Comparative neuroprotective effects of preischemic PACAP and VIP administration in permanent occlusion of the middle cerebral artery in rats

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ORIGINAL ARTICLE

Abstract

OBJECTIVES: Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) belong to the same peptide family, and both neuropeptides have been shown to exert *in vitro* and *in vivo* neurotrophic and neuroprotective effects. The aim of the present study was to investigate and compare the protective effects of PACAP and VIP in permanent focal cerebral ischemia in rats. The effect on the progression of the cerebral infarct was also studied.

METHOD: Male rats were injected 450 pmol PACAP or VIP dissolved in physiological saline intracerebroventricularly, preceding the occlusion of the middle cerebral artery. Control animals received vehicle treatment. Permanent focal ischemia was induced by the intraluminal filament occlusion of the middle cerebral artery. Animals were sacrificed 12 or 24 hours after the onset of ischemia, and infarcted brain areas were determined by staining bran sections with triphenyl-tetrazolium chloride.

RESULTS: Twelve hours after ischemia, the infarcted brain volume resulted to be 14.8% in the control group, 15.3% in the VIP-treated group and 5.8% in the PACAP-treated animals. Twenty-four hours after middle cerebral artery occlusion, the infarcted brain volumes were 21.5%, 20.7% and 14.3% in the control, VIP and PACAP-treated animals, respectively.

CONCLUSION: Our results provide further evidence for the neuroprotective effects of PACAP38 as given in form of a preischemic bolus. It slows down the progression of the evolution of the infarct and reduces the final infarct size. In contrast, a related peptide, VIP, does not have neuroprotective effects under the same experimental conditions.

Introduction

There is an increasing number of candidate therapeutic agents that are proven to be neuroprotective in animal models of stroke. In addition to classical neurotrophic factors, some neuropeptides have been shown to exhibit neurotrophic and neuroprotective effects [1–3], with vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) being among the main candidates [1]. They belong to the same peptide family (secretin/glucagon/VIP family), show closest structural homology among these related peptides and have numerous similar effects [4–6]. *In vitro*, both VIP and PACAP stimulate neuronal survival, regulate mitogenic activity and differentiation of embryonic neuronal cultures, and protect neurons against various toxic agents [1,4,6].

VIP and PACAP have also *in vivo* neurotrophic and neuroprotective effects. Both play a role in the development of the brain [7–10]. VIP analogs are protective in white matter lesions in leukomalacia [11,12], enhance cognitive functions in Alzheimer-related models [13,14], while antagonists retard neonatal behaviors and produce microcephaly [7,8]. Recent studies show neuroprotective effects of PACAP in animal models of other cerebral pathology. PACAP protects hippocampal neurons in global ischemia [15] and promotes the survival of forebrain cholinergic neurons after fornix transection [16].

We have previously demonstrated the protective effects of PACAP in a rat model of focal ischemia: PACAP reduced the infarct size when given postischemic, in transient Middle Cerebral Artery Occlusion (MCAO) [17]. This administration paradigm was in accordance with studies showing that most compounds investigated as possible treatments for stroke in animal models are maximally effective given as initial bolus followed by constant intravenous infusion [18]. However, for practical reasons it would be more acceptable to give treatment in the form of a single or intermittent bolus [18]; examination of the effectiveness of drugs as bolus injections is therefore reasonable. According to our previous observations, systemic administration of postischemic intravenous bolus injection of PACAP38 alone did not prove to be effective (unpublished observations). Similar observation was made in a model of global ischemia, due to a binding protein in the blood [15,19,20]. This, and the known potent peripheral vasodilator effect of PACAP restrict the systemic administration of higher doses of the peptide at present [6, 21]. Therefore, to investigate the effects of a single bolus injection, we chose to use intracerebroventricular administration in the present study.

The efficacy of a candidate neuroprotective drug may be very different under various experimental conditions: type of the occlusion (transient or permanent), beginning of administration, temperature and rat strain have been documented to influence the effects of drugs [22–27]. In order to extend our knowledge on the neuroprotective efficacy of PACAP in cerebral ischemia, we investigated the effects of PACAP38 in permanent focal ischemia, when given as a single intracerebroventricular bolus preceding the onset of the insult. A neuroprotective drug that is effective when given as a single preischemic bolus injection is especially important in certain neurosurgical interventions, where the time of the intervention is restricted to short intervals due to the risk of infarction [28]. In the present study we also examined the neuroprotective effect of the same doses of VIP, and the effect of both peptides on the progression of the infarct size, as measured 12 and 24 hours after the occlusion of the middle cerebral artery.

Materials and Methods

Male Wistar rats weighing 200–250 g were housed under diurnal lighting conditions. Animals were fasted overnight before the experiment, but were allowed free access to water. Animal housing, care and application of experimental procedures were in accordance with institutional guidelines under approved protocols.

Rats were anesthetized with intraperitoneal injection of 35 mg/kg pentobarbital. Animals were treated with PACAP (Sigma), VIP (Sigma) or vehicle using a stereotactic instrument. A hole with a diameter of 1 mm was drilled at 0.5 mm posterior, 1.5 mm lateral (left) from bregma point. The Hamilton needle was lowered to 3.5 mm deep from the dural surface, and 450 pmol PACAP38 (n=24) or VIP (n=24) dissolved in 2 μl sterile physiological saline was slowly injected into the lateral ventricle. Control animals (n=20) received 2 μl sterile physiological saline.

Immediately after the bolus injection of PACAP, the animals underwent occlusion of the middle cerebral artery, using the intraluminal suture technique described by Longa et al. [29]. Briefly, the left carotid region was exposed through a midline cervical incision. The external carotid and the common carotid arteries were ligated and a 4–0 nylon (Ethilon, Ethicon Inc., France) filament with its tip rounded was introduced from the carotid bifurcation into the internal carotid artery, until a mild resistance was felt (18–19 mm). The surgical procedure required 7–8 minutes. Those animals were excluded from further experiments, where MCAO surgery took longer times due to anatomical variance or difficulty in suture insertion, and thereby longer periods would have passed between treatment and MCAO. No recirculation was established, thereby the suture permanently occluded the middle cerebral artery. Control animals receiving vehicle treatment, un-

derwent the same procedures. The examiner performing the MCAO surgery was unaware of the treatment.

Temperature of the animals was kept in the normal range with a heating lamp during and after surgery. Among the physiological parameters, only systemic blood pressure was influenced by intravenous administration of PACAP in our previous study [17], therefore we measured blood pressure in 6 randomly chosen animals by catheterizing the left femoral artery. Blood pressure was monitored for 30 minutes following intracerebroventricular administration of PACAP or VIP. These animals were not included in the final analysis due to more extensive surgical intervention.

Animals prematurely died were not included in the final evaluation. Twelve or 24 hours after MCAO, animals were decapitated (PACAP: n=10 or 12, VIP: n=9 or 8, and control: n=10 or 15, respectively). Brains were removed and 2-mm-thick coronal sections were cut with the aid of a brain matrix (Braintree Sci.). The brain sections were stained with 2,3,5-triphenyl tetrazolium chloride (Sigma) and then fixed in formalin. Brain areas were traced and measured using an image analysis system, where unstained areas were defined as ischemic lesions. The areas of infarcted tissue and the areas of both hemispheres were calculated for each brain slice. An edema index was calculated by dividing the total volume of the hemisphere ipsilateral to MCAO by the total volume of the contralateral hemisphere. The actual infarct volume adjusted for edema was calculated by dividing the infarct volume by the edema index. Infarct volumes are expressed as percentage of the total brain volume ± SEM. The percentage of infarcted striatum and cerebral cortex were determined for each animal. Statistical analyses were conducted with a two-way ANOVA followed by a two-tailed Student *t-test*. Statistical significance was taken at $P < 0.05$ level.

Results

Body temperature was controlled for 6 hours following MCAO, and no significant difference was observed between temperatures of control and treated animals. Premature death was evenly distributed in the PACAP- or VIP-treated and the control groups: 1 control, 4 VIP-treated and 2 PACAP-treated animals died in the 12-hour-group, and 3 control, 2 VIP-treated and 3 PACAP-treated rats died prematurely in the 24-hour groups. This distribution of premature mortality suggests no specific relationship to the different drugs injected. Mean arterial blood pressure did not show any difference before and during the observed 30 minutes after the intracerebroventricular injection of 450 pmol PACAP38 or VIP in any of the 6 animals (data not shown).

The infarct volumes measured 12 hours after MCAO were 14.8±7.2% in the control animals, 15.2±7.3% in the VIP-treated group and 5.8±3.2% in the PACAP-treated group (Fig. 1). VIP treatment did not signif-
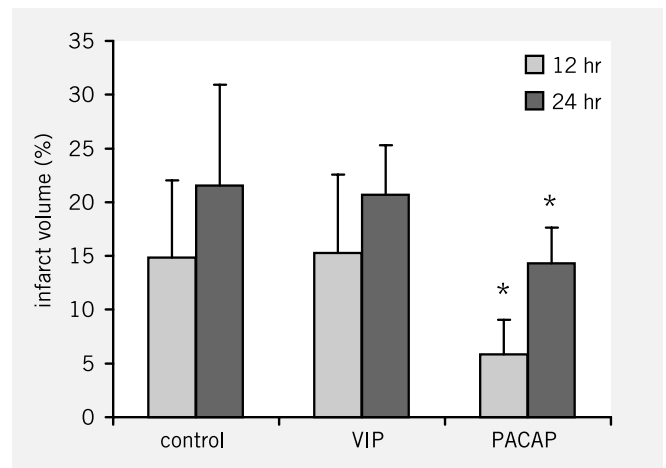
Figure 1. Effect of PACAP38 and VIP on the infarct volume measured 12 and 24 hours after permanent MCAO. Infarct volumes are expressed as the percentage of total brain volumes ± SEM. There was a significant reduction in infarct size in the PACAP-treated group versus the VIP- and vehicle-treated groups (*P< 0.05).

icantly alter the infarct size when compared to the vehicle-treated group, while PACAP treatment significantly reduced the infarct volume by approximately 60%. In animals killed 24 hours after MCAO, infarct volumes were 21.5±9.4%, and 20.6±4.6% in the control and VIP-treated groups, respectively. The infarct volume was 14.3±3.3% in the PACAP-treated rats Fig. 1). These results show that VIP did not change the infarct volume in the 24-hour group, while PACAP reduced it by 33%. This reduction resulted to be statistically significant. Fig. 2 and Fig. 3 show representative brain sections from control and VIP- or PACAP-treated animals. In summary, PACAP reduced the infarct volume measured at both time points.

Discussion

Our present study shows that PACAP reduces the infarct size and slows down the progression of the infarct in permanent occlusion of the middle cerebral artery. In contrast, a closely related peptide, VIP, does not alter the infarct size under the same experimental conditions.

PACAP and VIP belong to the secretin/glucagon/ VIP peptide family, and they share 60% of their amino acid sequences [4,6]. PACAP38 was isolated from ovine hypothalami on the basis of its ability to stimulate cAMP formation in rat pituitary cells [30]. Its potency of the adenylate cyclase activation is 1.000 to 10.000 times higher than that of VIP. A receptor, named PAC_1 binds only PACAP, while two other receptors $VPAC_1$ and $VPAC_2$ bind both PACAP and VIP with equal affinity [4]. PACAP and VIP have numerous similar effects, while others seem to be specific for PACAP or VIP only [4–6].

VIP was among the first candidate neurotrophic and neuroprotective neuropeptides *in vitro* [2]. It stimulates the release of neuronal survival factors from as-
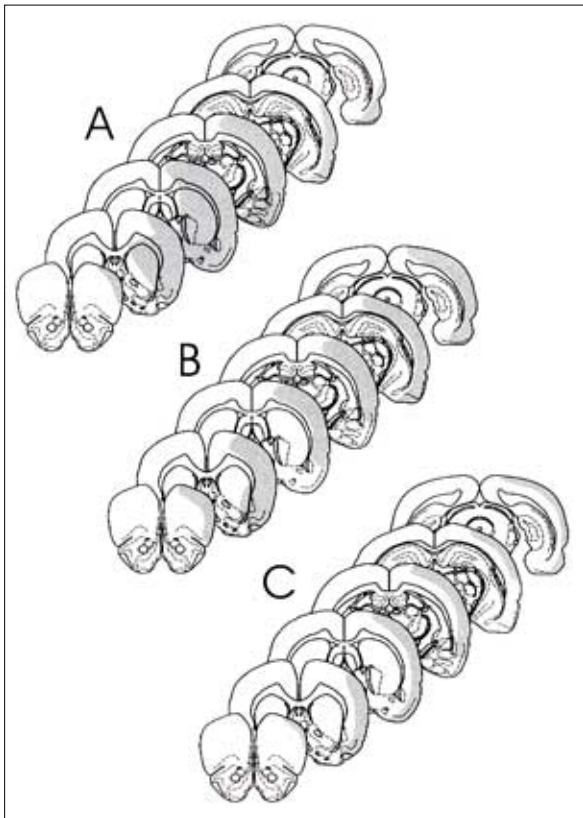
Figure 2. Representative distribution of ischemic damage (shaded areas) at different coronal levels in control (A), VIP- (B) and PACAP-treated (C) animals measured 12 hours after MCAO.
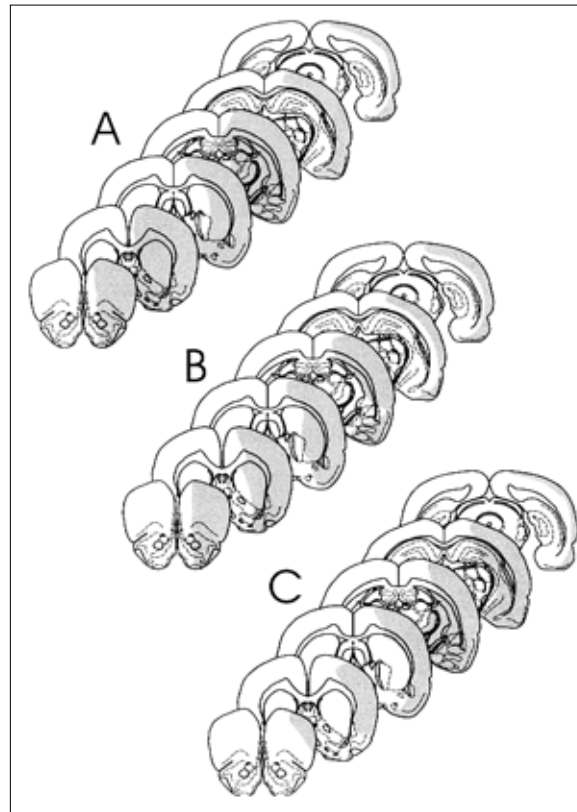
Figure 3. Representative distribution of ischemic damage (shaded areas) at different coronal levels in control (A), VIP- (B) and PACAP-treated (C) animals measured 24 hours after MCAO.

troglial cultures and regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts or glial cells [31,32]. VIP antagonists have been reported to decrease neuronal survival [33,34]. VIP provides neuroprotection against various neurotoxic agents, like beta-amyloid toxicity in models of Alzheimer's disease [13], dopamine toxicity in models of Parkinson's disease [35], and HIV envelope protein [36]. It also has protective effects against anoxia/glucopenia injury in peripheral nerves [37]. *In vivo*, it is suggested to have a role in the development of the brain: VIP antagonists produce severe microcephaly and retard the development of neonatal behaviors in the rat [7,8]. VIP analogs have therapeutic potential in human premature babies for being protective in white matter lesions in a mouse model of periventricular leukomalacia [11,12]. VIP derivatives have also been shown to enhance cognitive functions in Alzheimer's disease-related *in vivo* models [13,14].

The reports on the neuroprotective efficacy of VIP triggered similar investigations on PACAP soon after its discovery. Numerous studies have documented its neurotrophic activity *in vitro*: PACAP stimulates growth and survival of neurons, prevents apoptosis in various embryonic neuronal cultures [4,6] and protects neurons against neurotoxicity induced by various agents, such as glutamate, 6-hydroxidopamine, lipopolysaccharide and HIV envelope protein [4,38–41].

PACAP has important functions also during ontogenesis of the nervous system [9,10,42].

In vivo studies have reported that PACAP or its receptors can be upregulated after various neuronal insults, like focal ischemia in mice [43], contusion brain injury and peripheral nerve injuries [44,45]. These studies imply the possibility that endogenously occurring PACAP may attenuate neuronal damage resulting from certain types of insults. Only few reports have documented the efficacy of exogenously given PACAP against different types of neuronal injuries: it prevents ischemia-induced neuronal death of hippocampal neurons following global ischemia [15] and promotes survival of basal forebrain cholinergic neurons after fornix transection [16]. Recently, we have shown neuroprotection by low doses of PACAP in a transient MCAO in rats, when a systemic, 48-hour-administration was started with 4 hours of delay [17]. Although PACAP crosses the blood-brain barrier [46,47], administration of higher doses is restricted due to its vasodilator effects resulting in lowering the blood pressure [21,48], and the presence of a binding protein in the plasma [19, 20]. Intracerebroventricular administration allowed us to investigate the effects of higher doses of PACAP38, for lower doses of 50, 100 and 200 pmol PACAP did not prove to be effective under the same conditions (unpublished observations). The efficacy of the same drug may be very different when given pre- or post-

ischemic, or using it in permanent or transient occlusion [22,23,26,27,49]. In the present study, we showed that PACAP is also neuroprotective when given before permanent occlusion of the middle cerebral artery. This administration paradigm may be of clinical importance, since occlusion of arteries in certain neurosurgical interventions is limited due to the risk of infarction [28]. We also showed that PACAP slows down the progression of the infarct, as measured 12 hours after MCAO. This can be of clinical importance, since combining PACAP with other neuroprotective agents during the critical first hours after ischemia may attenuate neuronal damage to a greater extent.

Our present study shows that PACAP is more effective in reducing brain damage in focal ischemia than VIP. Although the exact mechanism of PACAP is not known, this difference could be due to PACAP exerting neuroprotection by acting on its specific receptors (PAC1-R) or by its 10.000 times more potent ability to activate adenylate cyclase [4,30]. As far as neuroprotective mechanisms are considered, numerous similar observations have been made for VIP and PACAP. Low doses of both peptides seem to have indirect effects through glial cells: they regulate the activity of glial cells and stimulate different chemokins and trophic factors from astrocytes [36,38,50–54]. Activity dependent neurotrophic factor (ADNF) has been isolated under the action of VIP, which, along with its shorter fragments exhibit even more potent neuroprotection [50]. Antiserum to ADNF has been reported to produce neuronal cell death in neuronal cultures [55]. Efforts have been made to develop VIP agonists and to map the active site of the peptide to increase its neuroprotective efficacy [14,56]. The protective mechanism of PACAP may include direct neuronal effects, which require higher doses of PACAP, as it has been shown on cerebellar granular cells [57,58]. According to our present results, it may be suggested that for preventive neuroprotection, higher doses may be required, which exceed the physiological concentrations [4]. This may also explain the difference between the neuroprotective effects of VIP and PACAP. Similar observations have been made on rat cerebral microvessels, where PACAP proved to be more potent in inhibiting the cyclooxygenase pathway than VIP [59]. Also, VIP has been shown to mimic the actions of PACAP in astrocytes at much higher concentrations [60]. However, based on the numerous observations on the efficacy of VIP to reduce neuronal injuries, further studies with VIP or its more potent analogues may reveal effective neuroprotection in cerebral ischemia.

Acknowledgements

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