylation and function. Our studies with protein phosphatase inhibitors suggest that protein phosphatases-1, 2A and 2B could be involved in modulating receptor phosphorylation. However, although these inhibitors show selectivity *in vitro*, their selectivity *in vivo*, is questionable. Therefore, we cannot define the type(s) of protein phosphatase(s) involved.

It is possible that basal PKC activity was sufficient to phosphorylate α_{1b}-ARs. This is supported by the observation

that PKC inhibitors decreased basal phosphorylation at concentrations above 1 μM. The marked increase in α_{1b}-AR phosphorylation in the presence of okadaic acid suggests that protein phosphatase activity exert a tonic action dephosphorylating these adrenoceptors. Alternatively, it is possible that inhibition of protein phosphatases may activate PKC; in fact, there is evidence that protein phosphatases reversibly inhibit PKC activity *in vitro* (Ricciareli & Azzi, 1998). The present data do not allow us to distinguish between these not mutually exclusive possibilities.

A particularly interesting finding was that despite the fact that okadaic acid increased α_{1b}-AR phosphorylation, neither the $[Ca^{2+}]_i$ increase nor the production of [^{3}H]inositol trisphosphate induced by noradrenaline were significantly altered. This is in marked contrast with the results obtained with PMA, which greatly reduced both receptor responses. We have previously observed that bradykinin induced α_{1b}-AR phosphorylation without leading to adrenoceptor desensitization (Medina *et al*., 1998); however, bradykinin induced only a 50% increase in receptor phosphorylation. Okadaic acid induced an increase in α_{1b}-AR phosphorylation of similar magnitude as PMA but the functional repercussions markedly differ.

The results on noradrenaline-stimulated [^{35}S]GTPγS binding indicate that the phosphorylation induced by the treatment with okadaic acid does indeed impair the adrenoceptor-G protein coupling, but to a much lesser extent than PMA or noradrenaline. Such okadaic acid-induced decrease of receptor-G protein coupling does not however seem to affect the adrenergic actions in whole cells, i.e. the intracellular calcium and inositol trisphosphate responses were not decreased.

The differences in the effect of PMA and okadaic acid on the receptor response may also reflect the fact that in addition to receptor phosphorylation other event may underlie desensitization. Among these, PKC-dependent phosphorylation of other molecular entities participating in signalling, such as G protein(s) or phospholipase C might result in desensitization.

The actions of both PMA and okadaic acid on α_{1b}-AR phosphorylation seem to involve PKC activity, but they had very different functional repercussions. This suggests that differences may exist in the sites phosphorylated under the action of PMA or okadaic acid. One possibility that may explain this puzzle is that PMA may activate PKC isoforms that are not active in the basal state or in the presence of okadaic acid. PKC is a multigene family of protein kinase with different sensitivity to activators and substrate selectivity (Newton, 1995). There is no data on the isoforms of PKC that participate in α_{1b}-AR phosphorylation.

As indicated, the sites involved in PKC-mediated α_{1b}-AR phosphorylation have been identified in the carboxyl terminus (Ser^{394} and Ser^{400}) although a third, yet unidentified, site seems to exist (Diviani *et al*., 1997). The sites where basal phosphorylation takes place and the kinase(s) involved have not yet been positively identified. The functional significance of such basal phosphorylation is unknown.

In summary, our data indicate that inhibition of protein phosphatases increase α_{1b}-AR phosphorylation. This effect seems to involve PKC activity. In contrast to the effect of PMA, okadaic acid does not block α_{1b}-adrenergic actions in whole cells and only marginally affect receptor coupling to G proteins as evidenced by the noradrenaline-stimulated [^{35}S]GTPγS binding.

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