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Contribution of Phospholipase C-β3 Phosphorylation to the Rapid Attenuation of Opioid-Activated Phosphoinositide Response

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ABSTRACT

Activation of the δ-opioid receptor in NG108–15 neuroblastoma X glioma hybrid cells results in a transient increase at the intracellular level of inositol-1,4,5-triphosphate [Ins(1,4,5)P3]. This time course in the transient increase in the Ins(1,4,5)P3 level is distinctly different from that observed in the homologous opioid receptor desensitization as measured by the inhibition of adenylyl cyclase activity. One probable mechanism for this rapid loss in Ins(1,4,5)P3 response is the feedback regulation of the phospholipase C activity. Regulation by protein phosphorylation was suggested by the observations that the opioid-mediated response was potentiated by calphostin C, an inhibitor of protein kinase C (PKC), and was abolished by either phorbol-12-myristate-13-acetate, a PKC activator, or calyculin A, a protein phosphatase½A inhibitor. The direct phosphorylation of phospholipase C was demonstrated by immunoprecipitation of PLC-β3 from metabolically labeled NG108–15 cells challenged with the δ-selective agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE). A time- and DPDPE concentration-dependent and naloxone-reversible increase in the PLC-β3 phosphorylation can be demonstrated. This PLC-β3 phosphorylation was mainly due to PKC activation because pretreatment of NG108–15 cells with calphostin C could block the DPDPE effect. Activation of the PLC-β3 by DPDPE was one of the prerequisites for agonist-mediated PLC-β3 phosphorylation because the aminosteroid phospholipase C inhibitor U73122 could block the DPDPE effect. In addition to DPDPE, lysophosphatidic acid (LPA) stimulated the PLC-β3 phosphorylation, but bradykinin did not. Furthermore, the LPA- and DPDPE-mediated PLC-β3 phosphorylation was additive and was much less than that observed with phorbol-12-myristate-13-acetate. The effect of DPDPE was specific to PLC-β3; the βγ-insensitive phospholipase C-β1 was not phosphorylated in the presence of either DPDPE or LPA. These results indicate that although PKC phosphorylation of PLC-β3 is not obligatory for the opioid receptor desensitization, it seems to play a significant facilatory role in the mechanisms allowing desensitization of opioid-activated phospholipase C response before that of adenylyl cyclase inhibition.

Opioids acting through the δ, κ, and μ GPCRs, which exert their effects through the G_i/G_o heterotrimeric G proteins, mediate potent analgesic effects in the nervous system (Loh and Smith, 1990). Opioid receptors, like other G_i/G_o-activating GPCRs, control multiple effectors, including inhibition of both adenylyl cyclase and voltage-dependent Ca^{2+} channels (Fiorillo and Willaims, 1996), activation of inward rectifying potassium channels (GIRKs) (Piros *et al*., 1996), mitogen-activated protein kinase cascade (Li and Chang, 1996), and PLC (Cheuh *et al*., 1995; Smart and Lambert, 1995, 1996a, 1996b; Connor and Henderson, 1996; Murthy and Makhlouf, 1996a).

Opioid-regulated effector responses desensitize in a manner that is typically homologous (receptor specific) in nature, as do most other GPCR-regulated effector responses (Premont *et al*., 1995). To date, most attention has been focused on the role of receptor phosphorylation or internalization in the process of GPCR desensitization (Premont *et al*., 1995), and such mechanisms seem to be similarly important for desensitization of the opioid-promoted inhibition of adenylyl cyclase (Law *et al*., 1983, 1985; Loh and Smith, 1990; Pei *et al*., 1995; Sternini *et al*., 1996). However, the opioid-activated phosphoinositide response turns-off/desensitizes within 2 min (Cheuh, *et al*., 1995; Smart and Lambert, 1995, 1996a, 1996b; Connor and Henderson, 1996; Murthy and Makhlouf, 1996a), as do all GPCR-activated responses (Berridge, 1993; Fischer, 1995). This time course is much faster than the desensitization of the opioid-mediated inhibition of adenylyl cyclase (Law *et al*., 1983, 1985) suggesting distinct mecha-

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ABBREVIATIONS: DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; GPCR, G protein-coupled receptor; Ins(1,4,5)P3, inositol-1,4,5-triphosphate; LPA, lysophosphatidic acid; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-acetate; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

nisms of desensitization. Activation of PLC leads to activation of calcium-dependent protein kinases (PKC), and it is entirely possible that PKC phosphorylates some postreceptor target leading to the turn-off/desensitization of opioid-activated PLC. This would have the advantage of allowing the opioid receptor to continue to activate G_i/G_o and the concomitant inhibition of adenylyl cyclase to continue unabated. Although considerable evidence exists for a PKC-mediated negative feedback loop acting on GPCR-activated PLC, this has never been adequately proved for any GPCR (Ryu *et al.*, 1990; Berridge, 1993; Fischer, 1995; Ali *et al.*, 1997). Surprisingly, studies on the potential ability of either PKC or agonist-activated GPCRs to effect phosphorylation of the G protein-regulated PLCs, PLC-β1–4, are extremely limited. PLC-β1 has been identified as a target of PKC activated by the nonphysiological activator PMA (Ryu *et al.*, 1990) but not, as yet, by any physiological route of PKC activation through the activation of a GPCR. Recently, however, in certain cells of the immune system, PLC-β3 has been identified as a target of PKC activated by platelet-activating factor receptor, a G_q-coupled GPCR (Ali *et al.*, 1997). It is thought that this phosphorylation of PLC-β3 contributes as one of several steps in the homologous desensitization of platelet-activating factor receptor-activated phosphoinositide response (Ali *et al.*, 1997). PLC-β2 seems not be a target of PKC (Liu and Simon, 1996), and PLC-β4 has not been investigated. Whether activation of any GPCR other than the platelet activating factor receptor results in the PKC-dependent phosphorylation of any PLC-β is unknown. If such responses occur in other tissues, and particularly such a response by G_i/G_o-activating GPCRs such as opioid receptors, which activate the PLC-β enzymes by a completely different mechanism, is unknown. In addition, G_i/G_o-activating GPCRs such as opioid receptor do not activate PLC in the robust manner of GPCRs, such as the platelet-activating factor receptor, which couples to G_q, and therefore the PKC activation often is less robust and prolonged. Differences in the possible occurrence of a PKC-mediated negative feedback loop may arise because of tissue-specific expression of isozymes of PLC-β or PKC (Exton, 1996); the amplitude or duration of PKC activation, which is dependent on the GPCR in question (Nishizuka, 1995), and the type of PKC activated, which also varies depending on the GPCR in question (Clerk *et al.*, 1994). Also, not all GPCR-activated phosphoinositide responses show signs of PKC sensitivity (Fischer, 1995).

With the above observations in mind, we postulated that the rapid turn-off/desensitization of opioid activated phosphoinositide response might involve a PKC-mediated negative feedback loop in the form of a PKC-dependent phosphorylation directed at the opioid-activated PLC. In the current study, we provide evidence for this hypothesis, and we demonstrate that PKC or a PKC-activated kinase phosphorylated the agonist-activated PLC-β3 which contributed to the subsequent inactivation of this enzyme.

Experimental Procedures

Materials. PMA, calphostin C, and calyculin A were obtained from Alexis (San Diego, CA). LPA, bradykinin, and ionomycin were obtained from Sigma Chemical (St. Louis, MO). A kit for the measurement of Ins(1,4,5)P3 was obtained from New England Nuclear Research Products (Boston, MA). Affinity-purified rabbit polyclonal antibodies specific for each of PLC-β1–4, based on carboxyl-terminal sequences, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. NG108–15 neuroblastoma X glioma hybrid cells were maintained in Dulbecco's modified Eagle's medium containing HAT media and 10% fetal calf serum at 10% CO_2 in a humidified atmosphere as described previously (Law *et al.*, 1985).

Mass measurement of Ins(1,4,5)P3. Determinations were performed using a radioimmunoassay kit, which was specific for the Ins(1,4,5)P3 isomer, as described previously (Smart and Lambert, 1995).

Immunoprecipitation. This was performed essentially as described previously (Ali *et al.*, 1997). Washed immunoprecipitated samples were incubated at 42° for 30 min in 200 mM dithiothreitol and 10% β-mercaptoethanol to avoid the appearance of PLC-β3 dimers on SDS-PAGE. Samples were run routinely on 7% SDS-PAGE gels and either transferred to PVDF or were stained with Coomassie blue to visualize PLC-β3, so the amounts of PLC-β3 present between bands were equalized. After immunoprecipitation and SDS-PAGE, and Coomassie blue staining, PLC-β3 was readily visible from one confluent 60-mm plate of cells. Samples transferred to PVDF were immunoblotted to confirm the identity of the band in question as that of PLC-β3 and to determine consistent recovery of PLC-β3 between samples within the same experiment. Radioactivity within each band was quantified by the use of a Molecular Dynamics (Sunnyvale, CA) PhosphoImager Storm 840.

Immunoblotting. After SDS-PAGE, samples were transferred to PVDF, blocked in the blocking buffer [5% bovine serum albumin, phosphate-buffered saline-Tween-20 (0.05%)] for 1 hr, and incubated in the presence of the primary antibody (0.1 μg/ml) in blocking buffer for 1 hr. Blots were washed three times in phosphate-buffered saline-Tween-20 (0.05%) for 10 min. The secondary antibody, which was anti-rabbit conjugated with alkaline phosphatase, was added (0.1 μg/ml) for 1 hr, washed three times as above, developed with alkaline phosphatase substrate (Vistra Systems; Amersham, Arlington Heights, IL), and visualized by immunofluoresence, on the PhosphorImager Storm 840.

Results

The time course of Ins(1,4,5)P3 generation, as shown in Fig. 1, measured by mass assay on challenge of the NG108–15 cells with the δ-opioid receptor agonist DPDPE showed a rapid burst and subsequent decline to base-line as described previously (Smart and Lambert, 1996a). This transient increase in the intracellular Ins(1,4,5)P3 level is typical for GPCR-activated phosphoinositide responses (Berridge, 1993; Fischer, 1995). Preincubation of the NG108–15 cells with the selective PKC inhibitor calphostin C before challenge with DPDPE increased the amplitude and duration of the response (Fig. 1). This result indicates that calphostin C either inhibits the action of a rapidly activated PKC or suppresses the activity of a tonically active PKC on PLC output. Preincubation of the hybrid cells with the potent PKC activator (PMA) or the potent inhibitor of protein phosphatases 1 and 2A, calyculin A, abolished the effect of DPDPE (Fig. 1). The effect of calyculin A implies that some component of the pathway exists in a prephosphorylated state that was rapidly dephosphorylated by protein phosphatase 1/2A. The rapid loss of DPDPE response can be demonstrated to be homologous. LPA, acting through its cognate GPCR, promotes both inhibition of adenylyl cyclase and activation of phosphoinositide response (Moolenaar *et al.*, 1997). As shown in Fig. 1, when the intracellular Ins(1,4,5)P3 level returned to the base-line level in the continued presence of DPDPE, LPA was able to induce the transient increase in the Ins(1,4,5)P3 level.

The phosphoinositide response due to G_i/G_o-coupled GPCRs usually is attributed to the large amount of $\beta\gamma$ subunits released by the activation of the relatively abundant G_i/G_o (Exton, 1996). Of the four mammalian PLCs regulatable by G proteins, PLC-β1–4, (Exton, 1996; Rhee and Bae, 1997) only PLC-β2 and -β3 are activated by $\beta\gamma$ subunits (Exton, 1996; Rhee and Bae, 1997). The expression of PLC-β2 is limited to certain cell types of the immune system (Exton, 1996; Ali *et al*., 1997; Rhee and Bae, 1997), but PLC-β3 apparently is ubiquitous (Exton, 1996; Ali *et al*., 1997; Rhee and Bae, 1997). Immunobloting of the whole-cell lysates and membrane and cytosolic fractions with antibodies specific to each PLC-β subtypes revealed that PLC-β2 and PLC-β4 were not present in NG108–15 hybrid cells as one would expect based on literature reports (data not shown) (Exton, 1996; Rhee and Bae, 1997). Previous studies indicate that PLC-β3 is responsible for the opioid-activated phosphoinositide response in smooth muscle tissue (Murthy and Makhlouf, 1996a) and for the responses due to other G_i/G_o-coupled GPCRs such as A_1 adenosine and somatostatin receptors (Murthy and Makhlouf, 1995; Murthy *et al*., 1996b). To identify whether PLC-β3 was the locus of the aforementioned phosphorylation/dephosphorylation cycle involved in the regulation of the opioid-stimulated phosphoinositide response, NG108–15 cells were labeled metabolically with [^{32}P]orthophosphate, and PLC-β3 was immunoprecipitated using an antibody specific for this isozyme of PLC. In these hybrid cells, PLC-β3 was found to be basally phosphorylated, and challenge with DPDPE resulted in a rapid increase in the phosphorylation state of the enzyme (Fig. 2, A and B). The fact that PLC-β3 exists in a basal state of phosphorylation is consistent with the observation that calyculin A inhibits DPDPE-stimulated PLC (Fig. 1). The ability of the phosphatase inhibitor to attenuate DPDPE effect implied a proportion of PLC exists in a prephosphorylated state. The increase in PLC-β3 phosphorylation was transient, falling back to the resting/basal level by 10 min (Fig. 2, A and B). The phosphorylation also was found to be dose dependent (Fig. 3, A and B) and could be blocked by naloxone (Fig. 5), indicating the receptor-dependent/physiological nature of this event. If this DPDPE-stimulated phosphorylation represents the PKC-mediated negative feedback deduced from the effects of calphostin C, PMA, and calyculin A on DPDPE-stimulated intracellular Ins(1,4,5)P3 production (Fig. 1), then calphostin C should abolish the PLC-β3 phosphorylation induced by DPDPE, which was found to be the case. As shown in Fig. 4, pretreatment of NG108–15 hybrid cells with 1 μM calphostin C for 10 min, but not for 2 min, resulted in a blockade of DPDPE-induced phosphorylation of PLC-β_3. These data supported the hypothesis that PKC phosphorylation represents a negative feedback loop of the opioid receptor-mediated phosphoinositide response.

If the phosphorylation of PLC-β3 is a negative feedback loop, then its phosphorylation should be dependent on its activation. It could be demonstrated that DPDPE-stimulated phosphorylation PLC-β3 was blocked by preincubation of cells with either the opioid-receptor antagonist naloxone (Fig. 5, A and B, *lanes 1–3*) or the aminosteroid phospholipase C inhibitor U73122 (Fig. 5, A and B, *lanes 1, 2, 7, and 8*). LPA, which promotes both the inhibition of adenylyl cyclase and the activation of PLC (Moolenaar *et al*., 1997), also stimulated phosphorylation of PLC-β3 to an extent similar to that stimulated by DPDPE (Fig. 5, A and B, *lanes 1* and *4*). Interestingly, the effects of DPDPE and LPA, when admin-

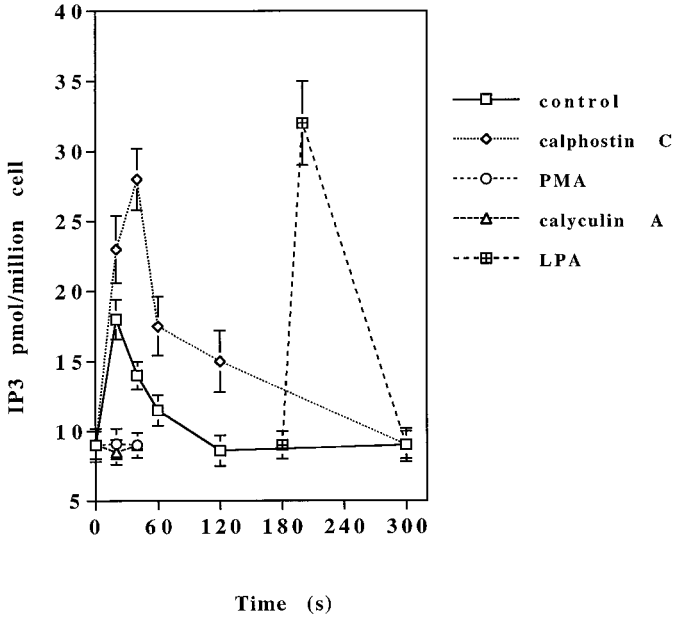

Fig. 1. Inhibition of PKC potentiates the DPDPE-stimulated phosphoinositide response. Whole-cell suspensions of NG108–15 cells were incubated at 37° for 10 min with buffer (*control*), 1 μM calphostin C, 100 nM PMA, or 20 nM calyculin A and subsequently challenged with 100 nM DPDPE for 0–300 sec. In addition, some cells were incubated with 100 nM DPDPE for 180 sec before the addition of 1 μM LPA and terminated 20 and 40 sec later. Ins(1,4,5)P3 was measured with a radioreceptor mass assay. Data are mean ± standard error of one of three similar experiments performed in triplicate.

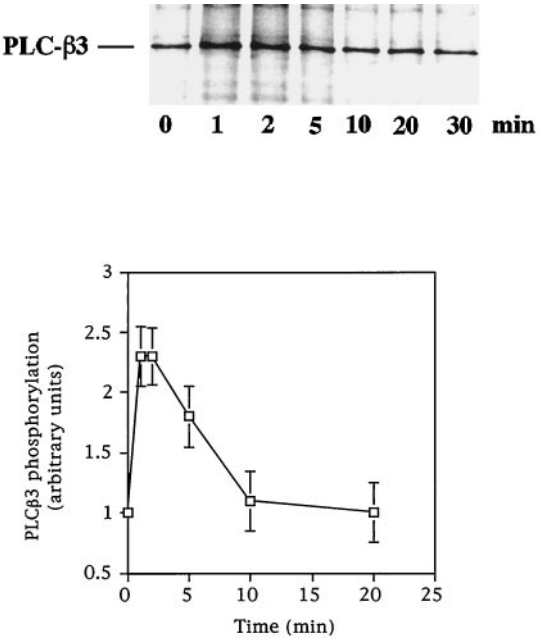

Fig. 2. Characterization of the effects of DPDPE on the phosphorylation state of PLC-β3. ^{32}P-labeled NG108–15 cells were challenged with drugs. A, Cells were challenged with 100 nM DPDPE for the indicated periods of time. Cells were subsequently lysed and immunoprecipitated with anti-PLC-β3 antibody, and samples were subjected to SDS-PAGE and autoradiography and visualized with a Molecular Dynamics PhosphoImager. B, Quantification of PLC-β3 phosphorylation from experiments illustrated in A. Values are mean ± standard deviation of three experiments.

istered concurrently, were additive (Fig. 5, A and B, *lane 5*). However, bradykinin, which activates a robust and transient Ins(1,4,5)P3 spike in NG108–15 cells (Cheuh *et al.*, 1995), did not increase the phosphorylation state of PLC-β3. PLC-β1 is not believed to be activated by $\beta\gamma$ subunits (Exton, 1996; Rhee and Bae, 1997) and was found to exist in a state of basal phosphorylation that was not altered by challenging cells with either DPDPE or LPA (Fig. 5C).

If PKC mediates the opioid-stimulated phosphorylation of PLC-β3, then direct activation of PKC should promote the phosphorylation of PLC-β3 in NG108–15 cells, and this was found to be the case (Fig. 6A, *lanes 1 and 2*). When NG108–15 cells were incubated with 100 nM PMA for 10 min, a robust increase in PLC-β3 phosphorylation was observed. Interestingly, the increase in phosphorylation was found to be much greater with the nonphysiological activator PMA (8-fold) than effected by the physiological activation of GPCRs for DPDPE and LPA (2-fold). Similarly, preincubation of the hybrid cells with 10 nM calyculin A also increased the phosphorylation of PLC-β3, in keeping with the effects of the drug on DPDPE-stimulated Ins(1,4,5)P3 and implying PLC-β3 is rapidly dephosphorylated by protein phosphatase

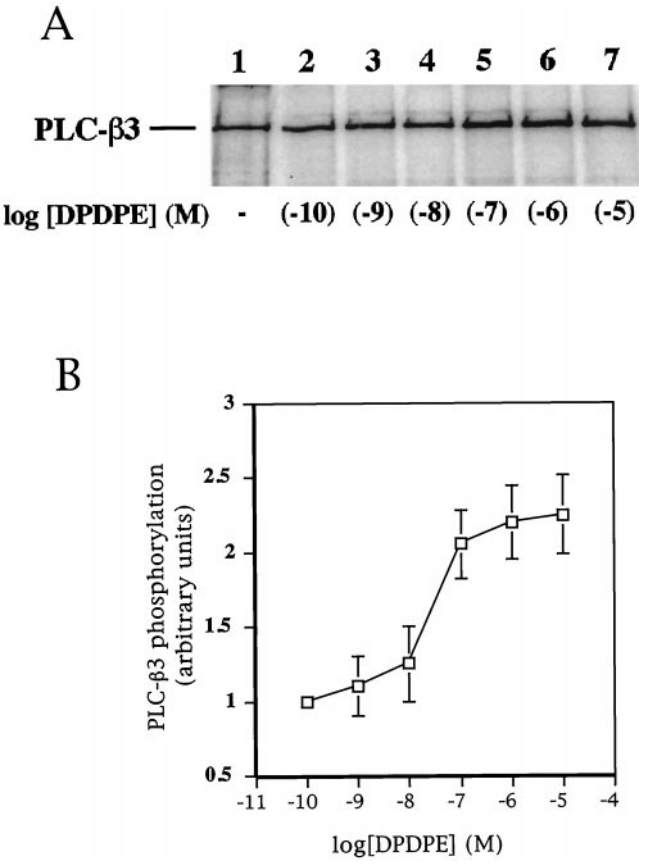

Fig. 3. DPDPE stimulation of PLC-β3 phosphorylation is dose dependent. A: *Lane 1,* no addition. *Lanes 2–7*, 0.1, 1, 10, 100, 1,000, and 10,000 nM DPDPE, respectively, for 1 min. Cells were subsequently lysed and immunoprecipitated with anti-PLC-β3 antibody, and samples were subjected to SDS-PAGE and autoradiography and visualized with a Molecular Dynamics PhosphoImager. B, Quantification of PLC-β3 phosphorylation from experiments illustrated in A. Values are mean ± standard deviation of three experiments.

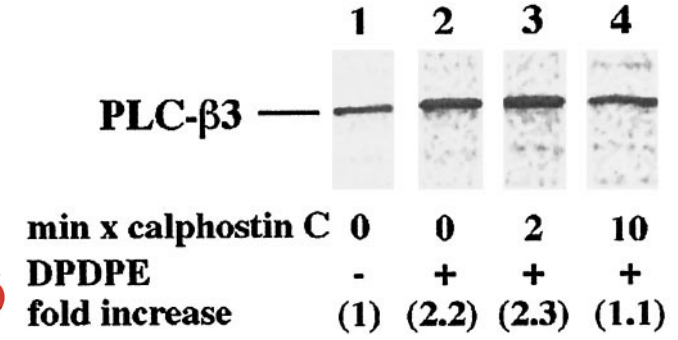

Fig. 4. DPDPE-stimulated phosphorylation of PLC-β3 is PKC dependent. ^{2}P-labeled NG108–15 cells were preincubated with either buffer (*lanes 1* and *2*) or 1 μM calphostin C for 2 and 10 min (*lanes 3* and *4*), respectively. Cells subsequently were challenged with either buffer (*lane 1*) or 100 nM DPDPE for 1 min (*lanes 2–4*). Cells were lysed and immunoprecipitated with anti-PLC-β3 antibody, and samples were subjected to SDS-PAGE and autoradiography and visualized with a Molecular Dynamics PhosphoImager. This result is a representative example of an experiment performed in triplicate.

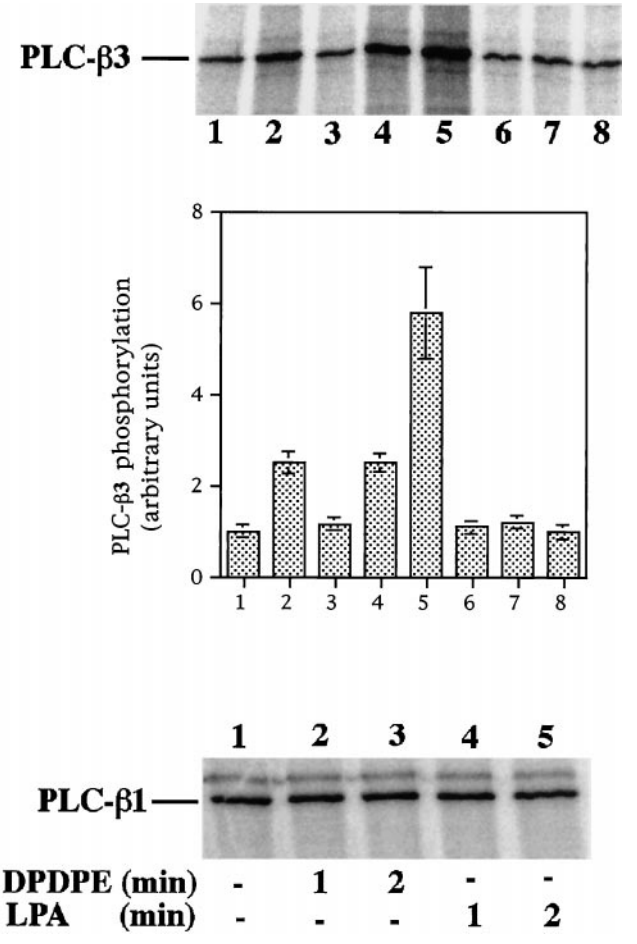

Fig. 5. Specificity of phospholipase C-β phosphorylation. ^{32}P-Labeled NG108–15 cells were incubated with drugs. A: *Lane 1*, no addition. *Lane 2*, 100 nM DPDPE for 1 min. *Lane 3*, as for *lane 2* but incubated for 10 min with 10 μM naloxone. *Lane 4*, 1 μM LPA acid for 1 min. *Lane 5*, 100 nM DPDPE and 1 μM LPA together for 1 min. *Lane 6*, 1 μM bradykinin for 1 min. *Lane 7*, as for *lane 2* but preincubated with 10 μM U73122. *Lane 8*, 10 μM U73122 for 10 min. C: *Lane 1*, no addition. *Lanes 2 and 3*, 100 nM DPDPE for 1 and 2 min, respectively. *Lanes 4 and 5*, 1 μM LPA for 1 and 2 min, respectively. Cells subsequently were lysed and immunoprecipitated with anti-PLC-β3 antibody (A) or anti-PLC-β1 (C), and samples were subjected to SDS-PAGE and autoradiography and visualized with a Molecular Dynamics PhosphoImager. Result shows a representative experiment repeated four times. B, Quantification of PLC-β3 phosphorylation of experiment shown in A; results are mean ± standard deviation of four independent experiments.

1 or 2A (Fig. 6A, *lanes 1 and 3*). The presence of the phosphatase activity also can be demonstrated by the ability of calyculin A to increase further the PMA-induced PLC-β3 phosphorylation (Fig. 6A, *lane 4*). The effects of these drugs were long lasting, not diminishing after 1 hr (data not shown). The Ca^{2+} ionophore ionomycin did not stimulate phosphorylation, implying that only the PKC arm of the phosphoinositide pathway exerts negative feedback inhibition at the level of PLC-β3 phosphorylation (data not shown). It could be demonstrated that this PKC-dependent phosphorylation only involves the activated PLC. As in the case of DPDPE-stimulated phosphorylation, the aminosteroid PLC inhibitor U73122 dramatically reduced the extent of PLC-β3 phosphorylation in the presence of 100 nM PMA (Fig. 6B). On the other hand, the less active aminosteroid analog U73343 also inhibited the affect of PMA but to a much lesser extent (Fig. 6B). Instead of the normal 8-fold increase in the PLC-β3 phosphorylation, PMA induced only a 5.5-fold increase in the phosphorylation in the presence of U73343 and a 2-fold increase in the presence of U73122. These data support the probable involvement of PKC in the feedback loop of the receptor-mediated phosphoinositide response.

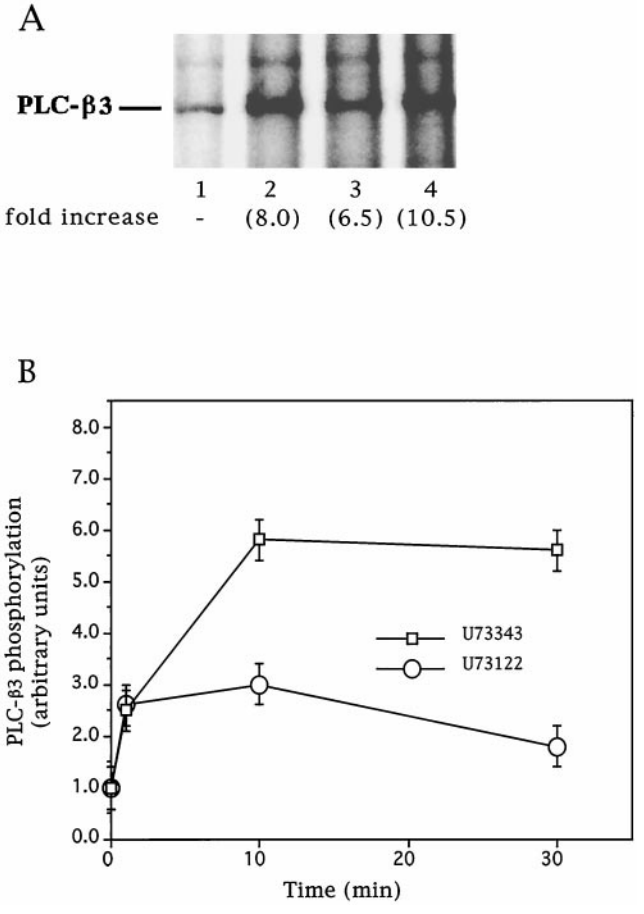

Fig. 6. Characterization of phorbol ester-stimulated phosphorylation of PLC-β3. ^{32}P-Labeled NG108–015 cells were incubated with drugs. A: *Lane 1*, no addition. *Lane 2*, 100 nM PMA for 10 min. *Lane 3*, 20 nM calyculin A for 10 min. *Lane 4*, 100 nM PMA and 20 nM calyculin A together for 10 min. B, Cells were incubated with either U73122 or U73343 and subsequently challenged with PMA for the indicated times. Cells subsequently were lysed and immunoprecipitated with anti-PLC-β3 antibody, and samples were subjected to SDS-PAGE and autoradiography and visualized with a Molecular Dynamics PhosphoImager. A, Representative example of an experiment repeated three times. B, Change in PLC-β3 phosphorylation; results are mean ± standard deviation of three separate experiments.

Discussion

The opioid-mediated phosphoinositide and adenylyl cyclase responses exhibit distinctly different time courses and extents of desensitization (Law *et al*., 1983, 1985; Loh and Smith, 1990; Cheuh *et al*., 1995; Pei *et al*., 1995; Smart and Lambert, 1995, 1996a, 1996b; Murthy and Makhlouf, 1996a). Because the opioid-mediated phosphoinositide response shuts down before the opioid adenylyl cyclase response, the termination of the former may require a separate unidentified mechanism. This mechanism would be at least initially independent of the receptor phosphorylation or internalization mechanisms believed to bring about desensitization of adenylyl cyclase response at later time points (Law *et al*., 1983, 1985; Pei *et al*., 1995; Sternini *et al*., 1996). We hypothesized that the unidentified mechanism terminating the phosphoinositide response may involve a PKC-mediated negative feedback loop, the like of which has been postulated but not proved for some other GPCRs that activate PLC (Ryu *et al*., 1990; Berridge, 1993; Ali *et al*., 1997).

Our data indicate that opioid receptor activation in NG108–15 hybrid cells resulted in an alteration in the PLC-β3 phosphorylation that involved a PKC/protein phosphatase 1/2A-dependent phosphorylation-dephosphorylation cycle at the level of effector itself. Thus opioid receptor and perhaps other receptors, including the LPA receptor, in NG108–15 hybrid cells seem to regulate PLC not only via G protein subunits but also by controlling the phosphorylation status of PLC-β3. However, it is clear that desensitization of the opioid phosphoinositide response is multifactorial, possibly with redundant mechanisms operating. Loss of PKC input does not prevent desensitization (Fig. 1), and DPDPE-stimulated phosphorylation of PLC-β3 is transient (Fig. 2), implying other mechanisms must act in addition to phosphorylation. This principle of facilitative but not obligatory role seems to be a common theme in the relationship between PKC and desensitization/attenuation of GPCR-activated phosphoinositide responses. Although stimulation of cells with PMA seems to universally block GPCR-activated phosphoinositide responses, inhibition of PKC by selective PKC inhibitors does not prevent desensitization (Fischer, 1995; Berridge, 1993). Similarly, loss of inactivation-no-afterpotential-c, the PKC known to function in the termination of the phosphoinositide response of the *Drosophila* phototransduction cascade, merely slows the onset of desensitization rather than preventing it (Zucker, 1996). This occurs despite the well recognized role of INAC in the turn-off/desensitization of the rhodopsin/G_q/PLC phototransduction cascade (Zucker, 1996; Chevesich *et al*., 1997). Recently, the desensitization of platelet-activating factor-activated PLC was shown to involve both phosphorylation of the receptor itself and PKC-dependent phosphorylation of PLC-β3. However, inhibitors of PKC do not prevent desensitization to platelet-activating factor (Fischer, 1995; Ali *et al*., 1997), implying again that the role of PKC is facilatory rather than obligatory, and the whole process of desensitization to the phosphoinositide response probably is multifactorial.

LPA, which like DPDPE activates both phospholipase C and adenylyl cyclase inhibitory pathways, stimulated phos-

phorylation of PLC-β3, but bradykinin did not (Fig. 5, A and B). In addition, DPDPE did not stimulate the phosphorylation of PLC-β1 (Fig. 5C). These results indicate that opioid receptor influences the phosphorylation of only a subset of the PLCs present and that phosphorylation of these PLC-β3 is GPCR specific. Desensitization of any GPCR-activated phosphoinositide response invariably is homologous (i.e., independent of other GPCRs capable of activating PLC in the same cell) (Fischer, 1995). Thus, it is no surprise that LPA could stimulate the production of Ins(1,4,5)P3 when the opioid receptor-mediated response was completely abolished (Fig. 1). Interestingly, the DPDPE- and LPA-induced PLC-β3 phosphorylations were additive, implying the possibility of different pools of PLC-β3 being regulated by these GPCRs. In contrast, the increase in PLC-β3 phosphorylation seen with PMA was much greater and persisted much longer than that observed with DPDPE (Fig. 6). This is to be expected because DPDPE only transiently and modestly activates PKC, whereas PMA is known to increase PKC activity to supraphysiological levels that are persistent (Nishizuka, 1995). Also, activation of the multiple PKCs present in any given cell is known to be dependent on the type of GPCR activated (Clerk *et al*., 1994), whereas phorbol esters activate most PKCs and attenuate all GPCR phosphoinositide responses (Berridge, 1993; Fischer, 1995; Nishizuka, 1995). It is tempting to speculate that limited duration, limited extent, and specificity of activated PKC underwrite the homologous desensitization observed with DPDPE, whereas PMA activation of the PKC resulted in the observed heterologous desensitization of PLC (Berridge, 1993; Fischer, 1995; Ali *et al*., 1997).

The PLC inhibitor U73122 attenuated DPDPE-stimulated phosphorylation of PLC-β3 (Fig. 5), implying the phosphorylation requires activation of the PLC. Furthermore, the inhibitor also attenuated PLC-β3 phosphorylation stimulated by PMA. These results imply that PKC may preferentially phosphorylate the active form of PLC-β3, just as some other protein kinases, such as G protein receptor kinases, are known to preferentially phosphorylate a particular conformation of their substrates (Premont *et al*., 1995). However, the specificity of U73122 is a crucial factor, and direct effect on the kinase or kinases involved in the phosphorylation, which seem at a minimum to involve some isoform of PKC, cannot be easily excluded. In any case, to conclude unambiguously that PKC phosphorylates only the active conformation of PLC-β3, a process that might be important in ensuring homologous desensitization, would at a minimum require detailed reconstitution experiments, which were beyond the scope of the present investigation.

It is tempting to hypothesize that phosphorylation of the PLC-β3 results in the inactivation of the enzyme itself. Our data with the PKC inhibitor calphostin C and phosphatase inhibitor calyculin A (Fig. 1) only suggested a role of PKC in the regulation of the opioid receptor-mediated activation of the PLC activity. Furthermore, reported studies have suggested phosphorylation of PLC-β did not result in its inactivation. Ryu *et al*. (1990) established that phorbol ester-stimulated PKC phosphorylation of PLC-β would not lead to its inactivation. Rather, the PKC-mediated phosphorylation of PLC-β resulted in the inhibition of receptor-mediated activation of the enzyme activity (Kellerer *et al*., 1990; Chen *et al*., 1995; Chen and Chen, 1996). It has been suggested by Ryu *et al*. (1990) that the phosphorylation of the PLC-β could interfere with the interaction of the enzymes with other cellular proteins, proteins that might be involved in the receptor-mediated activation. Because PLC-β3 can be activated by either G_q α subunit or the $\beta\gamma$ subunits of the heterotrimeric G proteins (Rhee and Bae, 1997), the PLC-β3 domains involved in these proteins interactions could be the probable sites for the receptor-mediated phosphorylation. Without detailed identification of the sites being phosphorylated, it is only a hypothesis that the PKC negative feedback loop involves the phosphorylation of these binding domains within the PLC-β3.

We have described, in essence, the occurrence of a postreceptor attenuation mechanism operating on opioid-activated PLC. This mechanism seems to contribute toward the process of desensitization; however, its exact function remains to be determined. The network of multiple effectors regulated by the opioid receptors may pose a significant problem in the coordination and flexibility of desensitization, which may not be adequately regulated solely by controlling the output of the opioid receptor alone. Indeed, the operation of postreceptor factor modulating opioid effector responses has been postulated previously (Prather *et al*., 1994), and recent progress involved the discovery of numerous G_i/G_o-directed inhibitory proteins, the RGS proteins (regulators of G protein signaling) (Dohlman and Thorner, 1997). Some of these have been shown to inhibit δ-opioid receptor-mediated inhibition of adenylyl cyclase *in vitro* (Hepler *et al*., 1997), and although their role in mammalian cells is not yet clear, SST2, the homologue of mammalian RGS proteins in *Saccharomyces cerevisiae,* is known to be crucial for desensitization of GPCR signaling systems in this organism (Dohlman and Thorner, 1997). Further understanding and characterization of any postreceptor acting factors modulating the output of opioid controlled effectors will be necessary to advance our understanding of the overall processes of desensitization to opioids. Much further work will be required to elucidate the relationship between opioid-induced phosphorylation of PLC-β3 and the rapid termination of opioid phosphoinositide response, which our data indicate is a multifactorial process.

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