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Peroxovanadate induces α_{1B}-adrenoceptor phosphorylation and association with protein kinase C

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Abstract

Peroxovanadate induced a marked increase in the phosphorylation state of α_{1B}-adrenoceptors. The effect was dose-dependent ($EC_{50} \approx 2$ μM) and rapid, reaching its maximum in 5 min and remaining at this level for 30 min. Hydrogen peroxide also increased α_{1B}-adrenoceptor phosphorylation but to a lesser extent, in an ephemeral fashion, and only at high (millimolar) concentrations. The effect of peroxovanadate was blocked by inhibitors of protein kinase C such as staurosporine and rottlerin and only partially reduced by genistein and inhibitors of phosphoinositide 3-kinase. Protein kinase C α, δ and ε are associated with the α_{1B}-adrenoceptor under basal conditions, as reflected by coimmunoprecipitation. Such association was increased by peroxovanadate for all isoforms. In contrast, hydrogen peroxide increased only the association of the ε isoform to the adrenoceptor. Peroxovanadate decreased the ability of noradrenaline to increase intracellular calcium, indicating that the receptor phosphorylation induced has functional consequences.

Keywords: α_{1B}-Adrenoceptor; Peroxovanadate; Phosphorylation receptor; Protein kinase C

1. Introduction

α_1-Adrenoceptors constitute a subfamily of the seven transmembrane domains-G protein-coupled receptor superfamily. It consists of three members: the α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, which mediate many of the physiologic actions of adrenaline and noradrenaline and participate in the pathogenesis of diseases such as hypertension and benign prostatic hypertrophy (García-Sáinz et al., 1999, 2000; Graham et al., 1996; Piascik and Perez, 2001). Phosphorylation via G protein-coupled receptor kinases and second messenger-dependent kinases such as protein kinase C (PKC) is one of the earliest processes that regulate receptor function (Casas-González et al., 2000, 2003; Diviani et al., 1997; García-Sáinz et al., 2000; Leeb-Lundberg et al., 1985; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997).

The hamster α_{1B}-adrenoceptor was the first receptor of this subfamily that was cloned (Cotecchia et al., 1988), it has been more extensively studied than the other subtypes and it is generally considered prototypic of the whole subfamily. G protein-coupled receptor kinases 2 and 3 appear to be involved in phosphorylation of agonist-occupied α_{1B}-adrenoceptors during homologous desensitization (Diviani et al., 1996; Iacovelli et al., 1999); the phosphorylation sites involved have been located at Ser^{404}, Ser^{408}, and Ser^{410} in the receptor carboxyl terminus (Diviani et al., 1997).

In heterologous desensitization PKC and phosphoinositide 3-kinase (PI3K) are key participants (reviewed in García-Sáinz et al., 2000). Direct activation of PKC with active phorbol esters markedly desensitizes α_{1B}-adrenoceptors and leads to a pronounced increase in receptor phosphorylation (Casas-González et al., 2000, 2003; Diviani et al., 1997; Leeb-Lundberg et al., 1985; Vázquez-Prado et al., 1997). Sites phosphorylated by PKC have been located at Ser^{394} and Ser^{400} of the α_{1B}-adrenoceptor carboxyl terminus (Diviani et al., 1997). It has been observed that activation of non-adrenergic receptors induce α_{1B}-adrenoceptor desensitization and phosphorylation. Thus, we have shown that activation of seven transmembrane domains receptors coupled to $G_{q/11}$ (such as endothelin ET_A (Vázquez-Prado et al., 1997)

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or bradykinin B2 receptors (Medina et al., 1998)), or G_i (such as lysophosphatidic acid receptors (Casas-González et al., 2000, 2003)), or receptors with intrinsic tyrosine kinase activity, such as epidermal growth factor (EGF), or platelet-derived growth factor receptors (Medina et al., 2000) can induce α_{1B}-adrenoceptor phosphorylation/desensitization.

We tested the hypothesis that oxidants such as peroxovanadate and hydrogen peroxide might affect the function and phosphorylation state of α_{1B}-adrenoceptors. Such hypothesis was based on a large series of experimental findings. On the one hand, PKC is an enzyme that can be redox-modified by oxidants; isoforms of this kinase contain zinc-binding, cystein-rich motifs with autoinhibitory function that can be oxidized readily by agents such as hydrogen peroxide (Gopalakrishna and Jaken, 2000; Konishi et al., 1997, 2001; Shibukawa et al., 2003). When oxidized, the regulatory function of this domain is compromised and, consequently, PKC is activated (Gopalakrishna and Jaken, 2000; Konishi et al., 1997, 2001; Shibukawa et al., 2003). On the other hand, protein phosphatase 2B, known also as calcineurin, is a Ca^{2+} and calmodulin-dependent serine/threonine phosphatase containing a dinuclear Fe^{3+}–Zn^{2+} center in the active site; this enzyme can be oxidized and inactivated with low concentrations of phenylarsine oxide and hydrogen peroxide (Bogumil et al., 2000). Peroxovanadate is a potent inhibitor of phosphotyrosine phosphatases, which induces the accumulation of numerous tyrosine-phosphorylated signalling proteins (Ruff et al., 1997). It has been demonstrated that peroxovanadate enhances desensitization and phosphorylation of adipocyte β_1-adrenoceptor by inhibiting the activity of protein phosphatase 2B (Bahouth et al., 1996). Furthermore, we have also shown previously that inhibition of serine/threonine phosphatases by okadaic acid and related agents markedly induces α_{1B}-adrenoceptor phosphorylation (Alcántara-Hernández et al., 2000). In addition, there is a large body of evidence suggesting that hydrogen peroxide could be a key physiological messenger that modulates protein phosphorylation through cysteine oxidation; among the many hormones and growth factors that elicit a hydrogen peroxide response are: platelet-derived growth factor, EGF, lysophosphatidic acid, bradykinin and endothelin (reviewed in Rhee et al., 2000). As mentioned, these agents are capable of inducing heterologous desensitization of α_{1B}-adrenoceptors associated to phosphorylation of these adrenoceptors (Casas-González et al., 2000, 2003; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997).

2. Materials and methods

2.1. Materials

(−)-Noradrenaline, staurosporine, bovine serum albumin, phospho-aminoacids, wortmannin, LY294002, protease inhibitors, lysophosphatidic acid and sodium vanadate were obtained from Sigma. Genistein was from Research Biochemicals International. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics, and others reagents used for cell culture were from Gibco BRL. Rottlerin, and Gö6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo-(2,3-a)-pyrrolo (3, 4-c)-carbazole), were from Calbiochem. [^{32}P]phosphate (8500–9120 Ci/mmol) was from Perkin Elmer Life Sciences Products. Sepharose-coupled protein A was from Upstate Biotechnology. FURA-2/acetoxymethyl ester was from Molecular Probes. Protein kinase C isoforms-selective antibodies were from Santa Cruz Biotechnology and secondary antibodies were from Zymed. Nitrocellulose membranes were from Bio-Rad and the chemiluminescence kits were obtained from Pierce. Stock solution and dilutions of hydrogen peroxide were prepared in phosphate buffered saline solution. To prepare peroxovanadate, a solution 5 mM of sodium vanadate in phosphate buffered saline solution was prepared previously by heating. Hydrogen peroxide was added to the orthovanadate solution 15 min prior to use, at room temperature.

2.2. Cell line, culture and receptor phosphorylation studies

Rat-1 fibroblasts transfected with the hamster α_{1B}-adrenoceptor (Cotecchia et al., 1988) were generously provided by Drs. R.J. Lefkowitz, M.G. Caron and L. Allen (Duke University) and were cultured as described previously (Casas-González et al., 2000, 2003; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997). Cells at confluence were serum-deprived in unsupplemented DMEM for 18–24 h, maintained in phosphate-free DMEM during 1 h and then incubated in 3 ml of the same medium containing 50 μCi/ml of [^{32}P]phosphate for 3 h at 37 °C. Labeled cells were stimulated with the agents and for the times 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 sulfate. 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}-adrenoceptor and protein A-Sepharose (Vázquez-Prado et al., 1997). The immunoprecipitates were subjected to electrophoresis and phosphorylated receptor was determined by PhosphorImager analysis, as described in detail (Casas-González et al., 2000, 2003; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997).

2.3. Intracellular calcium determinations

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were quantified as reported previously (Casas-González et al., 2000; Medina et al., 1998; Vázquez-Prado et al., 1997). In brief, cells were incubated overnight in G418-free DMEM without serum, loaded with 5 μM FURA2/acetoxymethyl ester a 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 peroxovanadate was used, the cells

were in contact with this agent for 15 min, washed and resuspended in Krebs–Ringer–HEPES buffer. 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 intracellular calcium concentration was calculated as described (Grynkiewicz et al., 1985) using the software provided by AMINCO-Bowman; traces were directly exported to the graphs.

2.4. α_{1B}-adrenoceptor-PKC coimmunoprecipitation assays

Coimmunoprecipitation studies were performed as described in detail (Alcántara-Hernández et al., 2001). In brief, cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h on ice in buffer containing 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 50 mM NaF, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phospho-serine, 1 mM phospho-threonine and protease inhibitors (20 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 500 μg/ml bacitracin, 50 μg/ml soybean trypsin inhibitor). Cells lysates were centrifuged at 12,700 × *g* for 15 min and the supernatants were incubated overnight at 4 °C with anti-α_{1B}-adrenoceptor antiserum and protein A-Sepharose. After four washes with 50 mM HEPES, 50 mM NaH_2PO_4, 100 mM NaCl, 1% Nonidet-P40 (pH 7.4), the immune complexes were denatured by boiling in Laemmli sample buffer containing 5% β-mercaptoethanol. Proteins were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. Incubation with the PKC isoform-selective antibodies was for 12 h at 4 °C and the secondary antibody for 2 h at room temperature. Super signal enhanced chemiluminescence kits from Pierce were used. PKC coimmunoprecipitation was assessed by densitometric analysis. PKC translocation (membrane association) was assayed as described (García-Sáinz and Alcántara-Hernández, 1998).

Statistical analysis between comparable groups was performed using ANOVA with Newman–Keuls analysis and was effected with software included in the GraphPad Prism program.

3. Results

As shown in Fig. 1, peroxovanadate increased α_{1B}-adrenoceptor phosphorylation in a dose-dependent fashion with an EC_{50} value of 2.0 ± 0.3 μM. The effect of 100 μM peroxovanadate was rapid reaching its maximum (approximately 2-fold) within 5 min, remained at this level up to 30 min and decreased afterwards (Fig. 1, upper right panel). Vanadate at similar concentrations hardly had any effect (data not shown). Hydrogen peroxide increased α_{1B}-adrenoceptor phosphorylation but several differences with the effect of peroxovanadate were evident, i.e., much higher concentrations were required to observe an effect (EC_{50} ≈ 1 mM), the magnitude of the α_{1B}-adrenoceptor phosphorylation induced was consistently smaller than that observed with peroxovanadate and the increase in receptor phosphorylation was ephemeral, i.e. rapid but decreasing in the same form (Fig. 1, lower panels).

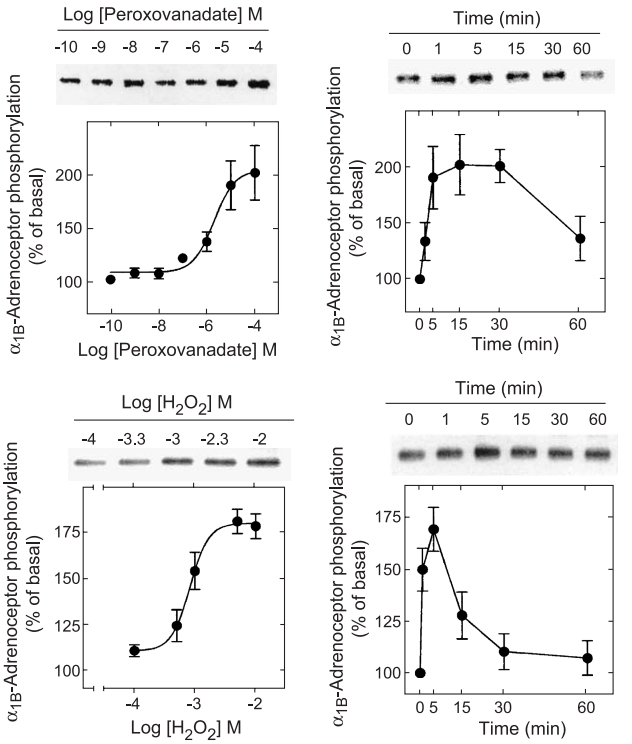

Fig. 1. Effects of peroxovanadate and H_2O_2 on α_{1B}-adrenoceptor phosphorylation. Left panels: cells were incubated with the indicated concentrations of peroxovanadate (upper panel) for 15 min or H_2O_2 (lower panel) for 5 min. Right panels: cells were incubated for the times indicated in the absence or presence of 100 μM peroxovanadate (upper panel) or 1 mM H_2O_2 (lower panel). In all cases data are presented as percentage of receptor phosphorylation observed in the absence of any agent and plotted are the means ± S. E. M. of three to five experiments using different cell preparations. Representative autoradiographs are shown.

In order to get some insights on the biochemical processes involved in the action of peroxovanadate, the effect of some protein kinase inhibitors was tested. It can be observed in Fig. 2 that staurosporine (1 μM), a protein kinase C inhibitor, markedly inhibited the effect of peroxovanadate on the adrenoceptor phosphorylation. In contrast, genistein (10 μM), a general tyrosine kinase inhibitor, and the phosphoinositide 3-kinase inhibitors, wortmannin (100 nM) and LY294002 (1 μM), only partially inhibited the effect of peroxovanadate (30–50% inhibition). The mentioned inhibitors were without effect by themselves on basal receptor phosphorylation (data not shown).

The ability of several PKC inhibitors to block the α_{1B}-adrenoceptor phosphorylation induced by peroxovanadate was tested. Staurosporine, a general PKC inhibitor, and

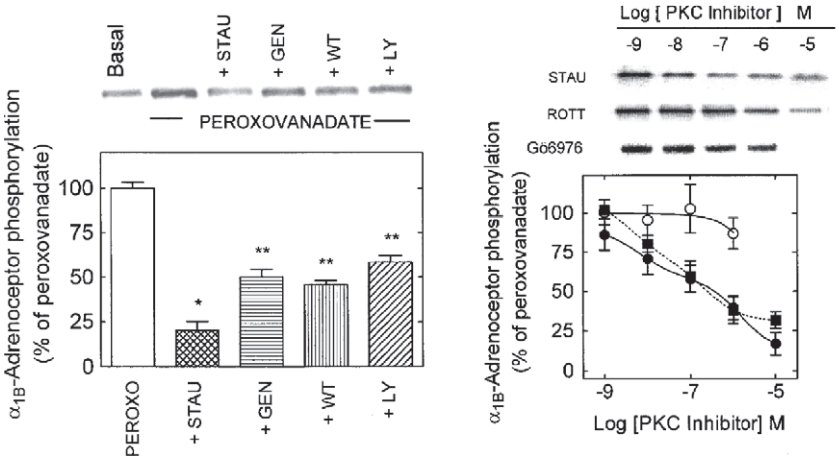

Fig. 2. Effect of kinase inhibitors on the α_{1B}-adrenoceptor phosphorylation induced by peroxovanadate. Left panel: cells were incubated in the absence (Basal) or presence of 100 μM peroxovanadate alone or with 1 μM staurosporine (+STAU), 10 μM genistein (+GEN), 100 nM wortmannin (+WT) or 1 μM LY94002 (+LY) for 30 min. Right panel: cells were incubated for 30 min in the absence or presence of 100 μM peroxovanadate and the indicated concentrations of staurosporine (STAU, solid circles), rottlerin (ROTT, solid squares, dotted line) or Gö6976 (open circles). Data are presented as percentage of the effect of peroxovanadate alone and plotted are the means ± S.E.M. of three to five experiments using different cell preparations. Representative autoradiographs are shown. *$p < 0.001$ vs. peroxovanadate alone, **$p < 0.001$ vs. peroxovanadate alone and vs. peroxovanadate with staurosporine.

rottlerin, a putative PKC δ-selective inhibitor (Way et al., 2000), blocked in a dose-dependent and nearly total fashion the effect of peroxovanadate; the IC_{50} values were very similar in the range of 100–300 nM (Fig. 2, right panel). In contrast, Gö6976, an inhibitor with selectivity for the classic PKC isoforms (α, β and γ) (Way et al., 2000) was essentially without effect up to 1 μM (Fig. 2, right panel). The inhibitors by themselves were without effect on basal α_{1B}-adrenoceptor phosphorylation at the concentrations used (data not shown).

We have previously shown that some PKC isoforms coimmunoprecipitate with α_{1B}-adrenoceptors and that such association is not static but dynamic, further increasing by agents that induce α_{1B}-adrenoceptor phosphorylation, such as tetradecanoyl phorbol acetate, noradrenaline, endothelin, lysophosphatidic acid or EGF (Alcántara-Hernández et al., 2001). We observed that incubation with peroxovanadate also increased the association of PKC α, δ and ε to α_{1B}-adrenoceptors (Fig. 3). The magnitude of the effect varied (PKC α, 3-fold; PKC δ, 4 to 5-fold and PKC ε, 1.5 to 2-fold). In Fig. 3, we showed that the amount of immunoprecipitated receptor, evidenced by Western blot, and the amount of IgG were not different, which served as internal controls of the experiments. In Fig. 3, it is also shown that peroxovanadate increased PKC membrane association (translocation). Hydrogen peroxide (1 mM for 5 min) also increased translocation of PKC α, δ, and ε isoforms but only increased association of PKC ε to α_{1B}-adrenoceptors in a consistent fashion (Fig. 4).

We next examined the functional repercussion of the α_{1B}-adrenoceptor phosphorylation induced by peroxovanadate. Peroxovanadate at a concentration of 100 μM decreased the effect of all agents tested on intracellular calcium concentrations. Nevertheless, at 10 μM a more selective effect was observed. As shown in Fig. 5, cells that were incubated with 10 μM peroxovanadate exhibited a markedly decreased response to noradrenaline; i.e., in control cells the maximal response was a ≈ 6-fold increase in intracellular calcium whereas in those treated with peroxovanadate the maximum was hardly 3-fold. Lysophosphati-

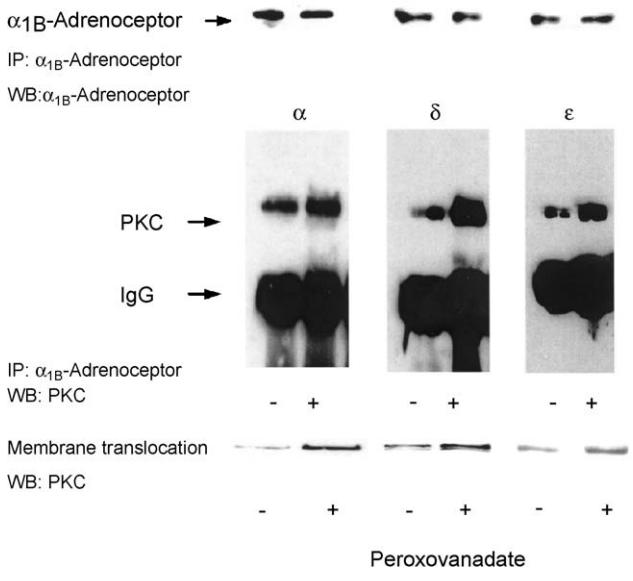

Fig. 3. Effects of peroxovanadate on the coimmunoprecipitation of α_{1B}-adrenoceptor and PKC isoforms. Cells were incubated for 15 min in the absence or presence of 100 μM peroxovanadate. Immunoprecipitated receptors or membranes were subjected to Western blotting using the anti-α_{1B}-adrenoceptor antiserum (upper panel) or PKC isoform-selective antibodies (middle and lower panels). Blots representative of six experiments using different cell preparations are shown. IP, immunoprecipitation; WB, Western blot.

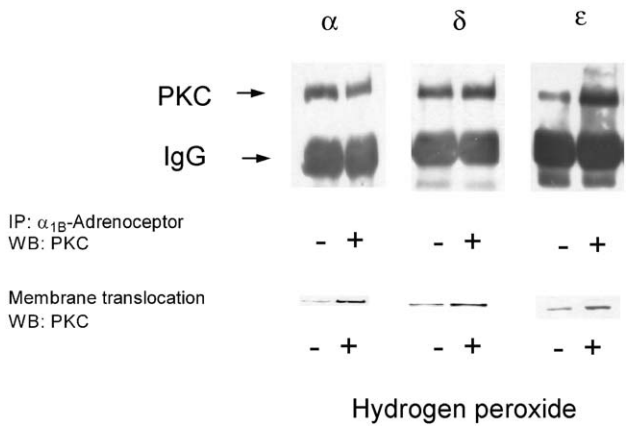

Fig. 4. Effects of hydrogen peroxide on the coimmunoprecipitation of α_{1B}-adrenoceptor and PKC isoforms. Cells were incubated for 5 min in the absence or presence of 1 mM hydrogen peroxide. Immunoprecipitated receptors or membranes were subjected to Western blotting using PKC isoform-selective antibodies (upper and lower panels). Blots representative of three experiments using different cell preparations are shown. IP, immunoprecipitation; WB, Western blot.

dic acid also increased intracellular calcium in these cells and its effect was also decreased by peroxovanadate but to a much lesser extent (Fig. 5, right panels).

The effect of hydrogen peroxide on intracellular calcium was also tested. This peroxide at millimolar concentrations abolished the effect of all the agents tested on this parameter. The increase in α_{1B}-adrenoceptor phosphorylation induced by hydrogen peroxide was blocked by staurosporine (data not shown). No further studies were performed with hydrogen peroxide due to the broad toxicity evidenced in the determinations of intracellular calcium.

4. Discussion

Our present data clearly show that peroxovanadate increases the phosphorylation state of α_{1B}-adrenoceptors. Such action seems to involve PKC as evidenced by the ability of inhibitors to block it. As mentioned, it is well known that activation of PKC with phorbol esters markedly desensitizes α_{1B}-adrenoceptors and leads to a pronounced increase in receptor phosphorylation (Casas-González et al., 2000, 2003; Diviani et al., 1997; García-Sáinz et al., 2000; Leeb-Lundberg et al., 1985; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997); this enzyme plays a key role in the desensitization/phosphorylation of α_{1B}-adrenoceptors induced by activation of non-adrenergic receptors (heterologous desensitization) (Casas-González et al., 2000, 2003; Diviani et al., 1997; García-Sáinz et al., 2000; Leeb-Lundberg et al., 1985; Medina et al., 1998, 2000; Vázquez-Prado et al., 1997).

Protein kinase C belongs to the serine/threonine protein kinase superfamily. Eleven PKC isoforms have been identified and classified into three groups based on their ability to be activated by Ca^{2+} and diacylglycerol. The classical PKC-α, -βI, -βII and -γ isoforms are activated by Ca^{2+}, phosphatidylserine and diacylglycerol or phorbol esters; the novel PKC-θ, -η, -δ, -ε which are Ca^{2+}-independent but diacylglycerol- and phosphatidylserine-dependent; and finally, the atypical isoforms, PKC-ζ PKCι (λ in murine

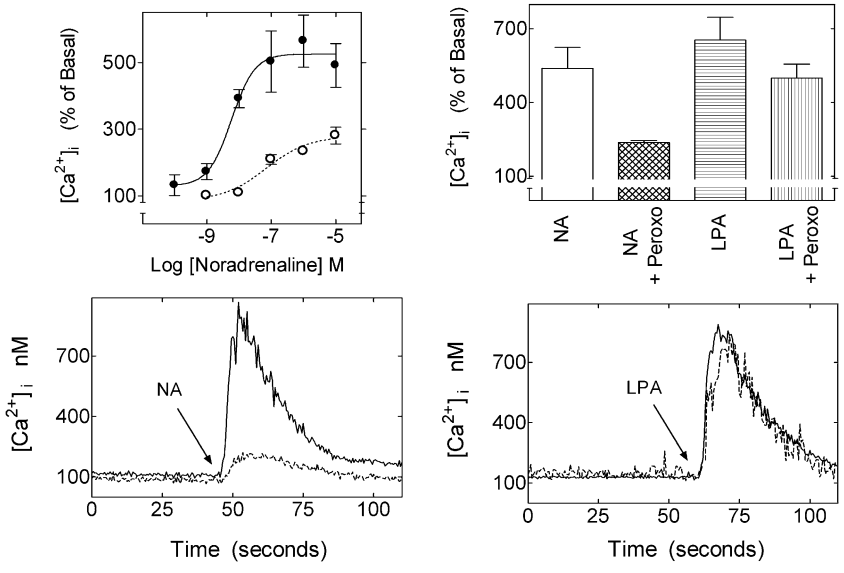

Fig 5. Effect of peroxovanadate on the effects of noradrenaline or lysophosphatidic acid on intracellular calcium concentration. Cells were preincubated in the absence of any agent (solid circles, solid line) or 10 μM peroxovanadate (open circles, dotted line) (+Peroxo) for 15 min and challenged with different concentrations of noradrenaline (upper left panel) or with 10 μM of noradrenaline (NA) or 1 μM lysophosphatidic acid (LPA) (upper right panel). Plotted are the means and vertical lines represent the S.E.M. of six determinations using different cell preparations (in some cases errors are within the symbols). Lower panels: representative tracings obtained from cells incubated in the absence (solid lines) or presence of 10 μM peroxovanadate (dotted lines) and challenged with 10 μM noradrenaline (NA) or 1 μM lysophosphatidic acid (LPA).

cells), which are Ca^{2+}- and diacylglycerol-independent (Newton, 1995). Rat-1 fibroblasts express PKC α, δ, ε and ζ isoforms (Alcántara-Hernández et al., 2001; Berti et al., 1994) and it has been observed that the first three isoforms associate with α_{1B}-adrenoceptors but that the ζ isoform, although it is easily detected in cell extracts does not associate with α_{1B}-adrenoceptors (Alcántara-Hernández et al., 2001). In the present study we confirmed that the α, δ and ε PKC isoforms associate with these adrenoceptors under basal conditions and extended the observation that such association is also markedly stimulated by peroxovanadate. These data and the ability of rottlerin to inhibit peroxovanadate-induced α_{1B}-adrenoceptor phosphorylation suggest a possible key role of PKC δ. Nevertheless, it should be considered that the selectivity of inhibitors is questionable (Davies et al., 2000). At this point, it is yet far from clear if the actions of one or more isoforms of PKC are physiologically relevant for the regulation of α_{1B}-adrenoceptor signalling; similarly it is not yet know to what extent the functions of the different isoforms are complementary or redundant. Therefore, our present data only suggest participation of PKC δ in the phosphorylation/desensitization processes; obviously different approaches will have to be used to define directly its precise role. Nevertheless, we would like to mention that PKC δ participates in the activation of tyrosine kinases by reactive oxygen species and that such effect is independent of tyrosine phosphatase inhibition (Frank et al., 2003). We cannot discard a role of PKC ε in the described effects since this isoenzyme also associates with the receptor in a dynamic fashion. As mentioned, hydrogen peroxide increased receptor association only of PKC ε and induced no more than a transient effect on receptor phosphorylation; the data suggest the possibility that different PKC isoforms might play different roles.

Data reported by us and other authors indicate that other mechanisms may likely participate in mediating the described effect of peroxovanadate. As indicated previously, peroxovanadate enhances desensitization and phosphorylation of adipocyte β_1-adrenoceptor by inhibiting the activity of protein phosphatase 2B (Bahouth et al., 1996). We have shown that inhibitors of serine/threonine protein phosphatases, such as okadaic acid markedly increase α_{1B}-adrenoceptor phosphorylation (Alcántara-Hernández et al., 2000) and recent evidence indicates that okadaic acid inhibits α_{1A}-adrenoceptor-mediated actions in cultured glia (Assari et al., 2003). Protein phosphatase 2B has a dinuclear Fe^{3+}–Zn^{2+} center whose oxidation results in inactivation (Bogumil et al., 2000). It is, therefore, likely that the actions of peroxovanadate described here could involve this mechanism. Protein kinases and phosphatases play very dynamic roles in modulating receptor functioning. In our studies using okadaic acid, we observed that the increase in the α_{1B}-adrenoceptor phosphorylation was blocked by inhibitors of PKC, which suggested that basal PKC activity may results in accumulation of the phosphorylated receptor when protein phosphatase activity is blocked (Alcántara-Hernández et al., 2000). Similarly, inhibitors of PKC blocked the effects of okadaic acid on α_{1A}-adrenoceptor-mediated actions in cultured glia (Assari et al., 2003), suggesting that the same process is probably involved. In other words, tonic protein phosphatase activity is likely to be important in maintaining a low phosphorylation state of these adrenoceptors.

It is likely that the effect of peroxovanadate on α_{1B}-adrenoceptor phosphorylation might also involve others sites of actions. Hydrogen peroxide and vanadate are powerful broad inhibitors of phospho-tyrosine phosphatase activities and their action in vivo results in the accumulation of large number of tyrosine-phosphorylated proteins (Ruff et al., 1997); hydrogen peroxide, on the other hand, can potentially oxidize cystein-rich domains in a very large number of proteins. The ability of genistein and PI3K inhibitors to block partially the effect of peroxovanadate on α_{1B}-adrenoceptor phosphorylation is also consistent with the idea that several processes might be involved. We have recently reported that lysophosphatidic acid induces α_{1B}-adrenoceptor phosphorylation through two mechanisms, one involving activation of PI3K through G protein βγ subunits and another also involving activation of the phospholipid kinase but through EGF receptor transactivation via the release of HB-EGF (Heparin-binding EGF-like growth factor) (Casas-González et al., 2003). It was recently shown that hydrogen peroxide induces shedding of HB-EGF and activation of EGF receptors (Frank et al., 2003). It is possible, therefore, that the ability of PI3K inhibitors to reduce the effect of peroxovanadate on α_{1B}-adrenoceptor phosphorylation may involve such process.

Finally, we would like to mention that reactive oxygen species, including hydrogen peroxide are known to act as second messengers (Frank et al., 2003; Rhee et al., 2000). Interestingly, reactive oxygen species mediate α_1-adrenoceptor-stimulated hypertrophy in adult rat ventricular myocytes (Amin et al., 2001). Our data suggest the possibility that in a physiological context such reactive oxygen species may play a role in modulating the actions of α_{1B}-adrenoceptors.

Acknowledgements

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