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Kinin-B2 Receptor Exerted Neuroprotection After Diisopropylfluorophosphate-induced Neuronal Damage

Wilmarie Torres-Rivera[1], **Dinely Pérez**[1], **Keon-Young Park**[2], **Marimée Carrasco**[1], **Manu O. Platt**[2], **Vesna A. Eterović**[1], **Pedro A. Ferchmin**[1], **Henning Ulrich**[3], and **Antonio H. Martins**[1]

[1]Department of Biochemistry, Universidad Central del Caribe, Bayamón, Puerto Rico 00956, USA

[2]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, USA

[3]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP 05508-000, Brazil

Abstract

The kinin-B2 receptor (B2BKR) activated by its endogenous ligand bradykinin participates in various metabolic processes including control of arterial pressure and inflammation. Recently, functions for this receptor in brain development and protection against glutamate-provoked excitotoxicity have been proposed. Here, we report neuroprotective properties for bradykinin against organophosphate poisoning using acute hippocampal slices as an in vitro model. Following slice perfusion for 10 min with diisopropylfluorophosphate (DFP) to initiate the noxious stimulus, responses of pyramidal neurons upon an electric impulse were reduced to less than 30 % of control amplitudes. Effects on synaptic-elicited population spikes were reverted when preparations had been exposed to bradykinin 30 min after challenging with DFP. Accordingly, bradykinin-induced population spike recovery was abolished by HOE-140, a B2BKR antagonist. However, the kinin-B1 receptor (B1BKR) agonist Lys-des-Arg^9-bradykinin, inducing phosphorylation of MEK/MAPK and cell death, abolished bradykinin-mediated neuroprotection, an effect, which was reverted by the ERK inhibitor PD98059. In agreement with pivotal B1BKR functions in this process, antagonism of endogenous B1BKR activity alone was enough for restoring population spike activity. On the other hand pralidoxime, an oxime, reactivating AChE after organophosphate poisoning, induced population spike recovery after DFP exposure in the presence of bradykinin and Lys-des-Arg^9-bradykinin. Lys-des-Arg^9-bradykinin did not revert protection exerted by pralidoxime, however when instead bradykinin and Ly-des-Arg^9-bradykinin were superfused together, recovery of population spikes diminished. These findings again confirm the neuroprotective feature of bradykinin, which is, diminished by its endogenous metabolites, stimulating the B1BKR, providing a novel understanding of physiological roles of these receptors.

1. Introduction

Organophosphates (OPs) have been used as pesticides and as chemical warfare nerve agents, representing a big threat to the population worldwide due to its toxicity. These

Corresponding Author: Antonio H. Martins, Ph.D. Department of Biochemistry, Universidad Central del Caribe, Ave. Laurel #100, Santa Juanita, Bayamon, Puerto Rico, 00956. Tel: 787-798-3001 exts. 2097, 2051, Fax: 787-786-6285. baccin31@gmail.com.

organophosphorus compounds are known to inhibit acetylcholinesterase (AChE) (Koelle, 1994); however, further mechanisms of actions have also been hypothesized (Zaja-Milatovic et al., 2009); (Terry, 2012). AChE blockade affecting acetylcholine-induced neurotransmission causes a cholinergic syndrome due to overstimulation and desensitization of nicotinic and muscarinic acetylcholine receptors, resulting in a series of symptoms such as seizures, respiratory failure and even death (Abdollahi and Karami-Mohajeri, 2011). The classical antidotal treatment against OP intoxication is based on the use of atropine to address muscarinic symptoms, oximes that reactivate inhibited acetylcholinesterase and benzodiazepines to ameliorate seizures (Petroianu et al., 2012). However, these treatments focuses on increasing survival of acute-intoxicated individuals, but do not protect against long-term neurological deficits, as shown in cases of Gulf War soldiers exposed to chemical warfare nerve agents and Japan terrorist attacks (Bajgar, 2004).

The kallikrein-kinin system generating the peptides bradykinin (BK) and kallidin (Lys-BK) and their bioactive metabolites, des-Arg^9-BK and Lys-des-Arg^9-BK has presented well-characterized functions in several physiological and pathophysiological conditions including control of arterial pressure, inflammation and stroke. The kallikrein-kinin system is comprised of precursor kininogens, generating bradykinin and kallidin upon cleavage by kallikrein proteases. The most studied peptides, bradykinin (BK) and des-Arg^9-bradykinin are released after tissue injury (Albert-Weissenberger et al., 2013). BK can be either degraded by angiotensin-converting enzymes or suffer removal of Arg^9 by carboxypeptidase M present in neurons or by carboxypeptidase N present in the blood stream releasing des-Arg^9-bradykinin (Walker et al., 1995); (Bryant and Shariat-Madar, 2009); Albert-Weissenberger et al., 2013). BK specifically activates the kinin-B2 receptor (B2BKR), while des-Arg^9-BK stimulates the B1 receptor subtype (B1BKR) both G protein-coupled.

As already suggested in a previous study by our group (Martins et al., 2012), a neuroprotective role for B2BKR and deleterious effect of B1BKR has been demonstrated. Reversion of the loss of population spikes in rat hippocampal neurons against the oxidative damage triggered by NMDA was observed when cells had been exposed to BK after initial challenge with the glutamate analogue (Martins et al. 2012). As a suggested underlying mechanism, induction of apoptosis was reversed in the presence of BK. BK-promoted protection depended on phosphatidylinositol kinase (PI-3K) activation, while inhibition of mitogen-activated protein kinase (MEK/MAPK) signaling did not interfere with the induced neuroprotective effects. However, MEK/MAPK activation was involved in kinin-B1 receptor (B1BKR)-mediated signaling which reverted BK-induced population spike recovery.

Here, we show that after exposure of hippocampal slices to diisopropylfluorophosphate (DFP), BK superfusion restates population spike amplitudes to those obtained in the absence of organophosphate. Co-application of BK and des-arg^9-BK abolished this neuroprotection. Nonetheless, superfusion of the B1BKR antagonist Lys-des-Arg^9-Leu^8-BK alone was able to revert the loss of population spikes showing that cleavage of bradykinin into des-Arg^9-BK in pyramidal neurons or in adjacent cells was enough to induce cell death.

2. Materials and Methods

2.1. Materials

Standard laboratory chemicals and bradykinin (BK), Lys-des-Arg^9-BK, HOE-140, Lys-des-Arg^9-Leu^8-BK, and 2-PAM were obtained from Sigma-Aldrich (St. Louis, MO). LY294002 and PD98059 were acquired from Calbiochem (La Jolla, CA) and EMD Chemicals (Gibbstown, NJ), respectively.

2.2. Slice Preparation and Electrophysiological Recordings

Acute hippocampal slices from male Sprague-Dawley rats (120–200g) from our colony were prepared and maintained using standard methods. All performed procedures involving animals have been reviewed and approved by the Institutional Animal Care and Use Committee of Universidad Central del Caribe, School of Medicine. Brains from rats sacrificed by decapitation were removed and the hippocampi dissected on ice. A standard artificial cerebrospinal fluid (ACSF), containing (in mM) 125 NaCl, 3.3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose, was used to irrigate hippocampi and for incubation. Transversal 400μm thick slices were cut with a manual slicer and immediately transferred to the incubation chamber consisting of a temperature-controlled bath surrounding an acrylic plate covered with nylon mesh (Hanes) and three lanes with independent perfusion to which slices were equally distributed. The lower part of the chamber was filled with water and kept at 37.4± 1°C and continuously bubbled with 95% O_2, 5% CO_2. The slices were kept in the lanes over the mesh at the interface between ACSF at 95% O_2, 5% CO_2 and 34± 1°C. The exterior of the chamber was kept at 30± 1°C. The temperature at the three levels (outside, nylon mesh, and water bath) was strictly controlled to minimize variability. Slices were allowed to recover their electrophysiological activity from dissection during one hour. A bipolar electrode placed in the stratum radiatum was used to stimulate the Shaffer collateral incoming fibers with a constant current of 0.2 ms. Synaptic-elicited population spikes (PSs) were recorded in stratum pyramidale with a glass electrode filled with 2M NaCl, having an impedance ranging from 1 to 5 M using a Grass S48 stimulator, Grass RPS 107 regulated power supply and the Grass photoeletric stimulator isolation unit.

2.3. Procedure for testing neurotoxicity

The testing procedure for neurotoxicity was performed according to (Schurr et al., 1995a); (Schurr et al., 1995b); and modified by (Ferchmin et al., 2000). About 30 slices from the hippocampi of two rats were distributed equally among the three lanes of the incubation chamber. A maximum of seven slices were analyzed per lane for each replication and 14 to 35 slices were tested per each experimental condition. The testing of slices started one hour after dissection. Each slice was stimulated with a pulse twice the strength required to elicit a threshold PS. The initial response was recorded as PS area (ms×mV) and compared with the response elicited by the same stimulus, recorded from the same position, after the completion of the experimental treatment. Neurotoxic insults and subsequent protection are given by percentages of the initial PS. The excitotoxic stimulus was delivered by perfusion for 10 minutes of 200μM DFP on each lane. The general experimental design was as demonstrated in Figure 1, with the initial PS being recorded after one hour of slice preparation. Following determination of the initial PS peak value (control measurement defining the 100% response), slices were perfused for 10 min with 200μM DFP. A 30 min washout with ACSF was performed. Then slices were perfused for 1h with drugs: BK (10nM and 1μM), 1μM of the B1BKR agonist Lys-des-Arg^9-BK, 1μM of the B2BKR selective inhibitor HOE-140, 1μM of the B1BKR antagonist Lys-des-Arg^9-Leu^8-BK, 10μM of the PI3 kinase inhibitor LY204002, 50μM of the MEK/MAPK inhibitor PD98059, or 100 μM of the pralidoxime (2-PAM), used as antidote for organophosphate poisoning. Before determining final PS recovery, hippocampal slices were washed with ACSF for 1h to eliminate remaining drugs as well as any short-lived drug effects.

2.4. Data Collection and Analysis

The areas of PSs (millivolts per millisecond) were acquired using a GRASS®P5 series AC–preamplifier and analyzed with the Labman program (a gift from Dr. T.J. Teyler, WWAMI Medical Education Program, University of Idaho, Moscow, ID). The data were statistically analyzed using SigmaStat version 2.03 (SPSS Science, Chicago, IL). One-way analysis of

variance (ANOVA) followed by the Student-Newman-Keuls test was used whenever the data were distributed normally; otherwise, Kruskal-Wallis one-way analysis of variance on Ranks was used followed by the appropriate post hoc test.

3. Results

3.1. Bradykinin (BK) protects the population spike from DFP toxicity through activation of the bradykinin-B2 receptor (B2BKR)

Acute hippocampal slices were perfused with the organophosphate DFP (200μM) by 10 min which resulted in a reduction of population spikes (PSs) of pyramidal neurons to 27.0±3.7% (mean ± S.E.M.) compared to control slices that did not receive DFP perfusion (Figure 1). Following 30 minutes after washout of DFP with ACSF, BK at 10nM and 1μM concentrations reversed DFP-induced neuronal damage, as shown by an increased recovery rates of 46.6±7.9% (n=21, *p<0.05 compared to DFP alone) and 59.6±9.0% (n=21, ***p<0.001 compared to DFP alone), respectively (Figure 1). One mM BK concentration presented a slightly better recovery rate compared to 10 nM of the peptide and was chosen for subsequent experiments. BK-exerted neuroprotection against DFP-induced cell death was mediated by B2BKR activation, since BK-mediated PS recovery was abolished in the presence of the selective B2BKR antagonist HOE-140. In these experiments slices were preincubated with HOE-140 (1μM) before being co-applied with BK in order to assure receptor inhibition. Recovery rates for this co-application was 35.2±3.4% (Figure 1) (#p<0.05 when compared to BK 1μM). HOE-140 by itself did not result in PS recovery (#p<0.05, when compared to BK 1μM).

3.2. Kinin-B1 (B1BKR) receptor activation abolishes B2BKR receptor-exerted protection against DFP neurotoxicity

Exposure to 200μM DFP caused a PS reduction (30.9±2.7% compared to untreated control slices) and was reversed by exposure to BK (1μM) for one hour following 30 min washout of DFP (68.4±6.2%; n=21, ***p<0.001, when compared to DFP alone, Figure 2). Perfusion of the B1BKR agonist Lys-des-Arg^9-BK (1μM) together with BK did not increase PS peak values compared to DFP alone 31.5±3.4% (n=35, ###p<0.001, compared to 1μM BK). However, when B1BKR activation was blocked by 10μM of the B1BKR antagonist Lys-des-Arg^9-Leu^8-BK, population spikes were reestablished in the presence of BK to 69.7±8.0% (n=21, ***p<0.001 compared to DFP alone and when compared with the combination of BK+Lys-des-Arg^9-BK, Figure 2). Activation of B1BKR completely abolishes BK-exerted neuroprotection against DFP, suggesting a detrimental role for B1BKR and a protective role for B2BKR in neuronal damage triggered by OPs.

3.3. BK stimulates the PI3K pathway, while MEK/MAPK pathway is needed for B1BKR activation and abolishment of protection against DFP

Perfusion of the MEK inhibitor PD98059 (50μM) together with BK did not affect PS recovery exerted by BK, indicating that BK exerted neuroprotection did not depend on MEK/MAPK activation (90.5±7.9%; n=14, ***p<0.001 when compared to DFP alone) (Figure 3). On the other hand, when slices were perfused with BK together with PI3K inhibitor LY294002 (10μM), BK-exerted neuroprotection was significantly attenuated. Here, PS recovery was 39.9±7.3% (n=14, ###p<0.001 compared to slices not treated with the inhibitor). As reported previously by our group (Martins et al. 2012), BK-induced neuroprotection depended was PI3K pathway dependent. However, following blockade of the MEK/MAPK pathway by PD98059 (50μM), co-application of BK and Lys-des-Arg^9-BK resulted in PS recovery (89.0±9.0%; n=14, ***p<0.001 when compared to 20±3.08%. DFP alone, and ***p<0.001 when compared to BK+Lys-des-Arg^9-BK (29±4.5%) in the absence of PD98059). In summary, B1BKR-mediated effects involved MEK/MAPK. The data in

Figure 3 revealed that BK is promoting neuroprotection against DFP by the activation of the PI3K pathway through constitutive B2BKR present in neurons, while the activation of MEK/MAPK pathway through B1BKR activated by its agonist Lys-des-Arg^9-BK was necessary to abolish BK protection.

3.4. Bradykinin cleavage into its metabolite des-Arg^9-BK causes detrimental effects

A possible mechanism responsible for the harmful effect of bradykinin *in vivo* could be the conversion of this peptide into the B1BKR agonist des-Arg^9-BK by endogenous carboxypeptidase activity. To test this hypothesis, Lys-des-Arg^9-Leu^8-BK (1μM) or PD98059 (50 μM) were employed to block B1BKR activity or the subsequent MEK/MAPK pathway, respectively (Figure 3). Following DFP (200μM) perfusion which decreased PSs recovery and 30 min ACSF washout, Lys-des-Arg^9-Leu^8-BK recovered population spikes (PSs) to 91.7±6.8% (n=14, ***p<0.001 when compared to DFP alone, Figure 3). Similarly, the presence of PD98059 resulted in PS recovery of 80.2±7.4% (n=14, ***p<0.001 compared to DFP alone). These results in figure 3 again demonstrate that activation of B1BKR led to noxious effects and support the hypothesis that BK naturally occurring in the system was breaking down to its active metabolite binding to the B1BKR and activating the MEK/MAPK pathway to cause neuronal damage. These results demonstrated that activation of B1BKR leads to noxious effects.

3.5. Interaction with 2-PAM and kinin receptors

Pralidoxime (2-PAM), known to revert inhibition of acetylcholine esterase (dos Santos et al. 2011) protected against neurotoxic effects of DFP that were not affected by the B1BKR agonist Lys-des-Arg9-BK (Figure 6). However, 2-PAM-induced PS recovery was reduced in the presence of BK and the B1BKR agonist, suggesting interference between B2BKR- and B1BKR-mediated signaling as reported previously (Martins et al. 2012).

4. Discussion

Measuring population spike (PS) recovery of pyramidal neurons in hippocampal slices is an accepted in vitro model for measuring physiological parameters, neurotoxicity, and subsequent neuroprotective features of diverse compounds (Wang et al., 2010; Kanak et al., 2011;Ferchmin et al., 2005 Ferchmin et al., 2013). Indeed, recovery of PS activity to similar values, as observed in control measurements in the absence of any drug, was also achieved by blocking apoptosis with caspase-9 or AKT-Glycogen synthase kinase inhibitor (Martins et al. 2012). BK functions in the CNS have been recently recognized including regulation of synaptic function, promotion of neurogenesis and neuroprotection against NMDA-induced excitotoxicity (Martins et al., 2005; Kohno et al., 2008). These data are in line with novel results showing that B2BKR expression is neither limited to cells directly involved in immune responses nor endothelial cells of blood vessels in the brain, but is expressed by neurons.

In our previous paper, we demonstrated B2BKR-mediated protection against NMDA-induced excitotoxicity. The neuroprotective signaling pathway involved activation of PI3-kinase and did not depend on MEK / MAPK signaling. Such as shown in our present work, BK-induced PS recovery was abolished in conditions of B1BKR co-activation (Martins et al. 2012).

Here, we show that BK-induced neuroprotection through B2BKR activation against DFP-poisoning was annulled by LY294002, a specific PI3 kinase inhibitor, while deleterious effects on BK-mediated neuroprotection induced by Lys-desArg^9-BK, a B1BKR agonist with equal pharmacological activity as desArg^{9}BK in rats (Regoli et al., 2001), were

inhibited in the presence of the MEK / MAPK blocker PD98059. Consequently, direct antagonism of B1BKR activity or inhibition of B1BKR-mediated signal transduction, enabled neuroprotection by B2BKR.

BK is released into extracellular space upon cell-death inducing insults. Released BK is then further processed into desArg^9-BK activating the B1BKR and exerting deleterious effects (Albert-Weissenberger et al., 2012). In line with endogenous BK and desArg^9-BK production, neuroprotection against DFP-mediated effects were observed in the presence of the B1BKR antagonist Lys-desArg^9-Leu^8-BK.

Experimental evidence points at different DFP-mediated mechanisms such as excessive cholinergic stimulation by AChE, but also suggests non-cholinergic effects such as activation of glutamatergic neurons and generation of reactive and nitrogen species, leading to neurodegeneration and cell death (Zaja-Milatovic et al., 2009). Such hypotheses are supported by our present study, since 2-PAM, known to revert DFP-induced inhibition of AChE (dos Santos et al., 2011), was neuroprotective, even in the presence of the B1BKR agonist desArg^9-BK, which abolished protection mediated by BK. The failure of BK inducing neuroprotective pathways in the presence of desArg^9-BK suggests that B2BKR-mediated PS recovery which is not directly connected with AChE inhibition by DFP. In agreement with separate mechanisms of action induced by 2-PAM and BK, no summative effects were observed in the presence of both drugs.

In fact, our work contributes to the controversy, whether BK-induced actions are beneficial or not in brain insults such as those resulting from strokes. For instance, kininogen production was identified as important for activation of thrombotic and inflammatory circuits and, participation in stroke development (Langhauser et al., 2012). Moreover, B2BKR activity has been associated with secondary brain damage after subarachnoid damage (Scholler et al., 2011). On the other hand, kallikrein gene transfer giving rise to BK production, resulted in neuroprotection in an animal model (Xia et al., 2004). Another *in vivo* study, using knock-out models and pharmacological approaches provided evidence that B2BKR provides protection from cerebral infarction and brain edema (Austinat et al., 2009). In agreement, (Danielisova et al., 2009) reported that more than 97% of CA1 pyramidal neurons survived after post-conditioning with BK following ischemia. However, in another work, genetic deletion or pharmacological inhibition of B2BKR did not reveal any significant impact on lesion formation nor on the development of brain edema, while B1BKR blockage resulted in decreased axonal damage and brain edema (Albert-Weissenberger et al. 2012).

Although further studies will need to be performed to elucidate molecular mechanisms of BK-induced effects resulting in neuroprotection, the data reported herein suggest a general function for BK in protecting the CNS against neurotoxic damage, and suggest pharmaceutical approaches with BK and its stable synthetic analogues, together with inhibition of B2BKR activity.

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REFERENCES

Abdollahi M, Karami-Mohajeri S. A comprehensive review on experimental and clinical findings in intermediate syndrome caused by organophosphate poisoning. Toxicol Appl Pharmacol. 2011; 258:309–314. [PubMed: 22177963]

Albert-Weissenberger C, Siren AL, Kleinschnitz C. Ischemic stroke and traumatic brain injury: the role of the kallikrein-kinin system. Prog Neurobiol. 2013; 101–102:65–82.

Albert-Weissenberger C, Stetter C, Meuth SG, Gobel K, Bader M, Siren AL, Kleinschnitz C. Blocking of bradykinin receptor B1 protects from focal closed head injury in mice by reducing axonal damage and astroglia activation. J Cereb Blood Flow Metab. 2012; 32:1747–1756. [PubMed: 22569191]

Austinat M, Braeuninger S, Pesquero JB, Brede M, Bader M, Stoll G, Renne T, Kleinschnitz C. Blockade of bradykinin receptor B1 but not bradykinin receptor B2 provides protection from cerebral infarction and brain edema. Stroke. 2009; 40:285–293. [PubMed: 18988906]

Bajgar J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. Adv Clin Chem. 2004; 38:151–216. [PubMed: 15521192]

Bryant JW, Shariat-Madar Z. Human plasma kallikrein-kinin system: physiological and biochemical parameters. Cardiovasc Hematol Agents Med Chem. 2009; 7:234–250. [PubMed: 19689262]

Danielisova V, Gottlieb M, Nemethova M, Kravcukova P, Domorakova I, Mechirova E, Burda J. Bradykinin postconditioning protects pyramidal CA1 neurons against delayed neuronal death in rat hippocampus. Cell Mol Neurobiol. 2009; 29:871–878. [PubMed: 19259804]

dos Santos AA, dos Santos DB, Ribeiro RP, Colle D, Peres KC, Hermes J, Barbosa AM, Dafre AL, de Bem AF, Kuca K, Farina M. Effects of K074 and pralidoxime on antioxidant and acetylcholinesterase response in malathion-poisoned mice. Neurotoxicology. 2011; 32:888–895. [PubMed: 21723318]

Ferchmin PA, Perez D, Biello M. Spermine is neuroprotective against anoxia and N-methyl-D-aspartate in hippocampal slices. Brain Res. 2000; 859:273–279. [PubMed: 10719074]

Ferchmin PA, Perez D, Castro Alvarez W, Penzo MA, Maldonado HM, Eterovic VA. gamma-Aminobutyric acid type a receptor inhibition triggers a nicotinic neuroprotective mechanism. J Neurosci Res. 2013; 91:416–425. [PubMed: 23280428]

Ferchmin PA, Hao J, Perez D, Penzo M, Maldonado HM, Gonzalez MT, Rodriguez AD, de Vellis J. Tobacco cembranoids protect the function of acute hippocampal slices against NMDA by a mechanism mediated by alpha4beta2 nicotinic receptors. J Neurosci Res. 2005; 82:631–641. [PubMed: 16247800]

Kanak DJ, Jones RT, Tokhi A, Willingham AL, Zaveri HP, Rose GM, Patrylo PR. Electrical and Pharmacological Stimuli Reveal a Greater Susceptibility for CA3 Network Excitability in Hippocampal Slices from Aged vs Adult Fischer 344 Rats. Aging Dis. 2011; 2:318–331. [PubMed: 22396884]

Koelle GB. Pharmacology of organophosphates. J Appl Toxicol. 1994; 14:105–109. [PubMed: 8027504]

Kohno T, Wang H, Amaya F, Brenner GJ, Cheng JK, Ji RR, Woolf CJ. Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. J Neurosci. 2008; 28:4533–4540. [PubMed: 18434532]

Langhauser F, Gob E, Kraft P, Geis C, Schmitt J, Brede M, Gobel K, Helluy X, Pham M, Bendszus M, Jakob P, Stoll G, Meuth SG, Nieswandt B, McCrae KR, Kleinschnitz C. Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation. Blood. 2012; 120:4082–4092. [PubMed: 22936662]

Martins AH, Alves JM, Perez D, Carrasco M, Torres-Rivera W, Eterovic VA, Ferchmin PA, Ulrich H. Kinin-B2 receptor mediated neuroprotection after NMDA excitotoxicity is reversed in the presence of kinin-B1 receptor agonists. PLoS One. 2012; 7:e30755. [PubMed: 22348022]

Martins AH, Resende RR, Majumder P, Faria M, Casarini DE, Tarnok A, Colli W, Pesquero JB, Ulrich H. Neuronal differentiation of P19 embryonal carcinoma cells modulates kinin B2 receptor gene expression and function. J Biol Chem. 2005; 280:19576–19586. [PubMed: 15767251]

Petroianu GA, Nurulain SM, Shafiullah M, Hasan MY, Kuca K, Lorke DE. Usefulness of administration of non-organophosphate cholinesterase inhibitors before acute exposure to organophosphates: assessment using paraoxon. J Appl Toxicol. 2012

Regoli D, Rizzi A, Perron SI, Gobeil F Jr. Classification of kinin receptors. Biol Chem. 2001; 382:31–35. [PubMed: 11258668]

Scholler K, Feiler S, Anetsberger S, Kim SW, Plesnila N. Contribution of bradykinin receptors to the development of secondary brain damage after experimental subarachnoid hemorrhage. Neurosurgery. 2011; 68:1118–1123. [PubMed: 21242838]

Schurr A, Payne RS, Heine MF, Rigor BM. Hypoxia, excitotoxicity, and neuroprotection in the hippocampal slice preparation. J Neurosci Methods. 1995; 59:129–138. [PubMed: 7475243]

Terry AV Jr. Functional consequences of repeated organophosphate exposure: potential non-cholinergic mechanisms. Pharmacol Ther. 2012; 134:355–365. [PubMed: 22465060]

Walker RJ, Lewis-Barned NJ, Edwards EA, Robertson MC. The effects of increasing doses of enalapril on insulin sensitivity in normotensive non-insulin dependent diabetic subjects. Aust N Z J Med. 1995; 25:698–702. [PubMed: 8770334]

Wang S, Dai ZG, Dong XW, Guo SX, Liu Y, Wang ZP, Zeng YM. Duplicate preconditioning with sevoflurane in vitro improves neuroprotection in rat brain via activating the extracellular signal-regulated protein kinase. Neurosci Bull. 2010; 26:437–444. [PubMed: 21113194]

Xia CF, Yin H, Borlongan CV, Chao L, Chao J. Kallikrein gene transfer protects against ischemic stroke by promoting glial cell migration and inhibiting apoptosis. Hypertension. 2004; 43:452–459. [PubMed: 14698996]

Zaja-Milatovic S, Gupta RC, Aschner M, Milatovic D. Protection of DFP-induced oxidative damage and neurodegeneration by antioxidants and NMDA receptor antagonist. Toxicol Appl Pharmacol. 2009; 240:124–131. [PubMed: 19615394]

- Bradykinin is neuroprotective with delay administration against DFP.
- Lys-des-Arg^9-bradykinin blocks the induced neuroprotection by bradykinin (BK).
- Lys-des-Arg^9-bradykinin does not abolish the neuroprotection induced by 2-PAM.
- Lys-des-Arg^9-bradykinin plus BK abolished the neuroprotection induced by 2-PAM.

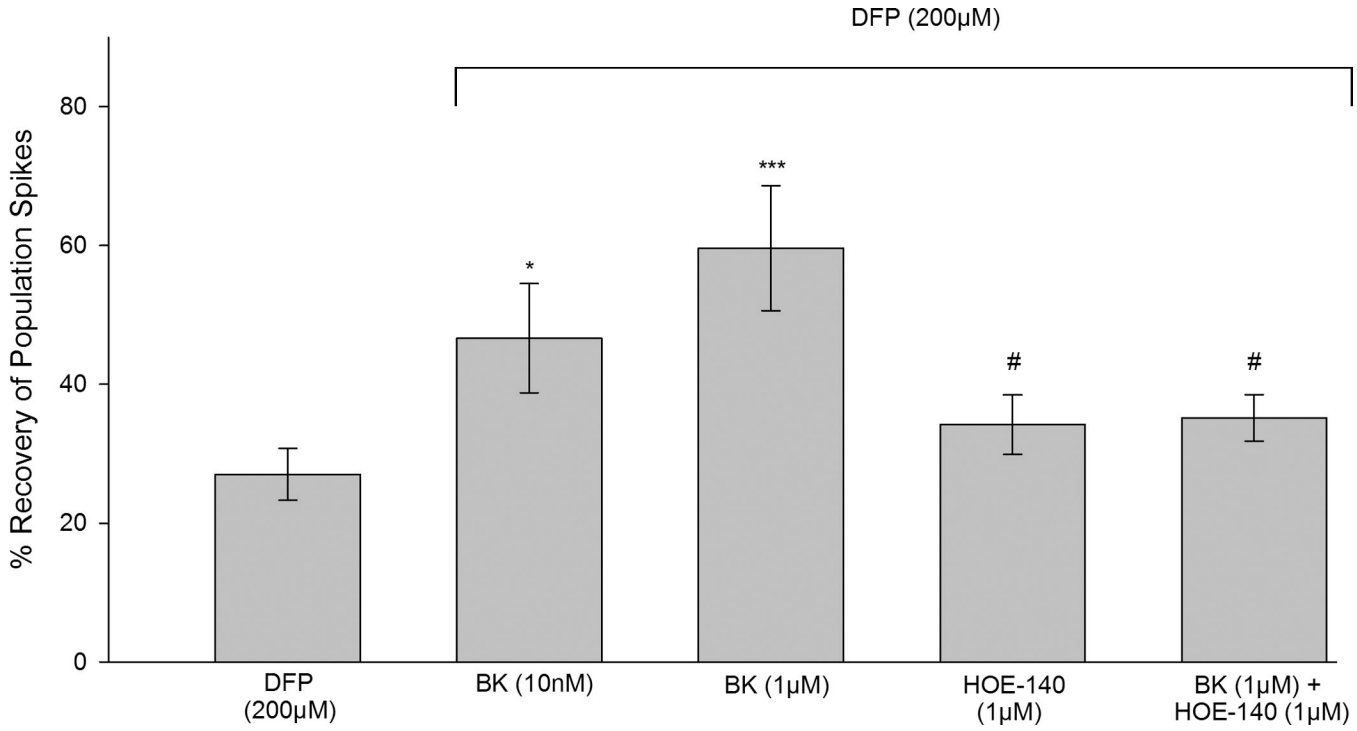

Figure 1. Kinin-B2 receptor (B2BKR)-mediated neuroprotection against neurotoxic effects of diisopropylfluorophosphate (DFP)-induced damage

Peak areas of synaptic-elicited population spikes (PSs) recorded in the stratum pyramidal region of rat hippocampal slices are reported as mean percentages ±S.E.M., compared to control measurements in the absence of any drug. DFP (200μM)-decreased PS recovery was recovered by posterior bradykinin (BK) application at 10nM and 1μM concentrations. Neuroprotective effects mediated by 1μM BK were abolished in conditions of pre- and co-application of the B2BKR receptor antagonist HOE-140 (1μM). HOE-140 (1μM) alone did not reveal any effects on PS recovery. Statistical analysis was done by one-way ANOVA followed by the Student-Newman-Keuls Method; n=21 per each treatment, *$p<0.05$, ***$p<0.001$ BK (1μM) vs DFP (200μM); #$p<0.05$ HOE-140 (1μM) vs BK (1μM) and HOE-140 +BK (1μM each) vs BK (1μM).

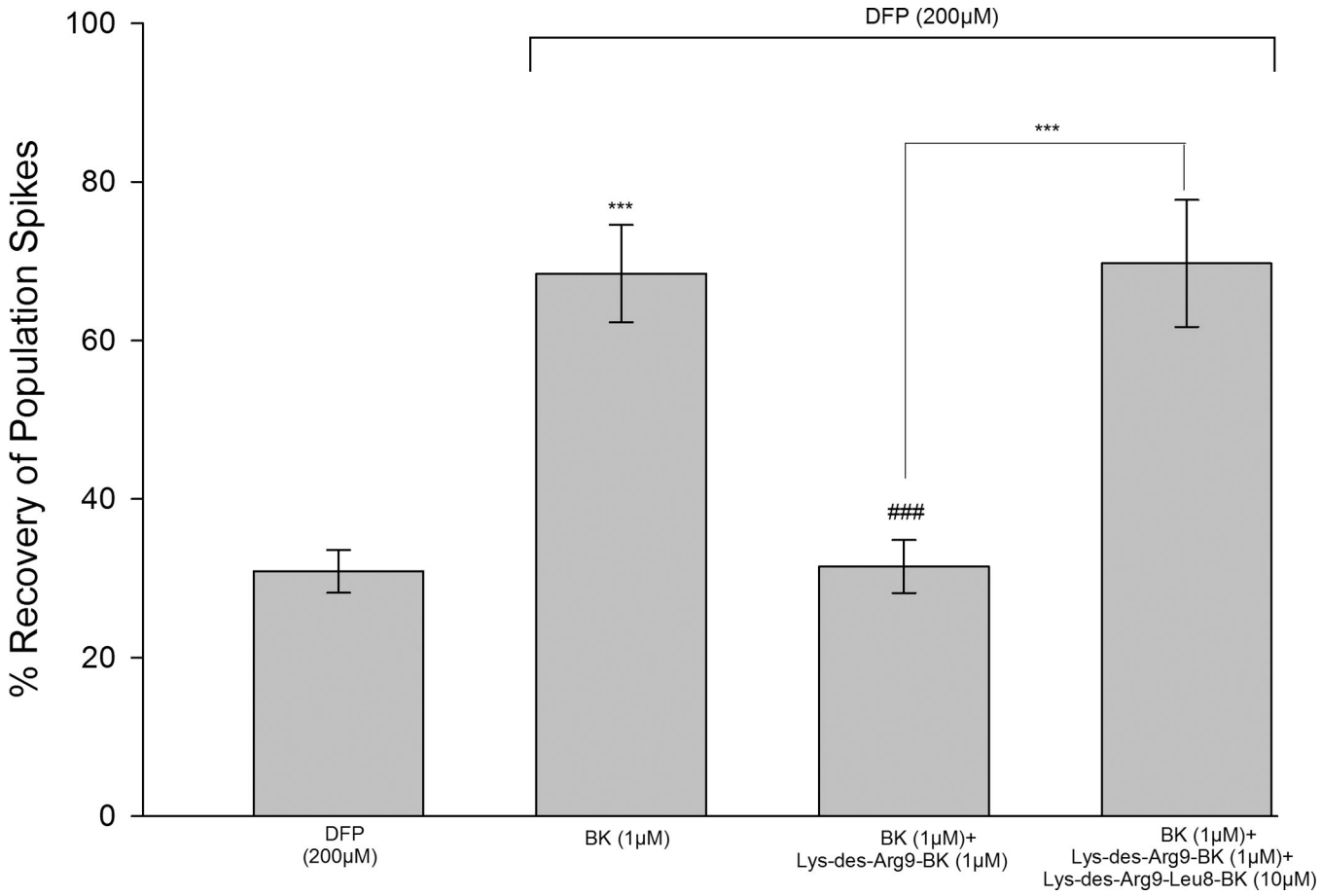

Figure 2. Reversion of B2BKR mediated neuroprotection against DFP by B1BKR activation

BK-mediated PS recovery from neurotoxic effects of DFP was abolished when the peptide was co-applied with the B1BKR agonist Lys-des-Arg^9-BK. Blockade of B1BKR with Lys-des-Arg^9-Leu^8-BK annulled the effects of the B1BKR agonist and enabled BK-induced neuroprotection. Data are presented as percentages of recovery ± S.E.M compared to control measurements in the absence of any drug. Statistical analysis was done by one way ANOVA followed by the Student-Newman-Keuls Method. ***$p<0.001$ BK (1μM) (n=21) vs DFP (200μM), BK+Lys-des-Arg^9-BK+Lys-des-Arg^9-Leu^8-BK (all drugs at 1μM concentration) (n=14) vs DFP (200μM) and BK+Lys-des-Arg^9-Leu^8-BK vs BK+Lys-des-Arg^9-BK (all drugs at 1μM concentration) (n=35); ### $p<0.001$ Lys-des-Arg^9-BK (1μM) vs DFP (200μM).

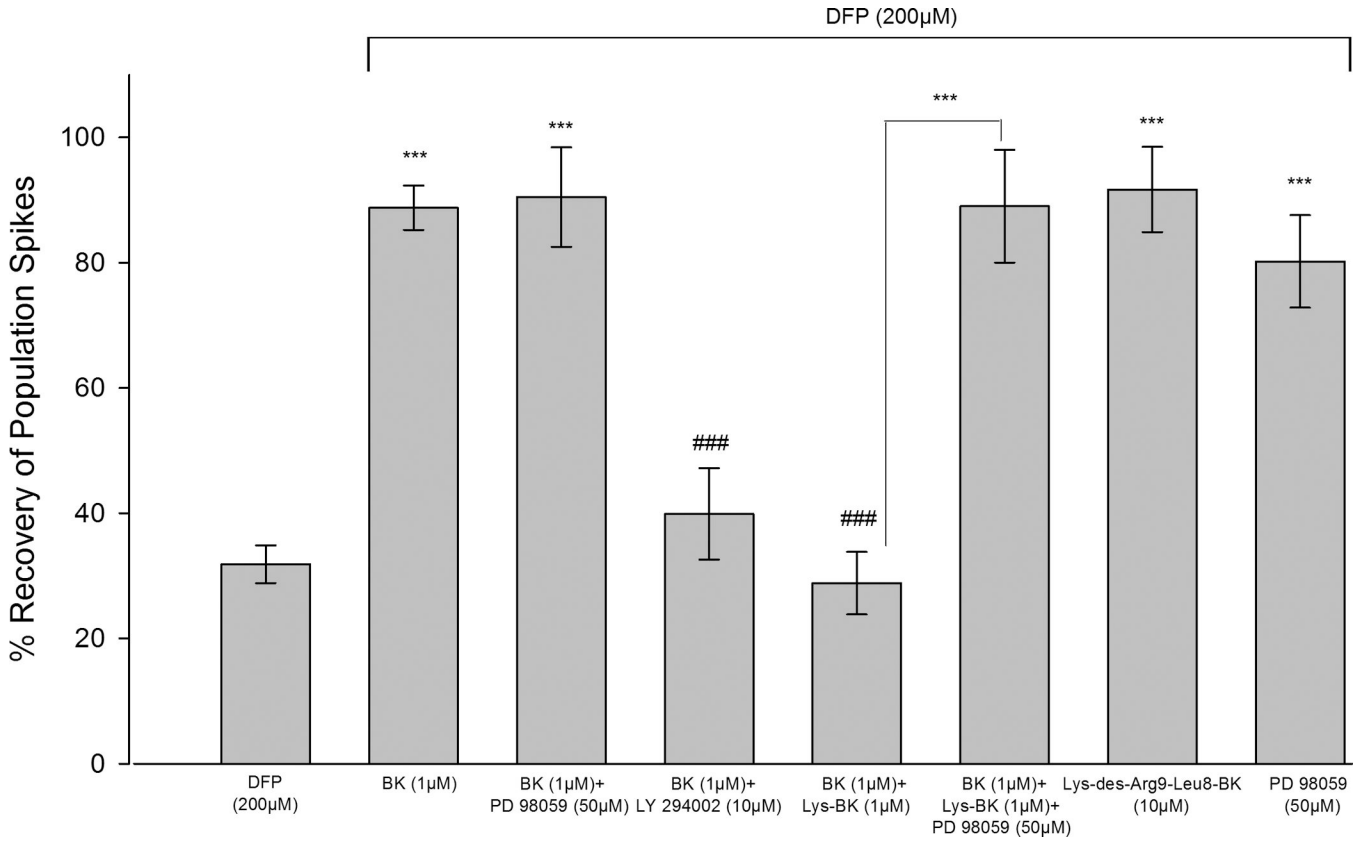

Figure 3. Population spikes recovery after inhibition of B1BKR and the PI3K and MEK/MAPK pathways

The MEK/MAPK inhibitor PD98059 perfused 15 min before BK did not interfere with BK-mediated neuroprotection. Co-application of the PI3K inhibitor LY294002 as well as Lys-des-Arg^9-BK abolished BK-induced neuroprotection. Inhibition of the MEK/MAPK pathway abrogated Lys-des-Arg^9-BK-induced effects, consequently enabling B2BKR-mediated protective actions. Lys-des-Arg^9-Leu^8-BK as well as PD98059 recovered PS peak values from DFP mediated neurotoxicity. Values are expressed as mean percentages of recovery ± S.E.M. compared to control measurements in the absence of any drug. Statistical analysis was done by Kruskal-Wallis one-way ANOVA on ranks followed by the Dunn's test. ***$p<0.001$ BK (1μM) (n=28) vs DFP (200μM), BK+PD98059 (n=14) vs DFP, BK +Lys-BK+PD98059 (n=14) vs DFP as well as vs BK+Lys-BK (n=14), Lys-des-Arg^9-Leu^8-BK (n=14) vs DFP and PD98059 (n=14) vs DFP. ###$p<0.001$ BK+LY294002 (n=14) vs BK (1μM) alone and BK+Lys-des-Arg^9-BK vs BK alone.

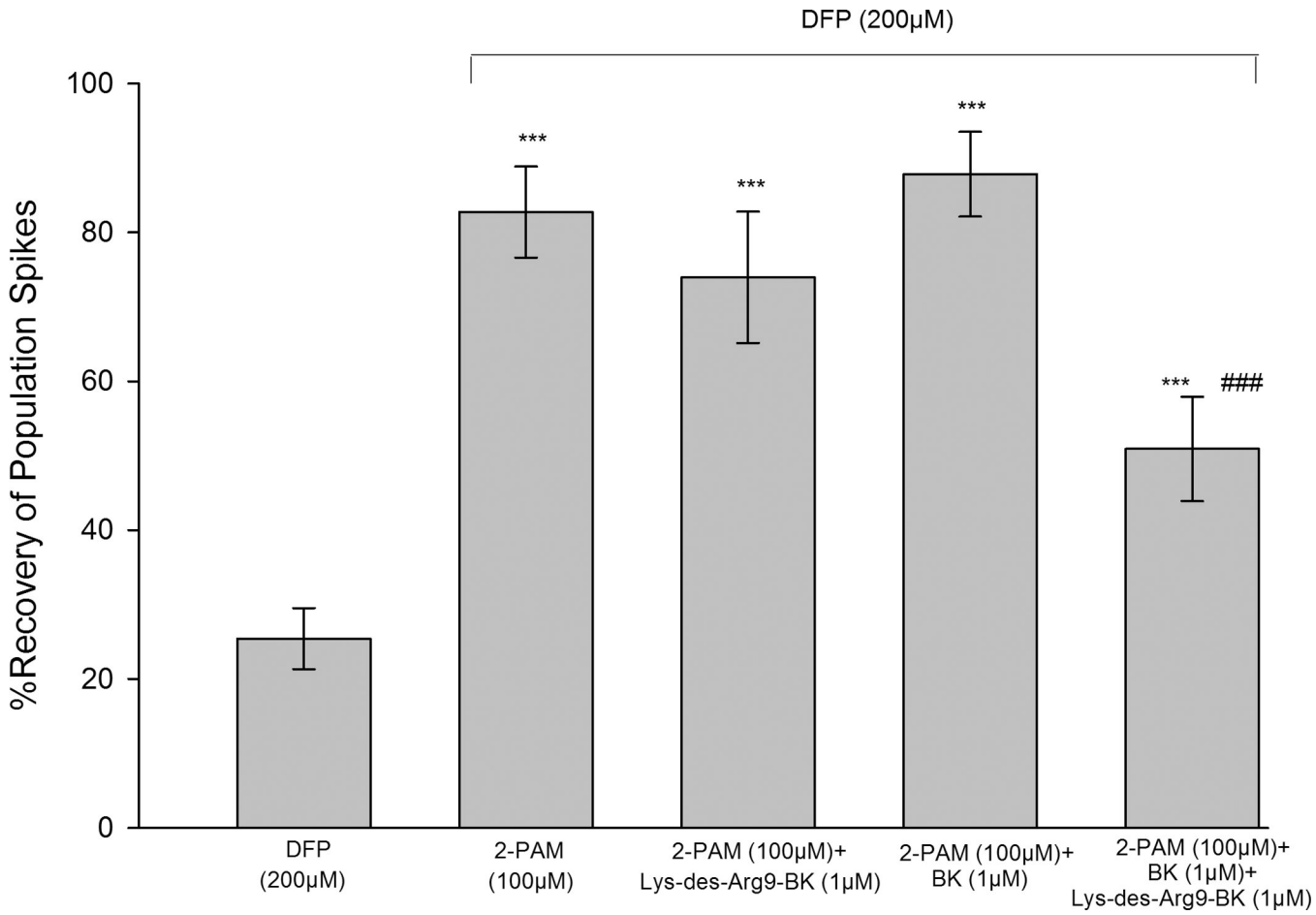

Figure 4. Neuroprotective effects exerted by 2-PAM in the presence of B2BKR and B1BKR activity

2-PAM (100μM) recovered PS activity when applied for 1h after exposure of hippocampal neurons to DFP (200μM), even in the presence of Lys-des-Arg^9-BK (1μM) (***$p<0.001$, n=21). No cooperative effects was observed in conditions of co-superfusion of 2-PAM and BK (1μM). However, 2-PAM-mediated effects were slightly reduced in the presence of BK (1μM) and Lys-des-Arg^9-BK (1μM) (###$p<0.001$). Values are expressed as mean percentage of recovery ± S.E.M. Statistical analysis was done by one way ANOVA followed by the Student-Newman-Keuls Method