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Short communication

Lidocaine increases phosphorylation of focal adhesion kinase in rat hippocampal slices

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Abstract

We examined the effect of lidocaine on phosphorylation of the tyrosine kinase focal adhesion kinase ($PP^{125}FAK$) in rat hippocampal slices by immunoblotting with both antiphosphotyrosine and specific anti-$PP^{125}FAK$ antibodies in the presence of tetrodotoxin (1 μM). Lidocaine induced a concentration-related increase in tyrosine phosphorylation of the 125-kDa band corresponding to $PP^{125}FAK$ phosphorylation (EC_{50} value = 0.39 ± 0.09 μM, maximal effect = 169 ± 28% of control, $P < 0.001$). This effect was sensitive to neither the *N*-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (MK 801, 10 μM) nor the inhibitor of the ryanodine receptor dantrolene (30 μM). In contrast, it was completely blocked by the protein kinase C (PKC) inhibitors chelerythrin, bisindolylmaleimide I (GF 109203X) and bisindolylmaleimide IX (RO-318220, 10 μM). We conclude that lidocaine increases phosphorylation of the tyrosine kinase $PP^{125}FAK$ in the rat hippocampus by a tetrotoxin (TTX)-insensitive mechanism which involves activation of PKC.

Keywords: Focal adhesion kinase; Lidocaine; Protein kinase C; Hippocampus; (Rat)

1. Introduction

Local anaesthetics are thought to act primarily via inhibition of electrophysiologic currents that travel through voltage-gated Na^+ channels (Butterworth and Strichartz, 1990). However, lidocaine can produce cellular actions not mediated by Na^+ channels by interacting with membrane phospholipids and/or proteins including G-proteins (Hageluken et al., 1994; Tan et al., 1999 Hollmann et al., 2002) and protein kinase C (PKC, Nivarthi et al., 1996; Do et al., 2002). Focal adhesion kinase ($PP^{125}FAK$) is a particularly important 125-kDa nonreceptor tyrosine kinase which may couple rapid events, such as action potential or neurotransmitter release, to long-lasting changes in synaptic strength or cell survival (Burgaya et al., 1995; Girault et al., 1999). Brain $PP^{125}FAK$ isoforms are regulated by various extracellular signals (Siciliano et al., 1996; Derkinderen et al., 1998). However, these stimuli utilize a restricted number of intracellular pathways, and the PKC family represents a major intracellular step for stimulating $PP^{125}FAK$ phosphorylation (Girault et al., 1999). Whether local anaesthetics affect tyrosine phosphorylation remains unknown. Therefore, in the present study, we examined the effect of lidocaine on $PP^{125}FAK$ phosphorylation in the rat hippocampus.

2. Materials and methods

The study complied with international guidelines of the European Community for the use of experimental animal and was approved by the institutional ethics committee. Experiments were performed on male Sprague–Dawley rats (Iffa-Credo, France) weighing 250 g and housed on a 12:12 light/dark cycle with food and water ad libitum.

2.1. Preparation of slices and homogenates and immunoblot analysis

Hippocampal slices (300-μm thickness each, three slices per tube) were incubated with 1 ml Ca^{2+}-free artificial

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cerebrospinal fluid (CSF, 60 min, 37 °C) containing 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% (v/v) oxygen/carbon dioxide mixture. Ca^{2+} was omitted from the CSF to avoid tyrosine kinase activation at this step of the experiment and added subsequently. Slices were incubated for 60 min at 37 °C with moderate agitation under a humidified atmosphere of O_2/CO_2 95%/5% (v/v) until $CaCl_2$ (1 mM) and pharmacological treatments were added. In experiments with the *N*-methyl-D-aspartate (NMDA) challenge, $MgCl_2$ was removed from the medium and replaced by $CaCl_2$ (final concentration: 1.93 mM). Tetrodotoxin (TTX, 1 μM) was added at the beginning of slice incubation to avoid indirect effects due to neuronal firing. Slices were frozen in liquid nitrogen and then homogenized by sonication in 200 μl of a solution of 1% (w/v) sodium dodecyl sulfate, 1 mM sodium orthovanadate and antiproteases (50 μg/ml leupeptine, 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. Protein concentration in the homogenates was determined with a bicinchoninic acid-based method, using bovine serum albumin as the standard. Equal amounts of protein (30 μg) were subjected to 6% (w/v) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and transferred electrophoretically to nitrocellulose. Immunoblot analysis was performed with affinity-purified rabbit anti-phosphotyrosine antibodies SL2. Primary antibodies were labeled with peroxidase-coupled antibodies against rabbit G immunoglobulins (IgG), which were detected by exposure of MP autoradiographic films in the presence of a chemiluminescent reagent (ECL, Amersham, UK). The specificity of the immunoreactivity was assessed by its competition in the presence of 50 μM *O*-phosphotyrosine. Identification of phosphorylated $PP^{125}FAK$ was performed with a rabbit anti-Y^{397} FAK phosphospecific antibody (Biosource International, diluted 1:1000) 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)/β-actin [quantified by using the specific monoclonal antiactin A5316 antibody (Sigma, France)] ratio (Cohu High-Performance CCD camera, Gel Analyst 3.01 pci, Paris, France).

2.2. Chemicals and data analysis

The effects of the following agents (alone or in combination, purchased by Sigma) on $PP^{125}FAK$ phosphorylation were studied: lidocaine hydrochloride (4.2×10^{-9}–4.2×10^{-4} M), NMDA (1 mM), dizocilpine (MK 801, 10 μM), phorbol 12-myristate 13-acetate (PMA, an activator of PKC, 0.1 μM), bisindolylmaleimide I (GF 109203X, 10 μM), bisindolylmaleimide IX (RO-318220, 10 μM) and chelerythrin (10 μM), three blockers of PKC and dantrolene (30 μM, a blocker of Ca^{2+} release from internal stores). Kinetics of lidocaine-induced phosphorylation measured at 1, 2 5 and 10 min displayed a linear increase in tyrosine phosphorylation with time between 1 and 5 min followed by a plateau until 10 min (data not shown). Therefore, a 5-min period of incubation was chosen for the agents tested to stimulate $PP^{125}FAK$ phosphorylation. Inhibitors of PKC, dantrolene and MK 801 were preincubated for 1 h before

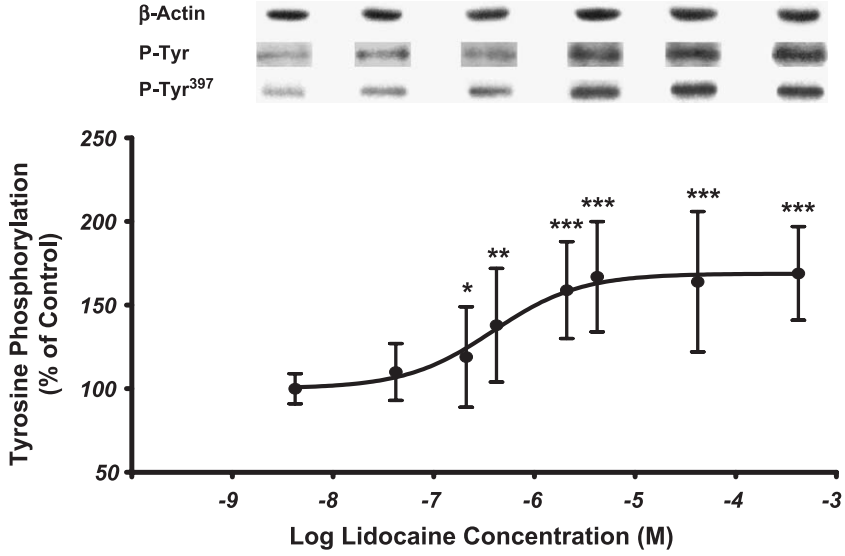

Fig. 1. Concentration–response curve of lidocaine effects on $PP^{125}FAK$ phosphorylation in rat hippocampal slices. Experiments were performed in the presence of tetrodotoxin (1 μM). Typical examples of Western blots show the effects of lidocaine concentrations on total tyrosine phosphorylation of the 125-kDa band (P-Tyr) and of $PP^{125}FAK$ phosphorylation labeled by the rabbit anti-Y^{397} FAK phosphospecific antibody (P-Tyr^{397}). β-Actin controls were quantified by using the specific monoclonal antiactin A5316 antibody (β-actin). Data (mean ± S.D.) are expressed as a percentage of basal phosphorylation (100%). $^*P<0.01$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. control.

adding any other 5-min treatment. Both linear and sigmoid models were tested to fit the curves to the data. Concentration–response data for lidocaine effects on tyrosine and $PP^{125}FAK$ phosphorylation was performed using the GraphPAD software (Intuitive Software for Science, San Diego, CA, USA). Normality of distributions was first assessed by the Fisher test for equality of variances. Statistical analysis was then performed by analysis of variance with Scheffé's post hoc correction for multiple comparisons. A P value <0.05 was considered the threshold for significance. Results (mean ± S.D.) are expressed as a percentage of control tyrosine phosphorylation (control = 100%).

3. Results

Lidocaine produced a significant, concentration-related, increase in phosphotyrosine immunoreactivity of the 125-kDa band corresponding to $PP^{125}FAK$, which was best fitted by a one site sigmoid model (EC_{50} value = 0.39 ± 0.09 μM, maximal effect = 169 ± 28% of control, $P<0.01$, Fig. 1). Consistent with previous findings (Siciliano et al., 1996), NMDA (1 mM) induced a MK801-sensitive increase in specific $PP^{125}FAK$ phosphorylation (177 ± 12% of control, $P<0.01$). PMA (0.1 μM) produced a marked increase in $PP^{125}FAK$ phosphorylation which was completely blocked by all PKC inhibitors tested (170 ± 14%, $P<0.01$). We found the effects of PMA (0.1 μM) plus lidocaine (4.2 μM) on $PP^{125}FAK$ to be nonadditive. The effect of a 4.2 μM lidocaine concentration on specific $PP^{125}FAK$ phosphorylation was completely blocked by GF 109203X, RO 318220 and chelerythrin (10 μM), but by neither tetrodotoxin (1 μM), MK801 (10 μM) nor dantrolene (100 μM, Fig. 2).

4. Discussion

We have shown that lidocaine increases phosphorylation of $PP^{125}FAK$ tyrosine kinase in rat hippocampal slices via a tetrodotoxin-insensitive, PKC-dependent mechanism.

We used the anti-Y^{397} FAK phosphospecific antibody to quantify $PP^{125}FAK$ phosphorylation The specificity of this antibody for the phosphorylated form of $PP^{125}FAK$ has been demonstrated previously (Derkinderen et al., 2001). At each lidocaine concentration tested, the increase in phosphorylation intensity of the phosphotyrosine 125-kDa band was of the same magnitude as that observed for specific $PP^{125}FAK$ phosphorylation. This indicates that the 125-kDa band phosphorylated by lidocaine on the phosphotyrosine immunoblotting indeed corresponds to $PP^{125}FAK$.

In the presence of tetrodotoxin, lidocaine's action is unlikely to be mediated via TTX-sensitive voltage-gated Na^+ channels. Elevation of intracellular Ca^{2+} is a key mechanism controlling tyrosine kinase activation. Lidocaine has been recently shown to increase $[Ca^{2+}]_i$ levels by preventing Ca^{2+} reuptake by intracellular stores (Johnson et al., 2002). However, these effects were reported for lidocaine concentrations within the millimolar range, while concentrations lower than the micromolar range were effective here. Therefore, a significant effect of lidocaine on Ca^{2+} stores was unlikely to account for the increase in tyrosine phosphorylation. The lack of effect of dantrolene is consistent with these findings. Since NMDA stimulates $PP^{125}FAK$ phosphorylation and lidocaine effect was MK801-insensitive, it is also likely not to be related to stimulation of NMDA receptors. The effects of lidocaine on $PP^{125}FAK$ phosphorylation were blocked by GF 109203X, RO 318220 and chelerythrin, three structurally distinct

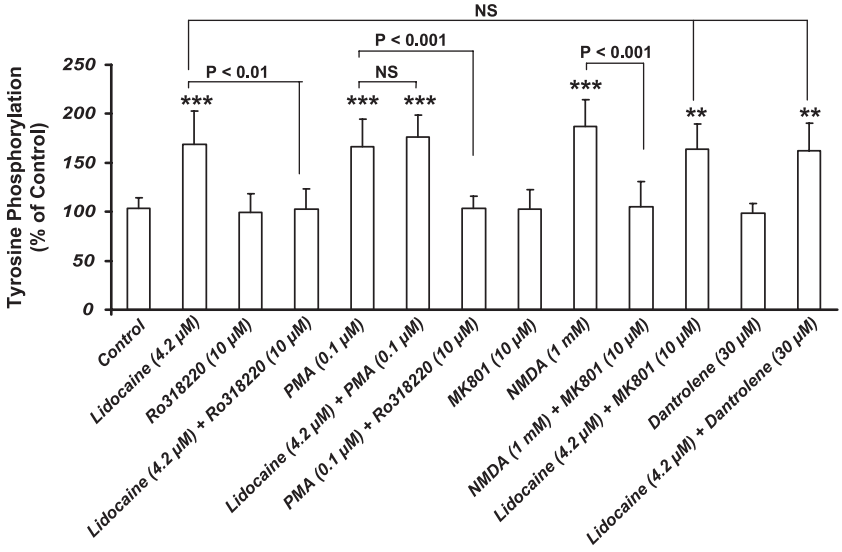

Fig. 2. Effects of various combinations of pharmacologic agents on $PP^{125}FAK$ phosphorylation in rat hippocampal slices. Experiments were performed in the presence of tetrodotoxin (1 μM). Lidocaine concentration (4.2 μM) was the lowest one achieving maximal effect in the dose–response curve (see Fig. 1). Similar effects to those obtained with RO 318220 were observed with GF 109203X and chelerythrin (10 μM). Data (mean ± S.D.) are expressed as a percentage of basal phosphorylation (100%).**$P<0.01$ and ***$P<0.001$ vs. control.

inhibitors of PKC (Way et al., 2000; Davies et al., 2000) which all blocked the effects of PMA, an activator of PKC. Taken together with the nonadditivity of lidocaine and PMA effects, these results strongly suggest that lidocaine-induced increase in $PP^{125}FAK$ phosphorylation was mediated via activation of PKC. Interestingly, PKC has recently been shown to be involved in lidocaine-induced enhancement of glutamate transporter subtypes expressed in Xenopus oocytes (Do et al., 2002). The nature (direct or indirect) of PKC activation by lidocaine remains to be delineated. The present findings might help to better understand some long-term effects of lidocaine in the central nervous system.

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