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Effects of Anesthetic Agents on Focal Adhesion Kinase (pp^{125}FAK) Tyrosine Phosphorylation in Rat Hippocampal Slices

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Background: **Tyrosine protein kinase proteins exert a prominent control on signaling pathways and may couple rapid events, such as action potential and neurotransmitter release, to long-lasting changes in synaptic strength and survival. Whether anesthetics modulate tyrosine kinase activity remains unknown. The aim of the current study was therefore to examine the effects of intravenous and volatile anesthetics on the phosphorylation of focal adhesion kinase (pp^{125}FAK), a functionally important nonreceptor tyrosine kinase, in the rat hippocampus.**

Methods: **Phosphorylation of pp^{125}FAK was examined in hippocampal slices by immunoblotting with both antiphosphotyrosine and specific anti-pp^{125}FAK antibodies. Experiments were performed in the absence (control) or presence of various concentrations of pharmacologic or anesthetic agents or both.**

Results: **Clinically relevant concentrations of thiopental, propofol, etomidate, isoflurane, sevoflurane, and desflurane induced a concentration-related increase in tyrosine phosphorylation. In contrast, ketamine (up to 100 μM) and the nonimmobilizer F6 (1,2-dichlorohexafluorocyclobutane, 25 μM) did not significantly affect pp^{125}FAK phosphorylation. The anesthetic-induced increase in pp^{125}FAK phosphorylation was blocked by GF 109203X, RO 318220, and chelerythrin (100 μM), three structurally distinct inhibitors of protein kinase C and U 73122 (50 μM), an inhibitor of phospholipase C. The propofol- and isoflurane-induced increase in pp^{125}FAK phosphorylation was reversible and showed nonadditivity of effects with phorbol 12-myristate 13-acetate (an activator of protein kinase C, 0.1 μM). In contrast, ketamine (up to 100 μM), MK801 (10 μM, an *N*-methyl-D-aspartate receptor antagonist), bicuculline (10 μM, a γ-aminobutyric acid type A receptor antagonist), and dantrolene (30 μM, an inhibitor of the ryanodine receptor) were ineffective in blocking anesthetic-induced activation of tyrosine phosphorylation.**

Conclusion: **Except for ketamine, anesthetic agents markedly increase tyrosine phosphorylation of pp^{125}FAK in the rat hippocampus, most likely *via* the phospholipase C–protein kinase C pathway, whereas the nonimmobilizer F6 does not. These results suggest that pp^{125}FAK represents a target for anesthetic action in the brain.**

PHOSPHORYLATION of proteins is the most common reversible posttranslational modification regulating their properties by numerous extracellular signals. Protein tyrosine phosphorylation was originally identified as a major step in the cellular actions of growth factors and oncogenes, but evidence has now been provided to support its role in the regulation of synaptic proteins, such as ion channels of excitable membranes. Nicotinic acetylcholine receptor desensitization and *N*-methyl-D-aspartate (NMDA) receptor and cation channel permeability have been found regulated by tyrosine phosphorylation.[1–3] A possible role of tyrosine phosphorylation in synaptic plasticity is suggested by the impairment of long-term potentiation in the hippocampus observed after administration of tyrosine kinase inhibitors or in mutant mice lacking a functional gene for the tyrosine kinase pp^{59}fyn.[4,5]

Focal adhesion kinase (pp^{125}FAK) is a particularly important 125-kd nonreceptor tyrosine kinase, which may couple rapid events, such as action potential or neurotransmitter release, to long-lasting changes in synaptic strength, cell motility, or adhesion-dependent survival.[6] It also seems to play critical roles in the signal transduction pathways that regulate cell activation, proliferation, and differentiation. Activation of the major specific neuronal isoform of pp^{125}FAK (named FAK^{+}6,7) is achieved by various extracellular signals, including stimulation of NMDA or acetylcholine nicotinic receptors, extracellular messengers (lysophosphatidic acid, arachidonic acid, or, to a lesser extent, depolarization).[7–9] Interestingly, these stimuli utilize a restricted number of intracellular pathways, and the phospholipid-dependent protein kinase C (PKC) family represents a major intracellular step for stimulating pp^{125}FAK phosphorylation[6,10] (fig. 1). pp^{125}FAK phosphorylation is increased in response to phorbol esters and inhibited by PKC antagonists.[6]

A large body of recent work suggests that production of the anesthetic state results from interactions between anesthetic molecules and a restricted number of ligand- or voltage-gated ionic channels located on presynaptic or postsynaptic nerve membranes.[11–15] However, it has been established that anesthetics may also act beyond the receptor level by modulating the activity of key intracellular enzymes of signal transduction, such as PKC.[16] PKC is activated by volatile anesthetics in various preparations, possibly *via* the regulatory domain of the enzyme at the diacylglycerol binding sites.[17] In synaptosomes, halothane and propofol activate both purified and endogenous PKC and potentiate activation-depen-

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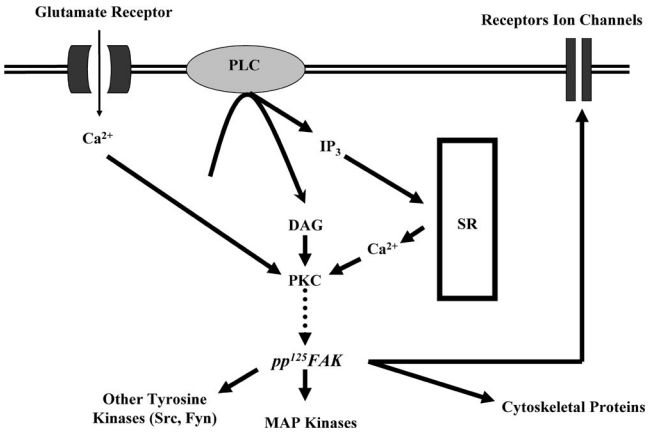

Fig. 1. Schematic representation of the putative pathways responsible for the increase in focal adhesion kinase (pp^{125}FAK) phosphorylation in the rat hippocampus. DAG = diacylglycerol; IP_3 = inositol triphosphate; MAP = mitogen-activated protein; PKC = protein kinase C; PLC = phospholipase C; SR = sarcoplasmic reticulum.

dent down-regulation for the enzyme.[17,18] It can therefore be hypothesized that anesthetics indirectly affect tyrosine phosphorylation *via* an action at the PKC level. The aim of the current study was to examine the effects of anesthetic agents on pp^{125}FAK phosphorylation in the rat hippocampus.

Materials and Methods

Handling procedures according to the *Guide for the Care and Use of Laboratory Animals* were followed throughout.[19] Experiments were performed in male Sprague-Dawley rats (Iffa-Credo, L'Arbresle, France) weighing 250 g and housed on a 12:12 light:dark cycle with food and water *ad libitum*. Approval was obtained from the Institutional Animal Care and Use Committee at Paris 7 University (Paris, France).

Preparation of Hippocampal Slices and Homogenates

Animals were killed by stunning (thoracic shock followed by reflex cardiac arrest) and decapitation. Brains were quickly removed, and hemispheres were separated until the corpus callosum was observed. The hippocampus of each hemisphere was carefully dissected and incubated in Ca^{2+}-free artificial cerebrospinal fluid (4°C, 126.5 mM NaCl, 27.5 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.93 mM $MgCl_2$, 0.5 mM Na_2SO_4, 10 mM glucose, and 11 mM HEPES adjusted to pH 7.4 with 95%/5% [vol/vol] oxygen–carbon dioxide mixture as previously described[7,9,20]). Hippocampal slices (each 300 μm thick) prepared with a MacIlwain tissue chopper were transferred to polypropylene tubes (three slices per tube) containing 1 ml artificial cerebrospinal fluid (60 min, 37°C). To avoid tyrosine kinase activation at this step of the experiment, Ca^{2+} was omitted from the medium from the dissection phase until the end of incubation. Slices were incubated for 60 min at 37°C with moderate agitation under a humidified atmosphere of 95%/5% [vol/vol] oxygen–carbon dioxide until pharmacologic treatments were added together with $CaCl_2$. Tetrodotoxin (1 μM) was added at the beginning of slice incubation to avoid indirect effects due to neuronal firing. In experiments with the NMDA or ketamine challenge, $MgCl_2$ was removed from the medium and replaced with $CaCl_2$ (final concentration, 1.93 mM). At the end of the experiments, cerebrospinal fluid was aspirated, and slices were frozen in liquid nitrogen and then homogenized by sonication in 200 μl of a solution of 1% (wt/vol) sodium dodecyl sulfate, 1 mM sodium orthovanadate, and antiproteases (50 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 μg/ml pepstatin) in water at 100°C and placed in a boiling bath for 5 min. Homogenates were stored at −80°C until processing.

Chemicals and Anesthetics

The effects of the following pharmacologic and anesthetic agents on pp^{125}FAK phosphorylation were studied alone or in combination: thiopental (1 nM–100 μM; Specia Rhône-Poulenc Rorer, Paris, France), propofol (1 nM–00 μM; AstraZeneca, Rueil, France), etomidate (1 nM–100 μM; Janssen Pharmaceuticals, Boulogne, France), ketamine (1 nM–100 μM; Sigma, St-Quentin Fallavier, France), isoflurane (10 nM–1 mM; Abbott, Rungis, France), sevoflurane (10 nM–1 mM; Abbott), desflurane (10 nM–1 mM; Baxter, Maurepas, France), F6 (1,2-dichlorohexafluorocyclobutane, 25 μM; Interchim, Montluçon, France), NMDA (1 mM; Sigma), tetrodotoxin (1 μM; Sigma), MK801 (10 μM; Sigma), bicuculline (10 μM; Sigma), inhibitors of PKC (100 μM): bisindolylmaleimide IX (GF 109203X; Sigma), chelerythrin and bisindolylmaleimide I (RO 318220; Merck Biosciences, Fontenay-sous-Bois, France), phorbol 12-myristate 13-acetate (PMA, an activator of PKC, 0.1 μM; Sigma), U 73122 (50 μM, an inhibitor of phospholipase C [PLC]; Sigma), dantrolene (an inhibitor of the ryanodine receptor, 30 μM; Sigma). Propofol, etomidate, and volatile anesthetics were dissolved in dimethyl sulfoxide (1/100 or less; Merck, Sharp and Dohme, Darmstadt, Germany). Desflurane solutions were prepared at 4°C. Anesthetics, PMA, and NMDA were applied for 5 min, whereas MK801, bicuculline, dantrolene, GF 109203X, RO 318220, chelerythrin, and U 73122 were preincubated for 1 h before adding any other pharmacologic or anesthetic treatment. Because diffusion into brain tissue is particularly slow for lipophilic compounds such as nonimmobilizers in comparison with volatile anesthetics, a 40-min period of application was selected for F6 application to the slices.[21]

Concentrations of Volatile Anesthetics and F6 in the Superfusion Chambers

Final solutions of volatile anesthetics were prepared in an obturated Sarsted tube (volume, 1.5 ml; diameter, 8 mm) and obtained by fresh buffer successive dilution of a primary 0.1 M solution (100-μl samples) in dimethyl sulfoxide (1/100 or less final dilution) using Teflon-sealed glass containers. Aqueous concentrations of volatile anesthetics in the incubation chambers were checked by gas phase chromatography according to a slightly modified version of the method of Brachet-Liermain *et al.*, as previously reported and routinely used in our laboratory.[22,23] Great attention was paid to minimize gas vaporization by leaving the preparations containing volatile agents covered throughout the experiments, the Teflon caps being removed immediately before starting the applications. Samples to be processed for injection into the chromatograph were withdrawn from the superfusion chambers using a gas-tight glass syringe (Hamilton Co., Reno, NV) equipped with a Teflon stopcock (Hamilton Co.).

Concentrations of F6 in the saturated solutions and the samples obtained from the chambers were also measured by gas chromatography. Saturated F6 solutions were freshly prepared by adding 100 μl F6 to 30 ml buffer in an airtight container. Solutions were incubated overnight at room temperature with mixing. The concentration of the F6 saturated solution was 260 $\pm$ 30 μM (mean $\pm$ SD). Saturated solutions were diluted immediately before use to the designed concentration (25 $\pm$ 2.7 μM) corresponding to approximately 1.5 times the predicted minimum alveolar concentration (MAC).[21] Loss of F6 between concentrations measured in the stock solution and the incubation chambers was 28%.

Immunoblot Analysis

Protein concentrations in the homogenates were determined with a bicinchoninic acid–based method, using bovine serum albumin as the standard. Equal amounts of protein (30 μg) were subjected to 6% (wt/vol) polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and transferred electrophoretically to nitrocellulose. Immunoblot analysis was performed with affinity-purified rabbit antiphosphotyrosine antibodies SL2. Primary antibodies were labeled with peroxidase-coupled antibodies against rabbit immunoglobulin G, which were detected by exposure of autoradiographic films in the presence of a chemiluminescent reagent (ECL; Amersham, Little Chalfont, United Kingdom). The specificity of the immunoreactivity was assessed by its competition in the presence of 50 μM O-phosphotyrosine. For each anesthetic tested, identification of phosphorylated pp^{125}FAK was performed with a rabbit anti-Y397 FAK phosphospecific antibody (Biosource International, Camarillo, CA; diluted 1:1,000) after pooling five to eight independent samples. Immunoreactive bands were quantified using a computer-assisted densitometer and expressed as a phosphotyrosine (pp^{125}FAK, respectively) to β actin (quantified by using the specific monoclonal antiactin A5316 antibody [Sigma]) ratio (Cohu High Performance CCD camera, Gel Analyst 3.01 pci; Paris, France).

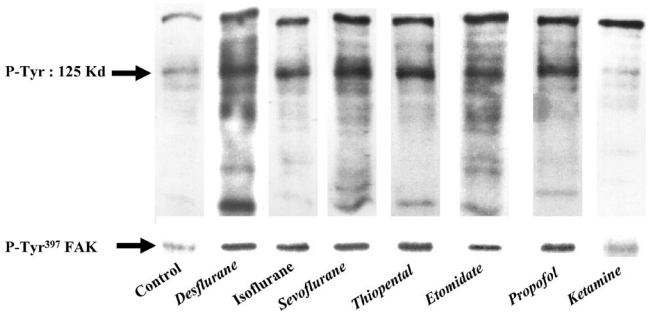

Fig. 2. Typical example of a Western blot showing the effects of anesthetics on total tyrosine phosphorylation (*upper panel*; *left row* = control [100%]) and specific focal adhesion kinase (pp^{125}FAK) phosphorylation (*lower panel*) in rat hippocampal slices. The concentrations of anesthetics used in this experiment (corresponding fractional increases from basal phosphorylation, respectively) were 0.3 mM (270%) for desflurane, isoflurane (220%), and sevoflurane (295%), 5 μM (270%) for thiopental, for etomidate (235%) and for propofol (208%). Ketamine concentration was 10^{-4} M.

Statistical Analysis

Normality of distributions was first assessed by the Fisher exact test for equality of variances. Statistical differences between control and experimental values were determined by analysis of variance with the Scheffé *post hoc* correction for multiple comparisons. A P value less than 0.05 was considered the threshold for significance. Results (mean $\pm$ SD) are expressed as a percentage of control tyrosine (pp^{125}FAK, respectively) phosphorylation.

Results

Tyrosine phosphorylation kinetics induced by propofol and isoflurane in the presence of phosphatase inhibitors linearly increased with time between 1 and 5 min until reaching a ceiling, which was maintained until at least 30 min of incubation. Therefore, unless specified, a 5-min duration of exposure to the stimulating agents was selected in the current experiments.

Figure 2 shows a typical example of the effects of clinically relevant concentrations of anesthetics on tyrosine and pp^{125}FAK phosphorylation. Anesthetics used at these concentrations also markedly increased the density of the immunoreactive band labeled by Y-397 phosphospecific antiphosphorylated pp^{125}FAK antibodies. The increases from basal phosphorylation were 270 $\pm$ 41% ($P < 0.001$), 220 $\pm$ 34% ($P < 0.001$), and 295 $\pm$ 48% ($P < 0.001$) for desflurane, isoflurane, and sevoflu-

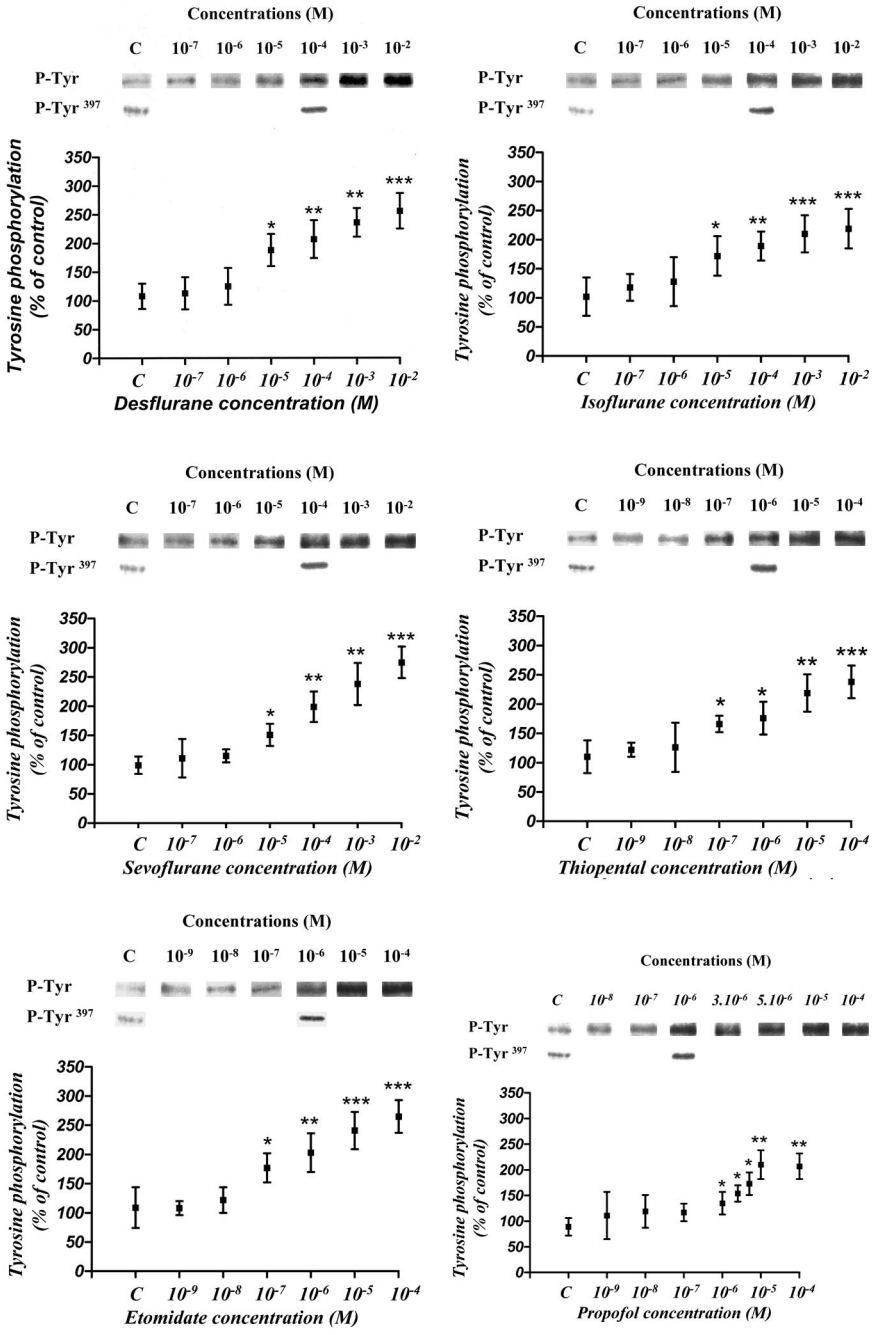

Fig. 3. Concentration–response curves of the effects of anesthetics on tyrosine phosphorylation in rat hippocampal slices. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). Western blots show the effects of anesthetic concentrations on total tyrosine phosphorylation (*upper panels*) and on specific focal adhesion kinase (pp^{125}FAK) phosphorylation (*lower panels*). ** $P < 0.05$ *versus* control. ** $P < 0.01$ *versus* control. *** $P < 0.001$ *versus* control.

rane used at 0.3 mM, respectively, and 270 ± 42% ($P < 0.001$), 235 ± 48% ($P < 0.001$), and 208 ± 36% ($P < 0.001$) for thiopental, etomidate, and propofol used at 5 μM, respectively. Ketamine up to 10^{-4} M had no effect on tyrosine phosphorylation. Except for ketamine, all anesthetics produced a concentration-related increase in phosphotyrosine immunoreactivity of the 125- kd protein band and pp^{125}FAK phosphorylation (fig. 3).

Consistent with previous findings,[9,20] NMDA (1 mM) application also resulted in a significant increase in tyrosine phosphorylation of the 125-kd band (177 ± 12%), whereas KCl (40 mM) produced a weak, nonsignificant increase from control response (fig. 4). The effect of NMDA was blocked by MK801 application (10 μM). Carbachol, an activator of PLC (100 μM), induced a marked increase in tyrosine phosphorylation of the 125-kd band (212 ± 31%), which is in agreement with previous reports.[7] This effect was significantly attenuated by the PLC inhibitor U 73122 (50 μM) (fig. 4). PMA (0.1 μM) induced a significant increase in phosphorylation of the 125-kd band (170 ± 14%), which was completely blocked by GF 109203X, chelerythrin, and RO 318220 (100 μM). Omitting tetrodotoxin from the incubation medium did not significantly change tyrosine phosphorylation (fig. 5). Dimethyl sulfoxide, MK801, dantrolene, chelerythrin, RO 318220, U 73122, and ket-

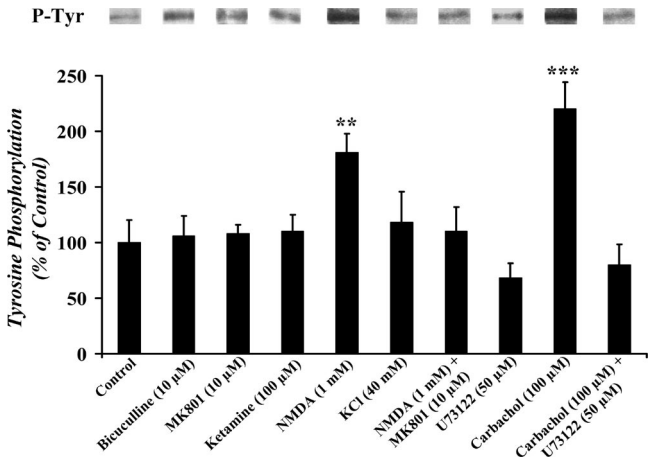

Fig. 4. Characterization of tyrosine phosphorylation induced by various pharmacologic agents in rat hippocampal slices. The following concentrations were used: 40 mM KCl, 1 mM NMDA in the absence of magnesium, 100 μM carbachol, 10 μM bicuculline, 30 μM U 73122, 10 μM MK801, 100 μM ketamine. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). Western blots show the effects of pharmacologic agents on total tyrosine phosphorylation. * $P < 0.05$ *versus* control. # $P < 0.01$ *versus* control.

amine (up to 10^{-4} M) did not significantly affect tyrosine phosphorylation of the 125-kd band *per se*, whereas GF 109203X used at the 10^{-4} M concentration significantly attenuated basal tyrosine phosphorylation (figs. 4–7).

The increase in tyrosine phosphorylation of the 125-kd band induced by anesthetics was affected neither by MK801 (10 μM) nor by bicuculline (10 μM; data not shown). In contrast, it was completely blocked by the PKC inhibitors GF 109203X, RO 318220, and chelerythrin (100 μM; fig. 6) and the PLC inhibitor U 73122 (30 μM; fig. 7). However, blockade of propofol-induced tyrosine phosphorylation by U 73122 was only partial (fig. 7). In contrast, dantrolene (100 μM) was ineffective (fig. 7). Anesthetic-induced increase in specific pp^{125}FAK phosphorylation was blocked by RO 318220, chelerythrin, GF 109203X (100 μM; fig. 6), and U 73122 (30 μM; fig. 7). The combination of PMA (0.1 μM) and either propofol (5 μM) or isoflurane (0.3 mM) showed nonadditivity of effects on tyrosine phosphorylation (fig. 8).

The effect of a 25-μM concentration of the nonimmobilizer F6 was compared to that of a 0.2-mM concentration of isoflurane. F6 used at approximately a 1-MAC predicted concentration[21] did not significantly affect tyrosine or pp^{125}FAK phosphorylation (109 ± 41% of control; not significant), whereas the effect of isoflurane was highly significant (191 ± 22% of control; $P < 0.01$).

The reversibility of the effects of one intravenous agent (propofol) and one volatile anesthetic (isoflurane) was examined by comparing the time course (7, 9, 12, 15, and 30 min) of tyrosine phosphorylation in the continuous presence of 5 μM propofol (or 0.2 mM isoflurane) and after washout of these anesthetics after 5 min of incubation. Maintenance of the anesthetic concentration in the medium resulted in an increase in tyrosine phosphorylation, which was not significantly different from that obtained at the fifth minute of incubation. In contrast, removal of the anesthetic at 5 min resulted in a significant reduction in tyrosine phosphorylation at all times tested between 7 and 30 min (fig. 9).

Discussion

In the current study, we have shown that clinically relevant concentrations of both intravenous (except for ketamine) and volatile anesthetics increase phosphorylation of pp^{125}FAK tyrosine kinase in rat hippocampal slices. These effects are likely to be mediated indirectly *via* activation of the PLC–PKC pathway. In contrast, the nonimmobilizer F6 had no significant effect on tyrosine phosphorylation. These findings support that phosphorylation of pp^{125}FAK represents a target for anesthetic action in the central nervous system.

In the current experimental protocol, activation of phosphatases after homogenization was prevented by orthovanadate, an inhibitor of phosphatases, which is necessary to block the dephosphorylation process.[7–9,20,24] Also, tetrodotoxin was used to prevent activation of tyrosine phosphorylation by depolarization. However, we found no significant difference in tyrosine phosphorylation, regardless of whether tetrodotoxin was present. Also, whole hippocampal slices were used in our experimental protocol. This precluded observing regional differences in the effects of anesthetics on pp^{125}FAK phosphorylation. However, because pp^{125}FAK is highly expressed in all hippocampal areas, including CA1, CA3,

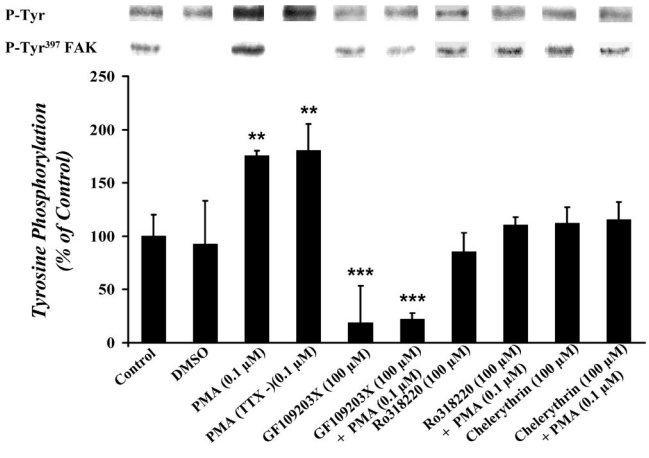

Fig. 5. Effects of protein kinase C inhibitors on phorbol 12-myristate 13-acetate (PMA, 0.1 μM)-induced tyrosine phosphorylation in rat hippocampal slices. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). Western blots show the effects of pharmacologic agents on total tyrosine phosphorylation (*upper panels*) and on specific focal adhesion kinase (pp^{125}FAK) phosphorylation (*lower panels*). Dimethyl sulfoxide (DMSO) was tested at a 1:100 dilution. TTX = tetrodotoxin. * $P < 0.05$ *versus* control. ** $P < 0.01$ *versus* control.

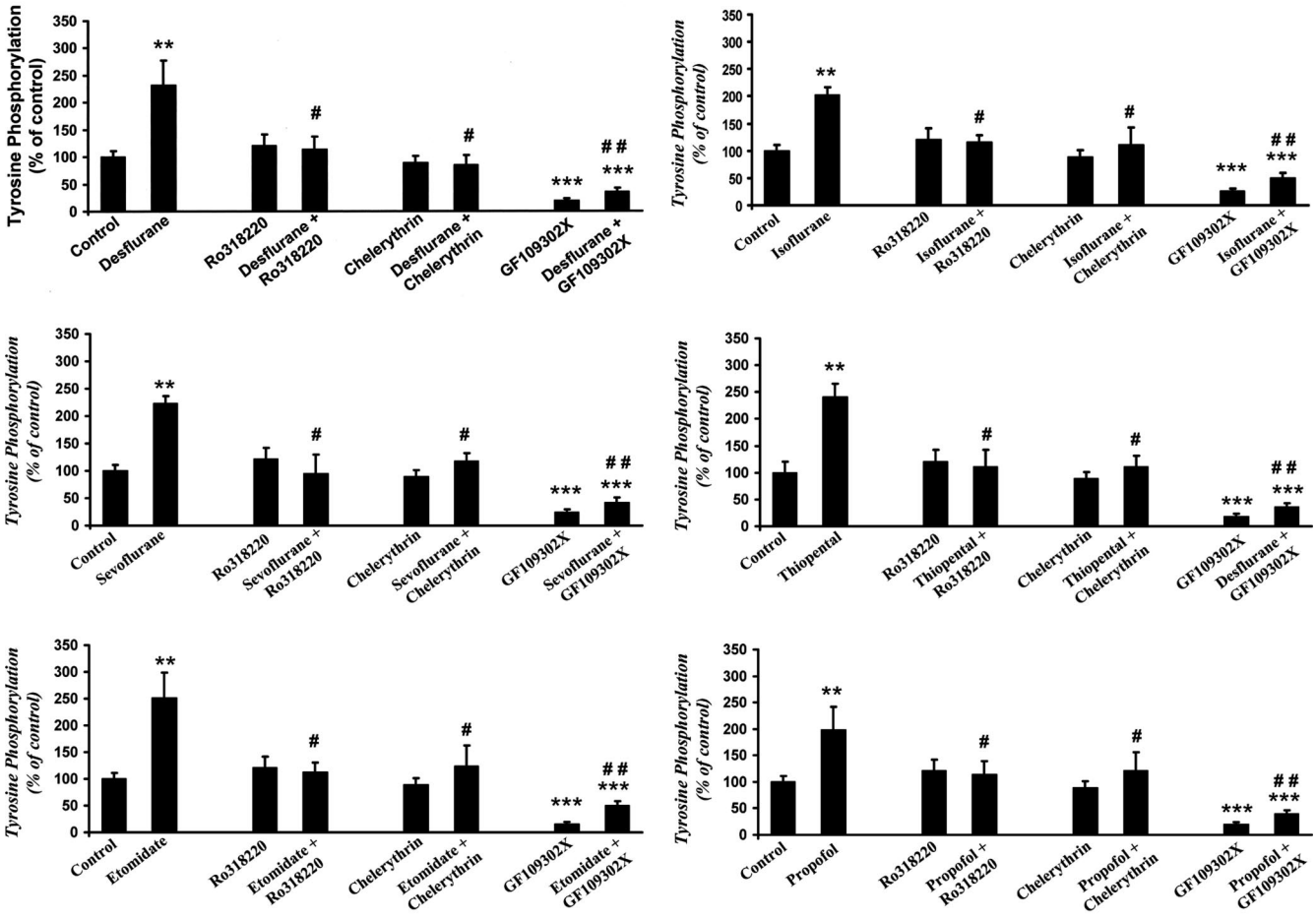

Fig. 6. Effects of protein kinase C inhibitors on anesthetic-induced increase in tyrosine phosphorylation in rat hippocampal slices. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). The anesthetics and pharmacologic agents were used at the following concentrations: desflurane, sevoflurane, and isoflurane: 0.3 mM; propofol, etomidate, and thiopental: 5 μM; GF 109203X, RO 318220, and chelerythrin: 100 μM. ** $P < 0.01$ and *** $P < 0.001$ *versus* control. # $P < 0.05$ and ## $P < 0.01$ *versus* anesthetic-induced tyrosine phosphorylation.

and the dentate gyrus, it can be speculated that a major regional dependence of anesthetic actions on tyrosine phosphorylation is unlikely.[24] Because comigrating bands could theoretically interfere with quantification of pp^{125}FAK within the 125-kd band identified with the nonspecific phosphotyrosine antibody, a crucial methodologic point was to ensure that the 125-kd band identified by the nonspecific antiphosphotyrosine antibody corresponded to pp^{125}FAK. For this purpose, we could have immunoprecipitated with specific anti-pp^{125}FAK antibody before immunoblotting for phosphotyrosine, as was performed in previous studies originating from part of the same group,[7,9,20,24] and demonstrated the comigration of the phosphotyrosine containing band with pp^{125}FAK by Western blotting of a duplicate blot. We chose an alternative approach, consisting of using the anti-Y397 FAK phosphospecific antibody to quantify pp^{125}FAK phosphorylation specifically induced by anesthetics and pharmacologic agents. The specificity of this antibody for the phosphorylated form of pp^{125}FAK has been shown previously.[8] The parallelism in phosphorylation intensity of the phosphotyrosine 125-kd band and pp^{125}FAK observed at clinically relevant concentrations of anesthetics supports that the 125-kd band phosphorylated by anesthetics on the phosphotyrosine immunoblotting indeed corresponds to pp^{125}FAK.

Interestingly, we found that the nonimmobilizer F6 had no effect on pp^{125}FAK phosphorylation. Nonimmobilizers are volatile compounds with lipophilicities that suggest that they should (but do not) prevent motor responses to surgical stimuli.[25] Therefore, the lack of F6 effect together with the marked increase induced by comparable MAC-equivalent volatile anesthetic concentrations supports that pp^{125}FAK phosphorylation represents a target for anesthetics in the central nervous system. However, in consideration of the highly nonpolar nature of F6, diffusion in the hippocampal slices is particularly slow, also depending on the slice thickness and the duration of incubation.[21] Based on the study of Chesney *et al.*[21] performed on 500-μm-thick slices, it can be anticipated that the 25 μM F6 concentration applied during the same period of time (40 min) to 300-μm-thick

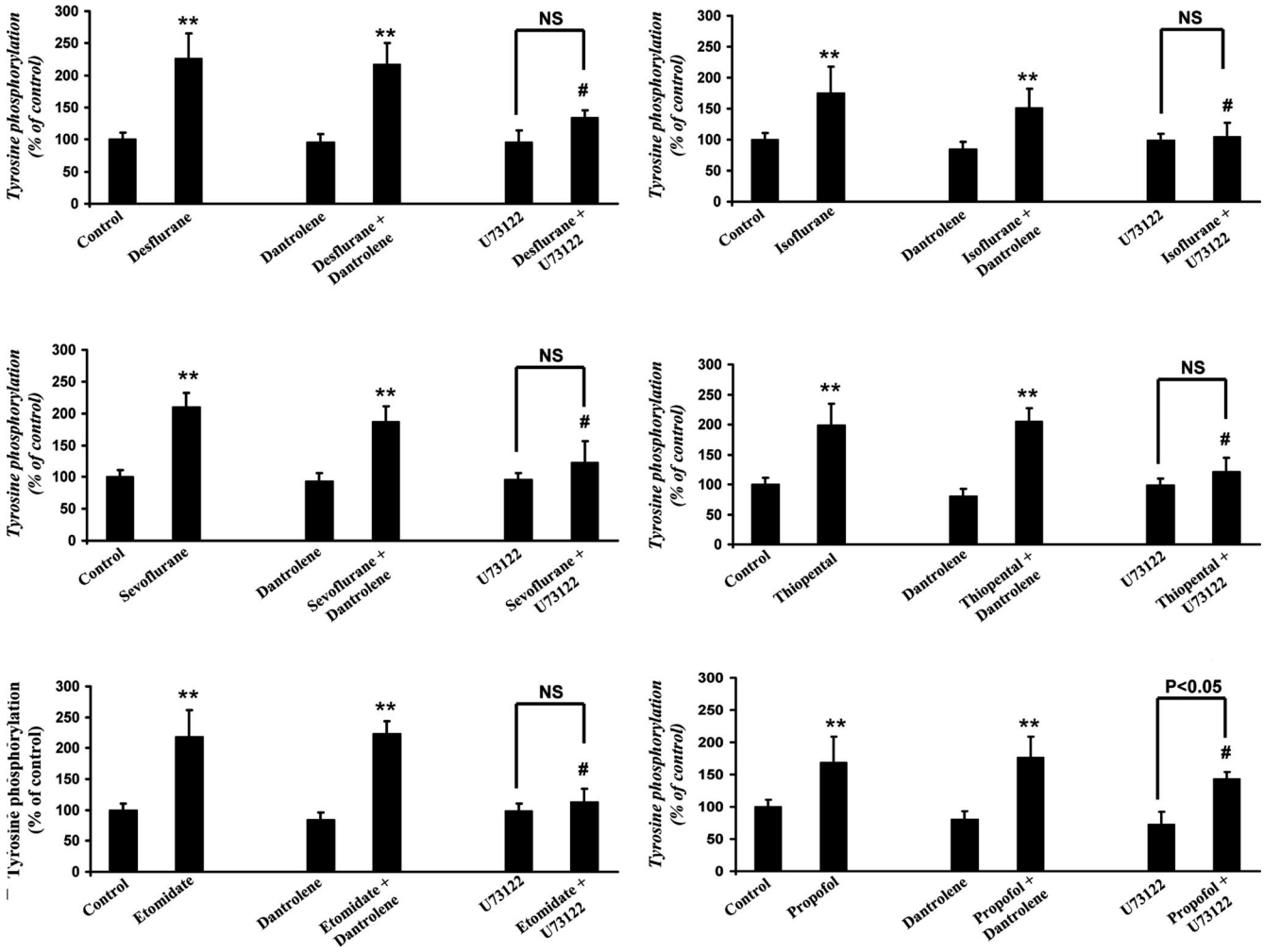

Fig. 7. Effects of dantrolene (50 μM) and U 73122 (30 μM) on anesthetic-induced tyrosine phosphorylation in rat hippocampal slices. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). The anesthetic and pharmacologic agents were used at the following concentrations: desflurane, sevoflurane, and isoflurane: 0.3 mM; propofol, etomidate, and thiopental: 5 μM. * *P* < 0.01 and ** *P* < 0.01 *versus* control. # *P* < 0.05 *versus* anesthetic-induced tyrosine phosphorylation. NS = not significant.

slices resulted in tissue concentrations at least equal, or more likely greater than, 14 μM aqueous (0.9 × predicted MAC). Therefore, the lack of F6 effect in our preparation is unlikely to be due to insufficient relevant tissue site concentrations. In the current study, the effects of both intravenous and volatile anesthetics were observed at particularly low, clinically relevant concentrations.[26] We also observed reversibility of anesthetic effects on tyrosine phosphorylation, at least for one intravenous agent (propofol) and one volatile anesthetic (isoflurane). Although direct extrapolation to all anesthetics cannot be made, the lack of desensitization of the effects over time and the reversibility of propofol- and isoflurane-induced tyrosine phosphorylation further support relevance of the current findings to anesthesia.

Because all anesthetics except ketamine increase the efficacy of the γ-aminobutyric acid type A ($GABA_A$) receptor–coupled chloride channel, it may be suggested that $GABA_A$ receptors are involved in tyrosine kinase activation by anesthetics. We found that bicuculline was ineffective in blocking the increase in phosphorylation elicited by anesthetics agents. Therefore, the involvement of $GABA_A$ receptors in this effect seems unlikely. Although the molecular basis for stimulation of tyrosine phosphorylation in response to Ca^{2+} is not known, anesthetics may have acted by stimulation of receptor-coupled ionic channels leading to an increase in $[Ca^{2+}]_i$ concentrations (intracellular calcium concentrations) or released $[Ca^{2+}]_i$ from endogenous stores. The lack of effect of the NMDA receptor antagonist MK801 in anesthetic-induced pp^{125}FAK phosphorylation support that NMDA receptor activation was not involved in these effects. This does not seem surprising because of the absence of stimulating properties of ionotropic glutamate receptors by anesthetic agents. The lack of efficacy of ketamine at concentrations up to 10^{-4} M is also consistent with these findings. Alternatively, anesthetics may have increased $[Ca^{2+}]_i$ concentrations by stimulat-

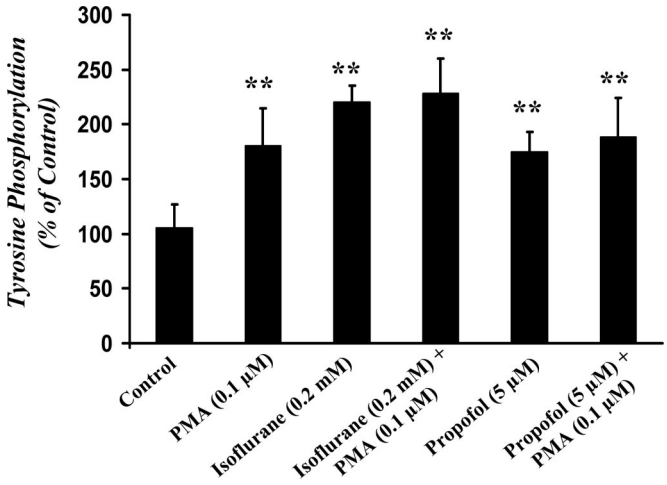

Fig. 8. Nonadditivity of effects of phorbol 12-myristate 13-acetate (PMA) with propofol or isoflurane on tyrosine phosphorylation in rat hippocampal slices. Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). The anesthetics and pharmacologic agents were used at the following concentrations: 0.1 μM PMA, 5 μM propofol, 0.2 mM isoflurane. $P < 0.01$ and ** $P < 0.01$ *versus* control.

ing Ca^{2+} release from intracellular stores *via* activation of inositol triphosphate receptors. Although indirect evidence suggests that this phenomenon may occur in neurons, elegant experiments performed in cultured rat hippocampal neurons in which $[Ca^{2+}]_i$ concentrations were directly measured by microfluorometry do not support this hypothesis.[27] The lack of effect of dantrolene, a blocker of the ryanodine receptor, is consistent with these findings. The lack of effect of KCl (40 mM) suggests that the increase in pp^{125}FAK phosphorylation is not due to an increase in $[Ca^{2+}]_i$ alone. Another possible pathway to activate tyrosine phosphorylation could be the decrease in cyclic adenosine monophosphate–dependent protein kinase A activity. For example, anandamide, an endogenous ligand of the cannabinoid receptors, has been reported to increase pp^{125}FAK phosphorylation *via* this mechanism.[8] So far, except for α_2-adrenoceptor agonists, anesthetics have not been reported to decrease adenylyl cyclase activity.

Our data support stimulation of the PLC–PKC pathway as the best candidate to account for the activation by anesthetics of pp^{125}FAK phosphorylation. In nonneuronal cells, tyrosine phosphorylation of pp^{125}FAK occurs in two steps[6]: First, Tyr-397 is autophosphorylated, allowing the high-affinity binding of the SH2 domain of the tyrosine kinases Src or Fyn. Second, these kinases phosphorylate tyrosine residues in the catalytic and carboxy-terminal domains of pp^{125}FAK. However, other steps, such as phosphorylation by serine/threonine kinases, could also be involved in pp^{125}FAK activation. PKC stimulation has been shown to potently activate pp^{125}FAK phosphorylation in the hippocampus.[6] Our results indicate that the effects of propofol and isoflurane on tyrosine phosphorylation were nonadditive with those of the PKC activator PMA. Also, the effects of all anesthetics on pp^{125}FAK phosphorylation were completely blocked by at least three PKC inhibitors (two bisindolylmaleimide, GF 109203X and RO 318220, and another structurally distinct one, chelerythrin). In contrast to RO 318220 and chelerythrin (100 μM), GF 109203X (100 μM) also inhibited the control response. This may suggest that pp^{125}FAK phosphorylation is more sensitive to inhibition of the α PKC subunit, which is more affected by GF 109203X than the other PKC inhibitors used in the current study.[10] Alternatively, PKC inhibitors lack specificity, and it cannot be excluded that GF 109203X might affect other protein kinases contributing to the control phosphorylation response.[28] Therefore, it can be speculated that activated PKC stimulates pp^{125}FAK phosphor-

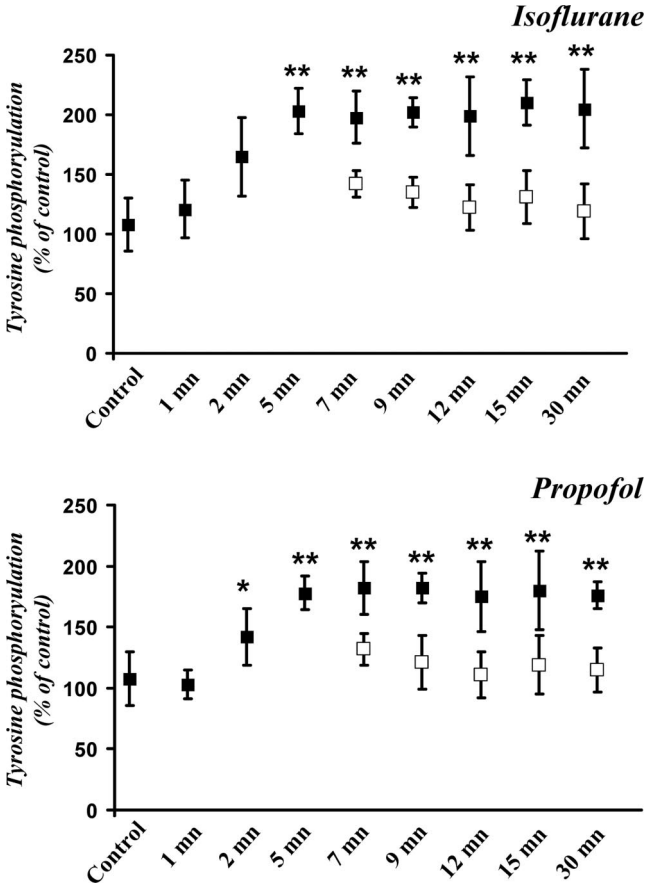

Fig. 9. Reversibility of isoflurane (0.2 mM; *upper panel*)- and propofol (5 μM; *lower panel*)-induced tyrosine phosphorylation in rat hippocampal slices. The phosphatase inhibitor orthovanadate was present in the preparation during the whole experiments. When anesthetic concentrations were maintained in the preparation throughout the experiment, no decrease in tyrosine phosphorylation was observed between the 5' and the 30' minute for both anesthetics (*filled squares*). In contrast, washout of the anesthetics at 5' resulted in a rapid and persistent decrease in tyrosine phosphorylation (*open squares*). Data (mean ± SD) are expressed as a fractional increase from basal phosphorylation (control = 100%). * $P < 0.05$ and ** $P < 0.01$ *versus* control.

ylation at serine/threonine residues, which subsequently triggers tyrosine autophosphorylation.

The complete blockade by the PLC inhibitor U 73122 of tyrosine phosphorylation induced by all anesthetics (except propofol) indicates that PLC stimulation plays a prominent role in anesthetic-induced PKC. Consistent with this hypothesis, PLC stimulation by various anesthetics has been observed in some tissues.[29,30] PLC activation may be elicited by stimulation of G protein–coupled receptors, such as the M1 muscarinic receptors, the glutamate metabotropic receptors, or the α_1 adrenoceptors. It may be elicited by stimulation of G protein-coupled receptors, such as the M1 muscarinic receptors, the glutamate metabotropic receptors, or the α_1 adrenoceptors. Consistent with the current findings, carbachol and trans-(1S,3R)-1-amino-1,3-cyclo-pentanedicarboxylic acid have been shown to potently stimulate pp^{125}FAK phosphorylation in the rat hippocampus.[7,9] However, anesthetics have been shown to depress muscarinic signaling either by an effect on the receptor or on receptor–G protein interaction, which makes the contribution of these receptors to the increase in pp^{125}FAK phosphorylation unlikely.[31] Alternatively, there is no evidence of activation of metabotropic glutamate receptors by anesthetics so far. Also, lysophosphatidic acid activates pp^{125}FAK phosphorylation,[6,7] but anesthetics differentially modulate lysophosphatidic acid signaling.[32,33] Whether anesthetics act directly at the enzyme level or in its vicinity cannot be inferred from our data. For propofol, reduction in anesthetic-induced tyrosine phosphorylation by U 73122 was only partial. This suggests that in this case, activation of the PLC–PKC pathway accounts only in part for PKC activation. Our data do not allow delineation of the precise molecular mechanisms involved in the PLC-independent part of PKC stimulation by propofol. Volatile and intravenous anesthetics activate purified and endogenous PKC and cause translocation of PKC to the membrane fraction.[17,18] Also, PKC enzyme activity is directly stimulated by volatile anesthetics in various preparations, possibly *via* the regulatory domain of the enzyme at the diacylglycerol binding sites.[18] Therefore, direct interaction between propofol and PKC cannot be ruled out. Although some PKC-dependent actions have been reported for ketamine in some tissues, our findings do not support that these mechanisms also apply to the effects of ketamine on tyrosine phosphorylation in the hippocampus.[34]

In summary, we have shown that pp^{125}FAK phosphorylation is increased by anesthetics (except ketamine) in the rat hippocampus *via* activation of the PLC–PKC pathway. The lack of effect of the nonimmobilizer F6 together with reversibility of anesthetic effects supports relevance of the current findings to anesthesia. Activation of the extracellular signal–regulated kinases and Src family kinases is triggered by pp^{125}FAK phosphorylation.[6] It can be speculated that indirect activation of the mitogen-activated protein kinase cascade suggests a possibility for anesthetics to modulate gene expression. Because $GABA_A$ receptor–mediated responses are enhanced by recruitment and activation of Src family kinases,[6] the actions reported here may indirectly contribute to enhance or maintain anesthetic-induced $GABA_A$ receptor–mediated responses in the brain.

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