

Reciprocal modulation of phospholipase C β isoforms: Adaptation to chronic morphine

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Phosphoinositide turnover and calcium mobilization are fundamental determinants of acute and chronic opioid effects. Phosphoinositide-specific phospholipase C (PLC) are key signaling enzymes that play a pivotal role in mediating opioid modulation of inositol trisphosphate production and cytosolic calcium distribution, substrates for many acute and chronic opioid effects. Notably, phosphorylation of the β isoforms of PLC, by kinases that are up-regulated after chronic morphine, is a potent modality for their regulation. Direct assessment of PLC β 1 and PLC β 3 phosphorylation in the guinea pig longitudinal muscle myenteric plexus tissue revealed substantial alterations after the induction of opioid tolerance. Notably, the direction of this modulation is isoform-specific. Phosphorylation of PLC β 1 is significantly reduced, whereas that of PLC β 3 is substantially augmented, changes not accompanied by altered content of PLC β 1 or PLC β 3 protein. In contrast to chronic morphine, acute morphine treatment of opioid naïve longitudinal muscle myenteric plexus tissue attenuates PLC β 3 phosphorylation, an effect also manifested by endogenous opioids that is reflected by the ability of acute naloxone to substantially augment PLC β 3 phosphorylation. This indicates that PLC β phosphorylation is dynamically regulated. PLC β 1 and PLC β 3 activities are negatively modulated by phosphorylation. Thus, their concomitant reciprocal phosphorylation would alter the relative contribution of these isoforms to PLC/Ca²⁺ signaling, a significant shift in light of their differential regulatory characteristics. Reciprocal modulation of the phosphorylation (activity) of two isoforms within the same subclass of signaling enzyme, proteins that have a high degree of structural similarity and subserved the same biological function, represents an adaptation modality to chronic morphine that has heretofore not been recognized.

opioid tolerance | phosphorylation

Opioids are known to produce a plethora of neurochemical perturbations. These include inhibition of adenylyl cyclase (1, 2), activation of inwardly rectifying K⁺ channels (3, 4), and inhibition of voltage-activated calcium (Ca²⁺) channels, predominantly of the N and P/Q types (5, 6). Additionally, there is growing evidence that modulation of phosphoinositide-specific phospholipase C (PLC), and consequently altered formation of inositol 1,4,5-trisphosphate/diacylglycerol/Ca²⁺ signaling, plays a pivotal role in mediating opioid effects.

PLC is one of only two signaling effector enzymes (the other being adenylyl cyclase) whose activity is directly modulated by opioids. PLC is positively regulated by opioids (7–11), and several physiological studies have implicated PLC-linked pathways in a diverse range of opioid-modulated events. These include pain regulation (12, 13), manifestation of opioid tolerance (14), and opioid withdrawal (15). More recently, facilitative interactions between vasoactive intestinal polypeptide and the δ -opioid [D-Pen^{2,5}] enkephalin to enhance cAMP accumulation in spinal tissue have been shown to involve the PLC signaling cascade (16).

PLC is the enzyme responsible for generating two intracellular second messengers via the hydrolysis of phosphatidylinositol 4,5-bisphosphate (17). Inositol 1,4,5-trisphosphate partitions in the cell cytosol and is considered to be universal Ca²⁺ mobilizing

second messenger. Diacylglycerol remains in the membrane and is an activator of PKC. Molecular cloning and biochemical characterizations have revealed the existence of four types of PLC: β , γ , δ , and ϵ . These isoforms range in molecular mass (kDa) from 230 to 260 (ϵ) and 150 (β and γ) to 85 (δ), which are immunologically distinct and products of separate genes. One ϵ , four β , two γ , and four δ isoforms have been described in mammalian systems (18).

Phosphorylation state is a critical regulatory parameter of PLC β isoforms, which are substrates for protein kinase A (PKA) and PKC. Phosphorylation by these kinases negatively modulates the activity of PLC β isoforms (see refs. 19 and 20 and references therein). Regulation of PLC β isoform activity by a PKA-/PKC-mediated phosphorylation suggests their modulation during opioid tolerance, a hallmark feature of which is enhanced phosphorylation of key components of signaling cascades. Up-regulation of adenylyl cyclase, i.e., cAMP overshoot (21, 22), and protein kinases, which include PKA (23–28) and PKC (29–31), which results in the augmented phosphorylation of multiple specific proteins (24, 26, 32, 33), are among the most frequently used neurochemical markers of opioid tolerance/dependence.

Based on the importance of (augmented) PKA and PKC activity to the transduction of chronic opioid effects, the ability of these kinases to also phosphorylate and thereby regulate PLC β activity and the established importance of phosphoinositide turnover and calcium mobilization to chronic opioid sequelae, we hypothesized that PLC β phosphorylation would be augmented as a function of opioid tolerance. The present results reveal, using the guinea pig longitudinal muscle myenteric plexus preparation (LMMP), that PLC β 1 and PLC β 3 phosphorylation is significantly altered after induction of opioid tolerance. Notably, however, the change in phosphorylation (and presumably activity) qualitatively differs between these PLC β isoforms. This is a demonstration of chronic morphine-induced reciprocal regulation among isoforms of an identified protein.

Materials and Methods

Materials. Morphine pellets were supplied by the National Institute on Drug Abuse. ³²P-labeled phosphoric acid (³²P_i) was obtained from Perkin-Elmer. All protein kinase and phosphatase inhibitors were purchased from LC Laboratories (Woburn, MA) except for cypermethrin, which was purchased from Calbiochem. All protease inhibitors were purchased from Sigma except for Complete Protease Inhibitor mixture, which was purchased from Roche Molecular Biochemicals. Antibodies against PLC β 1, PLC β 3, and their blocking peptides were obtained from Santa Cruz Biotechnology. Anti-phosphoPLC β 3 (Ser-1105) and its blocking peptide were obtained from Cell Signaling Technology (Beverly, MA).

Abbreviations: PLC, phosphoinositide-specific phospholipase C; LMMP, longitudinal muscle myenteric plexus; PKA, protein kinase A; IP, immunoprecipitate.

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LMMP Preparation and Incubation with $^{32}\text{P}_i$. Studies were carried out in accordance with the guide for the care and use of laboratory animals as adopted and promulgated by the National Institutes of Health. Morphine tolerance/dependence was induced by s.c. implantation of five morphine pellets (each containing a 75-mg morphine base) into male albino guinea pigs (375–450 g) as described (33, 34). For tolerant/dependent LMMP tissue, morphine (100 nM) was included in the buffer. LMMP strips were then incubated with 1 mCi/ml $^{32}\text{P}_i$ in phosphate-free Krebs (2 h at 35°C) with continuous gassing. To determine the effect of acute opioid treatment on phosphorylation of PLC β 1 and PLC β 3, opioid naïve LMMP strips were incubated with morphine (100 nM) during the last 5 min of $^{32}\text{P}_i$ incubation. The kinase(s)/phosphatases required for chronic morphine-induced change in phosphorylation of PLC β 3 and PLC β 1 were inferred from the effect of inhibitors on the incorporation of ^{32}P into LMMP protein in tissue obtained from morphine-treated animals. LMMP tissue from the same ileum was randomly divided into two samples, one of which contained a kinase or phosphatase inhibitor. Each was added during the last 30 min of the 2-h ^{32}P -labeling period. Concentrations of inhibitor were selected to maximize discrimination between classes of kinase or phosphatase.

After incubation with $^{32}\text{P}_i$, LMMP tissues were washed extensively with phosphate-free Krebs buffer and homogenized in Tris buffer (10 mM, pH 7.6) containing 10% sucrose, 2 mM DTT, 5 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, protease inhibitors (Bacitracin, 100 mg/liter; 20 mg/liter each of chloromethyl ketone, N^α -*p*-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride; 3.2 mg/liter each of leupeptin and soybean trypsin inhibitor; 1.0 mg/liter Aprotinin, 1 mM Benzamide, and complete inhibitor mixture, one tablet per 50 ml), protein phosphatase inhibitors, 25 nM calyculin A; 0.5 μM okadaic acid, 10 mM sodium fluoride and 100 μM sodium orthovanadate. Supernatant obtained from a low-speed centrifugation (1,000 $\times g$, 4°C, 10 min) was subjected to a high-speed centrifugation (30,000 $\times g$, 4°C, 30 min). Membranes obtained were resuspended in 50 mM Tris buffer containing 1 mM DTT, 10 mM sodium pyrophosphate, and the above-mentioned protease and phosphatase inhibitors.

Autoradiography of PLC β 1 and PLC β 3. Immunoprecipitation of PLC β 1 and PLC β 3 used rabbit polyclonal antibodies generated against the carboxyl terminus of bovine PLC β 1 or rat PLC β 3, respectively. Membranes were solubilized in buffer containing phosphatase and protease inhibitors, as described above, and 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, and 150 mM NaCl (60 min on ice), agitated, and centrifuged (at 14,000 $\times g$ for 30 min). Protein amounts in solubilized supernatants were determined by Bradford method (35) using BSA as a standard. Solubilized membrane protein (1 mg) from each sample was incubated with either 4 μg of anti-PLC β 1 or PLC β 3 antibodies (each for 60 min at 4°C), after which immunoprecipitation was performed as described (32). Samples were separated by NUPAGE (using 7% Tris-acetate gels) electrophoresis (Invitrogen). Gels were dried and exposed to PhosphorImager screens that were scanned in a PhosphorImager Storm 860 (Molecular Dynamics). The amount of ^{32}P incorporated into the phosphorylated protein bands was determined by using densitometric analysis (IMAGEQUANT, Molecular Dynamics).

Western Blot Analysis. Standard procedures were used for Western analyses as used previously by this laboratory (36). Proteins from gels were electrophoretically transferred onto nitrocellulose membranes. Selected lanes were incubated (overnight at 4°C) with anti-PLC β 1, PLC β 3, phosphoPLC β 3 (all at 1:1,000), or antibodies in the presence of their respective blocking peptides

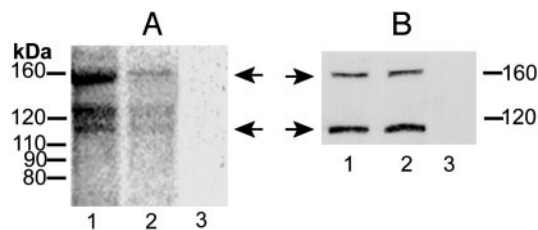


Fig. 1. Chronic morphine attenuates PLC β 1 phosphorylation without altering its protein content. (A) LMMP tissue was obtained from opioid naïve (lane 1) and chronic morphine-treated (lane 2) guinea pigs and incubated with ^{32}P for 2 h. Thereafter, membranes were prepared, solubilized, and immunoprecipitated by using antibodies selective for PLC β 1 or its preadsorbed control (lane 3). IPs were subjected to NUPAGE electrophoresis, and radiolabeled proteins were visualized by autoradiography (\approx 18-h exposure). Quantitative densitometric analysis was used to assess magnitude of ^{32}P incorporation. Chemical identity of proteins was based on electrophoretic molecular mass, calculated from a prestained protein ladder (10–200 kDa) that was included in each electrophoresis in combination with loss of signal after antibody preadsorption (representative of nine experiments). (B) LMMP membranes (50 μg) obtained from opioid naïve and chronic morphine-treated guinea pigs (lanes 1 and 2, respectively) were processed as described for A and subjected to Western blot analysis by using anti-PLC β 1 antibodies. Lane 3 represents Western blot analysis of LMMP membranes (50 μg) obtained from chronic morphine-treated tissue by using preadsorbed anti-PLC β 1 antibodies (representative of three experiments). Antibody–substrate complex was visualized by using ECL detection. Left arrows in A indicate phosphoPLC β 1, and right arrows in B indicate PLC β 1 protein. Chronic systemic morphine markedly reduces phosphorylation of PLC β 1 but does not alter its content.

(1:1; preadsorbed controls). The secondary antibody used was a peroxidase-labeled anti-rabbit antibody. Antibody–substrate complex was visualized by using an enhanced chemiluminescence detection kit (Amersham Pharmacia). Sample pairs, obtained from opioid naïve and chronic morphine-treated LMMP tissues, were processed, electrophoresed, and blotted in parallel, after which they were exposed concomitantly to the same Kodak X-Omat film (Perkin–Elmer). Intensity of signal was quantified by using NIH imaging software.

Statistical Analysis. Significance of autoradiographic and Western analyses was assessed by using a paired Student's *t* test.

Results

Effect of Chronic Morphine on PLC β 1 Phosphorylation and Protein Content. Immunoprecipitate (IP) was obtained by using PLC β 1 antibodies (PLC β 1 IP) from ^{32}P -radiolabeled opioid naïve and chronic morphine-treated LMMP tissue and subjected to autoradiographic analysis (Fig. 1A, lanes 1 and 2, respectively). PLC β 1 IP obtained from both preparations contained phosphorylated proteins of predominantly three molecular masses (\approx 155, 130, and 118 kDa). These radiolabeled proteins were not observable in IP obtained by using preadsorbed anti-PLC β 1-antibody (Fig. 1A, lane 3), underscoring the specificity of the immunoprecipitation. The \approx 155-kDa radiolabeled band is consistent with previously established values for the PLC β 1 isoform, whereas the \approx 130-kDa radiolabeled species is consonant with the molecular mass of its reported splice variant (20, 37). The radiolabeled signal of \approx 118 kDa most likely represents a degradation product of the \approx 155- or \approx 130-kDa single and was not visible in all experiments. Therefore, quantification of changes in PLC β 1 phosphorylation as a function of morphine treatment was confined to the \approx 155- and 130-kDa radiolabeled molecular mass species.

PLC β 1 IP obtained from opioid naïve and morphine-treated LMMP tissue contained analogous molecular mass profiles of radiolabeled proteins. However, the intensity of radiolabeling differed substantially among these two preparations. There was

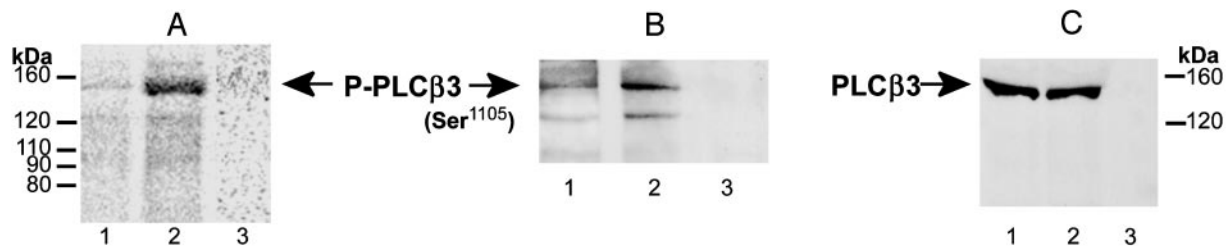


Fig. 2. Chronic morphine augments phosphorylation of PLC β 3 without altering its protein content. (A) Aliquots of solubilized LMMP membranes, prepared for experiments described in Fig. 1 A, were immunoprecipitated by using antibodies selective for PLC β 3 and subjected to autoradiographic analysis concomitantly with that of PLC β 1 IP. Lanes 1 and 2 represent IPs from LMMP tissue obtained from opioid naïve vs. chronic morphine-treated animals, respectively. Lane 3 represents IP obtained from opioid tolerant LMMP tissue by using preadsorbed anti-PLC β 3 antisera (representative of 10 experiments). (B and C) LMMP membranes obtained from opioid naïve and chronic morphine-treated guinea pigs (lanes 1 and 2, respectively) were processed as described for autoradiography and subjected to Western blot analysis by using either antibodies specific for phosphoPLC β 3Ser¹¹⁰⁵ (150 μ g of membrane, B) or PLC β 3 (100 μ g of membrane, C). Lane 3 in B and C represents Western analysis of chronic morphine-treated LMMP membranes using preadsorbed antibodies against phosphoPLC β 3 and PLC β 3, respectively. B and C are representative of four experiments each. Chronic morphine augments the phosphorylation of PLC β 3, assessed by ³²P incorporation or phosphoPLC β 3 Western analysis, but does not alter the content of PLC β 3. P-PLC β 3, phosphorylated PLC β 3.

a substantial reduction in the magnitude of ³²P incorporated into the \approx 155 ($63 \pm 1.73\%$; $P < 0.005$; $n = 9$) and 130-kDa proteins ($43 \pm 2.77\%$; $P < 0.05$; $n = 9$) present in PLC β 1 IP obtained from morphine-treated vs. opioid naïve tissue (Fig. 1A, compare lanes 1 and 2). These observations reveal that chronic morphine decreases PLC β 1 phosphorylation.

To investigate whether changes in PLC β 1 phosphorylation were accompanied by changes in protein content, quantitative Western analyses were performed. Two bands of \approx 155 and 118 kDa were visualized (Fig. 1B, lanes 1 and 2), comparable to that observed in autoradiography. However, the \approx 130-kDa band, visible in autoradiography, was not detected by Western analysis, possibly reflecting that it is present in minor amounts. Specificity of Western signals is indicated by their absence in Western blots by using preadsorbed antibody (Fig. 1B, lane 3). In contrast to ³²P incorporation, quantitative Western analyses revealed that the content of PLC β 1 protein (\approx 155 kDa) does not differ among LMMP membranes obtained from opioid naïve and chronic morphine-treated animals ($93 \pm 9\%$ of control; Fig. 1B, lane 1 vs. lane 2; $n = 4$).

Effect of Chronic Morphine on PLC β 3 Phosphorylation and Protein Content. Analogous experiments were performed by using antibodies selective for PLC β 3 (Fig. 2A and B). PLC β 3 IP obtained from opioid naïve and chronic morphine-treated ³²P-labeled LMMP tissue contained radiolabeled proteins of predominantly \approx 150 and 130 kDa (Fig. 2A, lanes 1 and 2). Neither was visible in autoradiographs of IP obtained by using preadsorbed anti-PLC β 3 antibody (Fig. 2A, lane 3). The \approx 130-kDa species is a relatively minor constituent. It was not observable in PLC β 3 Western analyses of LMMP membranes (Fig. 2C) unless the amount of membrane protein loaded was increased from 100 to 150 μ g or protein was immunoprecipitated by using anti-PLC β 3 antibody (thereby concentrating PLC β 3 protein) before commencing Western analysis (data not shown). Therefore, quantification of changes in PLC β 3 phosphorylation as a function of morphine treatment was confined to the \approx 150-kDa radiolabeled molecular mass species.

Chronic systemic morphine also had a substantial effect on the magnitude of ³²P incorporated into the \approx 150-kDa immunoprecipitated molecular mass species. Notably, however, the direction of the modulation was opposite from that observed for proteins present in PLC β 1 IP. Chronic morphine substantially increased ($227 \pm 19\%$ of control) the phosphorylation of PLC β 3 ($P < 0.05$; $n = 10$).

Consonant results were obtained when the phosphorylation state of PLC β 3 was assessed by using a PLC β 3 phosphospecific antibody. As was observed in autoradiographic analysis, quan-

titative Western analysis of PLC β 3 IP, obtained from control and chronic morphine-treated LMMP tissue, using an antibody selective for phospho-Ser-1105PLC β 3, revealed signals of \approx 150 and 130 kDa (Fig. 2B, lanes 1 and 2). Neither was observed in Western analysis using preadsorbed phosphoPLC β 3 antibody (Fig. 2B, lane 3). Higher amounts of protein (150 vs. 100 μ g) were used in the Westerns that used the anti-phosphoPLC β 3 antibody than did those using an anti-PLC β 3 common antibody, which would account for the presence of the \approx 130-kDa band in the former but not the latter (Fig. 2B vs. C). Notably, the intensity of the \approx 150-kDa signal was significantly augmented ($150 \pm 10\%$ of control, $P < 0.05$; $n = 4$) in phosphoPLC β 3 Western analysis of PLC β 3 IP obtained from chronic morphine-treated LMMP tissue. The differential magnitude of the increment in PLC β 3 phosphorylation observed by autoradiographic vs. Western analysis most likely indicates the chronic morphine-induced phosphorylation of sites in addition to Ser-1105, which would contribute to the autoradiographic but not the Western signal. These data reaffirm that chronic morphine augments PLC β 3 phosphorylation and underscore that potential metabolic effects of morphine on the specific activity of ATP pools do not confound interpretation of the chronic morphine-induced enhancement of ³²P incorporation into PLC β 3. Furthermore, they reveal that it results, at least in part, from increased phosphorylation at Ser-1105, the site recognized by the anti-phosphoPLC β 3 antibody.

Western analysis employing an anti-PLC β 3 common antibody also revealed a \approx 150-kDa molecular mass signal, analogous to that observed in autoradiographic and phosphoPLC β 3 Western analyses (Fig. 2C, lanes 1 and 2). This signal was obliterated in blots probed with preadsorbed antisera (Fig. 2C, lane 3). As was observed for PLC β 1, the content of PLC β 3 protein does not differ between LMMP membrane obtained from opioid naïve vs. chronic morphine-treated animals ($91 \pm 7\%$ of control; Fig. 2C, lane 1 vs. lane 2; $n = 4$).

Multiple considerations indicate that data interpretation is not confounded by cross reactivity of the anti-PLC β 1 and anti-PLC β 3 antibodies used for IP. First, chronic morphine-induced enhanced phosphorylation of PLC β 3 was demonstrated by using two different methodologies, which employ two different anti-PLC β 3 antibodies recognizing different epitopes. Second, because the direction of change in chronic morphine-induced phosphorylation of PLC β 1 is opposite that of PLC β 3, any residual cross reactivity would result in an underestimate of the magnitude of effect. Last, the peptide sequences used to generate anti-PLC β 1 and anti-PLC β 3 do not share any homology with PLC β 3 and PLC β 1, respectively.

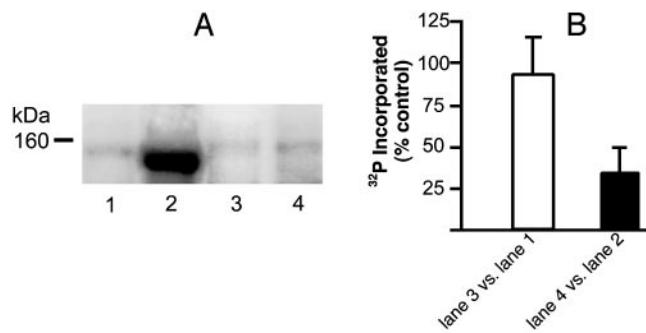


Fig. 3. Selective inhibition of PKC, but not PKA, abolishes the chronic morphine-induced increment in PLC β 3 phosphorylation. LMMP tissue from the same control and chronic morphine-treated guinea pig was randomly divided such that ^{32}P incorporation into immunoprecipitated protein could be concomitantly determined in the absence as well as the presence of chelerythrine (PKC-selective inhibitor, which was added during the last 30 min of the 2-h ^{32}P -labeling period). Thereafter, membranes were prepared, solubilized, and immunoprecipitated by using anti-PLC β 3 antibodies. IPs were subjected to electrophoresis, and radiolabeled protein was visualized by autoradiography (\approx 18-h exposure) by using storage phosphor imaging techniques. Quantitative densitometric analysis was used to assess magnitude of ^{32}P incorporation into an \approx 150-kDa signal. Radiolabeling of PLC β 3 in opioid naïve tissue is depicted without (lane 1) and with (lane 3) chelerythrine. Radiolabeling of PLC β 3 in chronic morphine-treated tissue is shown without (lane 2) and with (lane 4) PKC inhibition. The magnitude of ^{32}P incorporation was determined concomitantly in the presence vs. the absence of kinase inhibitor ($n = 3$). Chelerythrine abolished the increment in PLC β 3 phosphorylation produced by chronic morphine.

PKC Inhibition Abolishes the Chronic Morphine-Induced Increment in PLC β 3 Phosphorylation. The effect of PKC inhibition on the chronic morphine-induced increment in PLC β 3 phosphorylation is represented in Fig. 3. Pretreatment with the PKC-selective inhibitor chelerythrine failed to produce a significant reduction in ^{32}P incorporation into PLC β 3 obtained from opioid naïve tissue (Fig. 3A, lane 3 vs. lane 1). However, chelerythrine abolished the chronic morphine-induced increment in PLC β 3 phosphorylation (Fig. 3A, compare lanes 4 and 2), suggesting its mediation via PKC. In like fashion, we determined the effect of protein phosphatase inhibitors on the chronic morphine-induced decrement in PLC β 1 phosphorylation to ascertain whether or not enhanced phosphatase activity was a contributory factor. Neither okadaic acid, an inhibitor of protein phosphatases 1 and 2A (38), nor cypermethrin, an inhibitor of protein phosphatase 2B (39), altered the magnitude of the diminution of PLC β 1 phosphorylation.

Acute Treatment with Either Morphine or Naloxone Inhibits and Augments, Respectively, PLC β 3 Phosphorylation. To determine whether augmented PLC β 3 phosphorylation is a neurochemical sequela specifically associated with chronically administered morphine, the effect of its acute (5 min) administration on PLC β 3 phosphorylation was ascertained. In contrast to chronically administered morphine, its acute application (100 nM) inhibited PLC β 3 phosphorylation by $74 \pm 14\%$ ($n = 3$; Fig. 4, lane 2 vs. lane 1, gray bar). No effect of acute application of morphine was observed on phosphorylation of PLC β 1 ($n = 2$; data not shown). On the other hand, acute (30 min) treatment with 10 or $1 \mu\text{M}$ of the opioid antagonist naloxone substantially augmented phosphorylation of PLC β 3 ($309 \pm 11\%$ of control; $n = 2$; Fig. 4, lane 4 vs. lane 3, solid bar) and 257% of control, respectively (data not shown). These data indicate that morphine and its endogenous counterparts can acutely modulate (albeit negatively) PLC β 3 phosphorylation, but the augmentation of

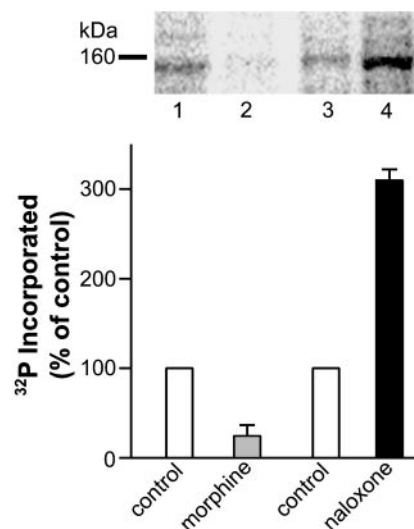


Fig. 4. Acute treatment with morphine and naloxone reciprocally modulates PLC β 3 phosphorylation. LMMP tissue from opioid naïve guinea pigs was randomly divided such that ^{32}P incorporation into immunoprecipitated protein could be determined concomitantly in the absence and presence of either morphine (100 nM; added during the last 5 min of the 2-h ^{32}P -labeling period) or naloxone (10 μM ; added during the last 30 min of the 2-h ^{32}P -labeling period). Thereafter, tissues were processed and analyzed as described for Fig. 3. The effect of acute opioid receptor activation on PLC β 3 phosphorylation is derived from comparing the intensity of signal in lane 1 (without morphine) vs. the signal present in lane 2 (with morphine) ($n = 3$); the effect of acute opioid receptor blockade on PLC β 3 phosphorylation is derived from comparing the intensity of signal in lane 3 (without naloxone) vs. the signal present in lane 4 (with naloxone). $n = 2$ in Lower. $P < 0.05$ for each comparison, which was determined in LMMP tissue obtained from separate animals. Data are expressed as percent control ^{32}P incorporation. Acute morphine substantially attenuates, whereas acute naloxone substantially enhances, PLC β 3 phosphorylation.

PLC β 3 phosphorylation that is observed after the development of tolerance requires their chronic administration.

Discussion

The present study reveals that chronic morphine alters the phosphorylation state of two PLC β isoforms, PLC β 1 and PLC β 3. Strikingly, their phosphorylation state is modulated in reciprocal fashion. Phosphorylation of PLC β 1 is diminished, whereas that of PLC β 3 is significantly augmented. There is a growing list of signaling proteins whose phosphorylation is modulated in response to chronic morphine. For example, in the locus coeruleus, the (cAMP-dependent) phosphorylation state of proteins of multiple molecular masses is augmented after chronic morphine (26). More recently, using the LMMP preparation, this laboratory demonstrated that chronic systemic morphine augments the phosphorylation of four signaling proteins, adenylyl cyclase (32), β -arrestin, G protein receptor kinase 2/3, and the G β subunit of G proteins (33). The diminished phosphorylation of another signaling protein, mitogen-activated protein kinase, has also been demonstrated during precipitated opioid withdrawal (40). The current demonstration of the reciprocal modulation of the phosphorylation of two isoforms within the same subclass of signaling enzyme (PLC β), proteins that not only have a high degree of structural similarity and amino acid identity (50%) but also subservise the same biological function, represents an adaptation modality that has heretofore not been recognized.

The augmented phosphorylation that results in response to chronic morphine is mediated via PKC. This is evidenced by the ability of chelerythrine, a PKC-selective inhibitor, to abolish the

chronic morphine-induced phosphorylation increment. This is consonant with the reported up-regulation of PKC in spinal cord (29, 30) and LMMP preparation (41) during opioid tolerance development. Notably, the chronic morphine-induced augmented phosphorylation of adenylyl cyclase, G protein receptor kinase2/3, β -arrestin, and G_{β} , previously reported by this laboratory, is also PKC-mediated (32, 33).

The mechanism(s) underlying the chronic morphine-induced diminution in phosphorylation of PLC β 1 remains to be elucidated. Pretreatment with an inhibitor of either protein phosphatase 1/2A or 2B, classes of phosphatase that act on Ser/Thr phosphorylation sites and should therefore counter the action of PKA and PKC, failed to diminish the decrement in PLC β 1 phosphorylation. This notwithstanding, both okadaic acid and cypermethrin produced a proportionately similar increase (2–3-fold) in ^{32}P incorporation into PLC β 1 obtained from either opioid naïve or morphine-treated LMMP preparations, indicating their effectiveness as phosphatase inhibitors under the current experimental conditions (data not shown). Thus, it seems unlikely that phosphatase activation is involved in this phenomenon, although the participation of other classes of protein phosphatase remains a viable possibility. Alternatively, the ability of G protein $\beta\gamma$ subunits to antagonize PKC-dependent phosphorylation of PLC β 1 *in vitro* (42) could be applicable to regulating the phosphorylation state of this isoform *in vivo*. Negative modulation of PLC β 1 phosphorylation by $G_{\beta\gamma}$ would be augmented in the opioid tolerant state because chronic morphine augments phosphorylation of G_{β} , which decreases its association with GRK2/3 and thus enhances the availability of $G_{\beta\gamma}$ for interaction with its varied effectors (33).

G protein mediation, pertussis toxin-insensitive (via the α subunit of G_q) or sensitive (via the $G_{\beta\gamma}$ subunit of G_i/G_o) is a prerequisite for receptor activation of PLC β isoforms, a property not observed for δ or γ types (19, 20). G_{α_s} , $G_{\beta\gamma}$, or both subunits bind to PLC β 1 and PLC β 3, increasing their catalytic activity. This mechanism of activation of the PLC β isoforms is of particular relevance to opioid neurochemical sequelae, as all known opioid effects are G protein mediated.

PLC β 1 and PLC β 3 are both negatively modulated by phosphorylation. PKC phosphorylates PLC β 1 (43), which inhibits its Ca^{2+} -dependent stimulation (44) as well as its activation via $G_{\beta\gamma}$ subunits (42). PKC can also phosphorylate, *in vivo*, PLC β 3 (45, 46), which inhibits activation by $G_{\alpha q}$ as well as $G_{\beta\gamma}$ (46). Inhibition of activation by $G_{\alpha q}$ by PKC is attributable to phosphorylation of Ser-1105, whereas inhibition of $G_{\beta\gamma}$ stimulated activity results from phosphorylation at another site (46). Thus, concomitant reciprocal phosphorylation of PLC β 1 and PLC β 3 by PKC, such as that which occurs after chronic systemic morphine, would be expected to alter the relative contribution of these isoforms to PLC signaling.

Exchange of one isoform for another with comparable regulatory and catalytic properties might not be expected to have notable physiological consequences. The chronic morphine-induced shift in the relative predominance of PLC β 1 vs. PLC β 3, however, should have significant opioid tolerance-associated signaling consequences because each manifests several unique regulatory characteristics. PLC β 1 and PLC β 3 are both activated by $G_{\alpha q}/11$ subunits (derived from G_q), which as expected, is insensitive to PTX. The concentration dependence of the activation, however, differs among these isoforms. Activation of PLC β 1 by $G_{\alpha q}/11$ subunits occurs over a broad concentration range, whereas its activation of PLC β 3 exhibits a relatively steep dependence on concentration (47). It is interesting to note that although all mammalian PLC β isoforms are G_q GTPase-activating proteins, GTPase-activating protein activity varies among isoforms. PLC β 1 increases GTPase activity about twice as much as PLC β 3 (G. Biddlecome and E. Ross, personal

communication). This underscores potential PLC β isoform-specific differences in signaling.

Efficacy of activation of PLC β 1 and PLC β 3 by $G_{\beta\gamma}$ subunits is also isoform-selective. $G_{\beta\gamma}$ is clearly more effective in stimulating PLC β 3 than PLC β 1 (ED_{50} for $G_{\beta\gamma}$ activation of PLC β 3 and PLC β 1 is 90 nM and >300 nM, respectively) (47). Additionally, concomitant regulation by $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits is also β -isoform subtype-selective. $G_{\alpha q}/11$ activation of PLC β 1 and PLC β 3 is reduced in the presence of $G_{\beta\gamma}$. However, whereas $G_{\alpha q}/11$ fails to activate the β 1 isoform in the presence of $G_{\beta\gamma}$, activation of PLC β 3 remains detectable. Moreover, the less than additive regulation of PLC β 3 by $G_{\alpha q}/11$ and $G_{\beta\gamma}$ becomes additive or even synergistic at higher Mg^{2+} concentrations (≈ 5 mM), whereas concomitant G protein subunit regulation of PLC β 1 remains less than additive at all Mg^{2+} concentrations (47). These observations indicate that PLC β 3, in contrast to PLC β 1, is able to integrate coincident signaling via pathways coupled to G_q and G_i/G_o .

These isoform-specific differences indicate that chronic morphine-induced diminution of the activity of PLC β 3 (via its increased state of phosphorylation) concomitant with augmented activity of PLC β 1 (via its diminished phosphorylation) results in the prevalence of PLC β signaling that is more muted and not able to act as a coincident signal detector. Chronic morphine shifts PLC β signaling from a high gain $G_{\alpha q}/G_{\beta\gamma}$ -regulated PLC isoform to one that is more graded in G protein subunit responsiveness. The sustained enhanced activity of PKC that has been associated with opioid tolerance might be more compatible with a new steady state in which there are less precipitous fluctuations in intracellular calcium content. Alternatively, or additionally, PLC β 1 and PLC β 3 isoforms may differ their associated assembly and organizational components that have been shown to be members of the PLC signaling family (48). These could include A-kinase anchoring protein, caveolin, and postsynaptic density disk-large ZO-1 proteins. These recognize specific motifs in various signaling components such as tyrosine and Ser/Thr protein kinases, phosphatases, ion channels, etc. Consequently, the temporal and spatial aspects of signaling as well as their relationship to disparate signaling pathways could differ between PLC β 1 and PLC β 3. These possibilities notwithstanding, the specific relevance of PLC isoform-specific adaptations to the formation of opioid tolerance requires further elucidation. The necessity of PLC activity for the tolerance development, albeit isoform-specific, is underscored by previous observations that i.c.v. application of ET-18-OCH $_3$, a selective inhibitor of PLC, reversed tolerance formation (14).

Neither the phosphorylation of PLC β 3 nor that of PLC β 1 was augmented or diminished, respectively, after acute morphine (5-min treatment), as was observed after its chronic administration. Thus, tolerant-associated changes in PLC β 3 and PLC β 1 phosphorylation represent adaptations unique to the chronic administration of morphine. Acute morphine did, however, diminish PLC β 3 phosphorylation. This is consistent with the established ability of opioids to stimulate PLC β (7–10, 12, 40, 49, 50) and suggests the relevance of dual mechanisms underlying the opioid activation, i.e., generation of $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits as well as acute negative modulation of PLC β 3 phosphorylation, which would augment its stimulatory responsiveness to them.

Interestingly, the opioid antagonist naloxone was also able to modulate PLC β 3 phosphorylation, but the direction was opposite to that of morphine, i.e., acute naloxone treatment augmented PLC β 3 phosphorylation. This indicates that negative modulation of PLC β 3 phosphorylation by exogenous opioids also occurs via their endogenous counterparts and that physiological levels of PLC β 3 phosphorylation are dynamically regulated, i.e., constrained, by tonic opioid activity.

Comparison of acute morphine responsiveness of PLC β 3-null mice with their wild-type counterpart has suggested that PLC β 3 constitutes an intrinsic negative modulatory pathway by which opioid responsiveness is constrained (13). This conclusion would seem to be the antithesis of the current demonstration that diminution of PLC β 3 activity, i.e., its increased phosphorylation (concomitant with enhanced activity of PLC β 1), is associated with tolerance formation. This apparent dichotomy underscores that divergent mechanisms subservise adaptations to acute vs. long-term exposure to opioids.

Chronic morphine also induces adenylyl cyclase isoform-specific changes in the LMMP tissue (36, 51, 52) and locus coeruleus (53). These observations, in combination with present findings of a reciprocal shift in PLC β isoform activity in response to chronic morphine, suggest that altered activity among isoforms of signaling proteins could have more generalized applicability to opioid tolerance-producing mechanism than has thus far been appreciated. Such considerations underscore that opioid tolerance is not simply the offset of acute, analgesic mechanisms but involves the emergence of new signaling strategies.

1. Childers, S. R. (1991) *Life Sci.* **48**, 1991–2003.
2. Sharma, S. K., Nirenberg, M. & Klee, W. A. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 590–594.
3. Armstrong, D. L. & White, R. E. (1992) *Trends Neurosci.* **15**, 403–408.
4. North, R. A., Williams, J. T., Surprenant, A. & Christie, M. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5487–5491.
5. Moises, H. C., Rusin, K. I. & Macdonald, R. L. (1994) *J. Neurosci.* **14**, 5903–5916.
6. Schroeder, J. E., Fischbach, P. S., Zheng, D. & McCleskey, E. W. (1991) *Neuron* **6**, 13–20.
7. Smart, D., Smith, G. & Lambert, D. G. (1994) *J. Neurochem.* **62**, 1009–1014.
8. Smart, D., Smith, G. & Lambert, D. G. (1995) *Biochem. J.* **305**, 577–581.
9. Jin, W., Lee, N. M., Loh, H. H. & Thayer, S. A. (1994) *J. Neurosci.* **14**, 1920–1929.
10. Miyamae, T., Fukushima, N., Misu, Y. & Ueda, H. (1993) *FEBS Lett.* **333**, 311–314.
11. Smart, D. & Lambert, D. G. (1996) *J. Neurochem.* **66**, 1462–1467.
12. Sanchez-Blazquez, P. & Garzon, J. (1998) *J. Pharmacol. Exp. Ther.* **285**, 820–827.
13. Xie, W., Samoriski, G. M., McLaughlin, J. P., Romoser, V. A., Smrcka, A., Hinkle, P. M., Bidlack, J. M., Gross, R. A., Jiang, H. & Wu, D. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 10385–10390.
14. Smith, F. L., Lohmann, A. B. & Dewey, W. L. (1999) *Br. J. Pharmacol.* **128**, 220–226.
15. Pellegrini-Giampietro, D. E., Ruggiero, M., Giannelli, S., Chiarugi, V. P. & Moroni, F. (1988) *Eur. J. Pharmacol.* **149**, 297–306.
16. Liu, N. J. & Gintzler, A. R. (2003) *Brain Res.* **959**, 103–110.
17. Berridge, M. J. & Irvine, R. F. (1989) *Nature* **341**, 197–205.
18. Rhee, S. G. (2001) *Annu. Rev. Biochem.* **70**, 281–312.
19. Fisher, S. K. (1995) *Eur. J. Pharmacol.* **288**, 231–250.
20. Rebecchi, M. J. & Pentyala, S. N. (2000) *Physiol. Rev.* **80**, 1291–1335.
21. Sharma, S. K., Klee, W. A. & Nirenberg, M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3092–3096.
22. Sharma, S. K., Klee, W. A. & Nirenberg, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3365–3369.
23. Kogan, J. H., Nestler, E. J. & Aghajanian, G. K. (1992) *Eur. J. Pharmacol.* **211**, 47–53.
24. Nestler, E. J. (1992) *J. Neurosci.* **12**, 2439–2450.
25. Duman, R. S., Tallman, J. F. & Nestler, E. J. (1988) *J. Pharmacol. Exp. Ther.* **246**, 1033–1039.
26. Guitart, X. & Nestler, E. J. (1989) *J. Neurosci.* **9**, 4371–4387.
27. Nestler, E. J. & Tallman, J. F. (1988) *Mol. Pharmacol.* **33**, 127–132.
28. Self, D. W., McClenahan, A. W., Beitner-Johnson, D., Terwilliger, R. Z. & Nestler, E. J. (1995) *Synapse* **21**, 312–318.
29. Mao, J., Price, D. D., Phillips, L. L., Lu, J. & Mayer, D. J. (1995) *Brain Res.* **677**, 257–267.
30. Mayer, D. J., Mao, J. & Price, D. D. (1995) *Pain* **61**, 365–374.
31. Mayer, D. J., Mao, J. & Price, D. D. (1995) *NIDA Res. Monogr.* **147**, 269–298.
32. Chakrabarti, S., Wang, L., Tang, W.-J. & Gintzler, A. R. (1998) *Mol. Pharmacol.* **54**, 949–953.
33. Chakrabarti, S., Oppermann, M. & Gintzler, A. R. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 4209–4214.
34. Wang, L. & Gintzler, A. R. (1997) *J. Neurochem.* **68**, 248–254.
35. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
36. Chakrabarti, S., Rivera, M., Yan, S.-Z., Tang, W.-J. & Gintzler, A. R. (1998) *Mol. Pharmacol.* **54**, 655–662.
37. Bahk, Y. Y., Lee, Y. H., Lee, T. G., Seo, J., Ryu, S. H. & Suh, P. G. (1994) *J. Biol. Chem.* **269**, 8240–8245.
38. Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K. & Uemura, D. (1989) *Biochem. Biophys. Res. Commun.* **159**, 871–877.
39. Enan, E. & Matsumura, F. (1992) *Biochem. Pharmacol.* **43**, 1777–1784.
40. Schulz, S. & Holtt, V. (1998) *Eur. J. Neurosci.* **10**, 1196–1201.
41. Wang, L., Medina, V. M., Rivera, M. & Gintzler, A. R. (1996) *Brain Res.* **723**, 61–69.
42. Litosch, I. (1997) *Biochem. J.* **326**, 701–707.
43. Ryu, S. H., Kim, U. H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K. P. & Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 17941–17945.
44. Litosch, I. (1996) *Recept. Signal Transduct.* **6**, 87–98.
45. Ali, H., Fisher, I., Haribabu, B., Richardson, R. M. & Snyderman, R. (1997) *J. Biol. Chem.* **272**, 11706–11709.
46. Yue, C., Ku, C. Y., Liu, M., Simon, M. I. & Sanborn, B. M. (2000) *J. Biol. Chem.* **275**, 30220–30225.
47. Smrcka, A. V. & Sternweis, P. C. (1993) *J. Biol. Chem.* **268**, 9667–9674.
48. Pawson, T. & Scott, J. D. (1997) *Science* **278**, 2075–2080.
49. Johnson, P. S., Wang, J. B., Wang, W. F. & Uhl, G. R. (1994) *NeuroReport* **5**, 507–509.
50. Yeo, A., Samways, D. S., Fowler, C. E., Gunn-Moore, F. & Henderson, G. (2001) *J. Neurochem.* **76**, 1688–1700.
51. Rivera, M. & Gintzler, A. R. (1998) *Mol. Brain Res.* **54**, 165–169.
52. Gintzler, A. R. & Chakrabarti, S. (2000) *Mol. Neurobiol.* **21**, 21–33.
53. Lane-Ladd, S. B., Pineda, J., Boundy, V. A., Pfeuffer, T., Krupinski, J., Aghajanian, G. K. & Nestler, E. J. (1997) *J. Neurosci.* **17**, 7890–7901.