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Short communication

Vasoactive intestinal peptide protects guinea-pig detrusor nerves from anoxia/glucopenia injury

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Abstract

Vasoactive intestinal peptide (VIP) was tested for its capability to protect the intrinsic nerves of guinea-pig urinary bladder from damage due to anoxia/glucopenia and reperfusion. Guinea-pig detrusor strips were mounted for tension recording in small organ baths and the nerves were subjected to electric field stimulation. VIP (0.3 μM) improved significantly the response of strips to electrical field stimulation either during anoxia/glucopenia or thereafter during reperfusion, as compared to untreated tissues. The antioxidant activity of VIP assessed as its capability to scavenge peroxyl radicals during linoleic acid oxidation corresponded to 6.42 ± 0.13 pIC_{50} M, i.e. close to the concentration proved to protect strips against the anoxic–glucopenic and reperfusion damage.

Keywords: Vasoactive intestinal peptide (VIP); Antioxidant; Detrusor; Ischaemia–reperfusion injury; Protection

1. Introduction

Clinical and experimental data suggest that ischaemic neuronal damage is partly induced by free radicals and/or lipid peroxidation, during both ischaemia and subsequent reperfusion phase (Maxwell and Lip, 1997). Many different drug treatments have been tried to curtail this damage, but, even if some calcium blocking agents or some K^+ openers have been shown to protect the neurogenic response following ischaemic injury (Levin et al., 1999), none has proved universally satisfactory.

Periodic bladder ischaemia during obstructed micturition has been suggested to result in the partial denervation of the detrusor smooth muscle, through ischaemia and reperfusion injury to the post-ganglionic parasympathetic neurones within the bladder wall (Brading, 1997). Pessina et al. (1997) have demonstrated that in conditions mimicking ischaemia and reperfusion, the intrinsic nerves were more susceptible than the detrusor smooth muscle.

The urinary bladder neuromodulator vasoactive intestinal peptide (VIP) possesses the ability to scavenge oxygen free radicals (Misra and Misra, 1990; Kalfin et al., 1994), and to reduce the intracellular Ca^{2+} overloading in ischaemic and reperfused heart (Kalfin et al., 1994).

The purpose of this study was, therefore, to examine the efficacy of VIP, to counteract the damage suffered by neurones in urinary bladder strips exposed "in vitro" to conditions mimicking ischaemia–reperfusion.

2. Materials and methods

2.1. Detrusor preparation

The experiments were conducted on 3- to 5-weeks-old Charles River guinea-pigs of either sex (350–500 g), anaesthetised with Ketavet®, killed by cervical dislocation. The bladders were removed, opened along the ventral surface, and the urothelium was removed from the underlying smooth muscle. Strips (approximately 1-mm wide, 0.5-mm thick, 8-mm long) were dissected from the detrusor following the direction of the muscle bundles. Fine silk ligatures were tied to each end of the strips and were mounted in small (0.2 ml) organ baths between two platinum ring electrodes 1 cm apart. Strips were continuously superfused with Krebs solution pumped by a peristaltic pump at a constant rate of 1.5 ml/min. The Krebs solution consisted of the following ion concentrations (in mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.5, NaH_2PO_4

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1, $CaCl_2$ 2.5, glucose 11.5. Strips were placed under an initial tension of 1 g and allowed to equilibrate for at least 60 min. Four organ baths were mounted in parallel above an enclosed water bath, maintaining the solution temperature at 37 °C.

Contractions were measured isometrically using mechanoelectrical transducers (Basile, Italy) and a four-channel pen recorder (Linseis, Germany). Electrical field stimulation was delivered via an Harvard stimulator (USA) previously calibrated with a Tektronix's digital oscilloscope (TDS, 754 D).

Under normal conditions, the Krebs solution was bubbled with 95% O_2, 5% CO_2, while under ischaemia-like conditions, glucose was replaced isosmotically with NaCl, the solution was gassed with 95% N_2, 5% CO_2. Oxygen tension in the organ baths was measured using a fine platinum oxygen electrode, previously calibrated in solutions of known oxygen tension (Pessina et al., 1997).

2.2. Effects of the VIP on detrusor muscle tone and electrical field stimulation-induced contraction under normal conditions

After 60-min equilibration period, detrusor strips were stimulated with 100 μM carbachol (applied for 10 s), after which strips were washed for up to 30 min. Subsequently, tissues were subjected to electrical field stimulation, 5 s trains of square voltage pulses (0.05 ms duration, 40 Hz, 50 V) with 15-min intervals allowed between stimulation. As soon as a reproducible response was obtained, VIP was added to the perfusing Krebs solution at fixed concentrations and kept for 90 min. Its effect on the muscle tone was observed for 30 min before stimulating electrically the strips every 30 min for 210 min to reproduce the ischaemia–reperfusion experiments in normal conditions. At the end of the experiment, carbachol was applied again.

2.3. Effect of ischaemia- and reperfusion-like conditions

After the equilibration period and the carbachol application (see above), the tissue was subjected to electrical field stimulation every 15 min until the response was reproducible. Then, detrusor strips were subjected to 60 min of ischaemic-like conditions followed by 150 min of reperfusion. During this 210-min period, intrinsic nerves (as the response was abolished in the presence of tetrodotoxin, see below) were stimulated electrically every 30 min. The response of the strips to electrical field stimulation was expressed as a percentage of the initial response in Krebs solution, taken to be 100%. VIP, at different concentrations, was added to the perfusion medium during the ischaemic conditions and the first 30 min of reperfusion. At the end of the experiment, muscle response of the strips was tested again applying 100 μM carbachol.

In a collateral experiment, to block nerve impulse propagation (Pessina et al., 2001), six strips ($n = 3$) were perfused with Krebs solution containing 1 μM tetrodotoxin during both the ischaemic- and the reperfusion-like conditions.

2.4. Inhibition of lipid peroxidation

The present experimental model system was based on the oxidation of linoleic acid initiated by 2,2′-azobis-2-amidinopropane hydrochloride (ABAP), a thermolabile azo compound which, on decomposition, forms radicals that remove hydrogen atoms from linoleic acid. O_2 consumption was monitored for approximately 5 min (control), five increasing concentrations VIP, dissolved in water were added to the peroxidising system. Oxygen consumption due to ABAP decomposition was determined separately and subtracted from that measured in the presence of linoleic acid (Sgaragli et al., 1993). Experiments were repeated at least three times. Results were expressed as percentage decrease with respect to control values and the pharmacological response to VIP was described as mean ± S.E.M. pIC_{50} M.

2.5. Chemicals

ABAP was purchased from Polysciences (USA). Carbachol, linoleic acid, tetrodotoxin and VIP were purchased from Sigma (Milan, Italy).

Stock solutions of VIP and tetrodotoxin in Type I Reagent Grade water (resistivity of 18 MΩ) were stored at −20 °C.

2.6. Data analysis

Results were expressed as mean ± S.E.M. Statistical analysis of the data was performed by Student's *t*-test for unpaired data or by one-way analysis of variance (ANOVA) followed by Dunnet's post-test. *P*-values < 0.05 were considered significant. The antioxidant effect of VIP was described by pIC_{50} M value, calculated by non-linear regression analysis.

Data were also expressed as mean of area under curves (AUCs) (GraphPad Prism program) of strips untreated or treated with the compound at various concentrations, calculated by processing separately data obtained when strips were exposed to the anoxia/glucopenia or the reperfusion phase.

3. Results

3.1. Effects of VIP on detrusor muscle tone and on electrical field stimulation-induced contraction under normal and ischaemia- and reperfusion-like conditions

The effect of VIP on either the resting tone or the response to electrical field stimulation in normal condi-

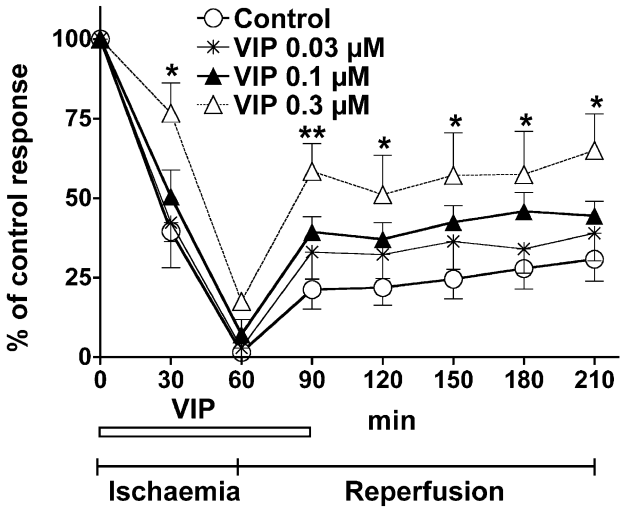

Fig. 1. Electrical field stimulation-induced contractile responses of guinea-pig detrusor strips subjected to 60 min of ischaemia and subsequent 150 min of reperfusion. Experiments carried out in the absence or presence of VIP. VIP was applied for the first 90 min of the experiment. Results are expressed as mean ± S.E.M. ($n = 5$–17). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-test. Significant differences from the control group are indicated; $^{*}P < 0.05$; $^{**}P < 0.01$. Horizontal bar represents the perfusion time with the compound added to the Krebs perfusing solution.

tions was investigated. When VIP was added at different concentrations in the perfusing medium, no alteration of the resting tone was observed. VIP was perfused for 90 min and the strips stimulated electrically every 30 min. VIP (0.03, 0.1 and 0.3 μM) did not affect the electrical field stimulation induced response under normal conditions both when present and when removed from the perfusing solution (data not shown).

The response to electrical field stimulation declined rapidly in the combined absence of oxygen and substrate, being abolished within an hour. After reintroduction of normal conditions, the recovery of the response to electrical field stimulation was poor, reaching a maximum of about 25% of the initial response in 2 h. At this time, however, the response of the muscle to carbachol had fully recovered.

To see if VIP could partially reduce the nerve damage described above, the peptide has been perfused during anoxia/glucopenia phase and the first 30 min of reperfusion as it is supposed that the major damage to the tissue develops not only during ischaemia, but also when the oxygen reaches again the tissue.

In order to distinguish the neurogenic response from any myogenic one, in a collateral experiment, some muscle strips were perfused with 1 μM tetrodotoxin throughout the experimental procedure. The only component involved in the electrical field stimulation-induced response detected in strips not exposed to tetrodotoxin was neurogenic in nature, as the latter was abolished in the presence of 1 μM tetrodotoxin both in anoxia/glucopenia and reperfusion phase (data not shown).

The time course of the change in response of detrusor muscle strips to electrical field stimulation in the control and the VIP-treated groups is shown in Fig. 1. VIP at 0.3 μM concentration improved significantly the electrical field stimulation-induced contractile response both in anoxia/glucopenia and in reperfusion phase, reaching 65.00 ± 11.49% ($n = 5$) at 210 min, as compared to 30.81 ± 6.97% of control, ($n = 9$; $P < 0.05$). At 0.03 μM, it did not exert any effect, while at 0.1 μM, it showed to increase the electrical field stimulation contractile response even though the latter being significantly different from control only at 90 min (39.40 ± 4.77%, $n = 5$ and 21.25 ± 6.19%, $n = 9$, respectively; $P < 0.05$).

The AUCs which relate muscle contraction on electrical field stimulation vs. time either in anoxia/glucopenia and in reperfusion phases, are shown in the inset of Fig. 2. Both the anoxia/glucopenia and the reperfusion phase AUCs of 0.3 μM VIP treated strips were significantly higher ($P < 0.05$) than AUCs of control strips. This effect, however, was not present for any of the other concentrations tested.

3.2. Peroxyl radical scavenging activity

The antioxidant activity of VIP was assessed for its capability to prevent linoleic acid peroxidation. VIP exhibited remarkable antiperoxidant activity with a pIC_{50} M value of 6.42 ± 0.13, i.e. close to the concentration proved

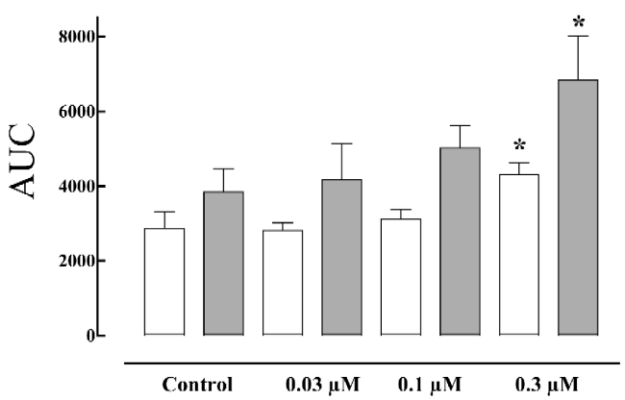

Fig. 2. Electrical field stimulation-induced contractile responses of guinea-pig detrusor strips subjected to 60 min of anoxia/glucopenia and subsequently to 150 min of reperfusion with normal Krebs solution. Experiments carried out in the absence or presence of VIP. VIP was applied at 5 μM concentration during the anoxia/glucopenia phase and the first 30 min of reperfusion. Results are expressed as mean of the area under curve (AUC) ± S.E.M. ($n = 5$–17), calculated in the anoxia/glucopenia (white bars) and reperfusion (shaded bars) phase separately. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-test. Significant differences from the control group are indicated; $^{*}P < 0.05$.

to protect strips against the anoxic–glucopenic and reperfusion damage.

4. Discussion

The structure and function of detrusor smooth muscle may be altered by a series of *noxae* including hypoxia, over distension, diabetes, and ischaemia. In a recent study, it is shown that both experimental ischaemia and partial outlet obstruction of the urinary bladder induce similar dysfunction with regard to the contractile responses to electrical field stimulation (Zhao et al., 1997). It has been demonstrated that in vitro ischaemia-like conditions (anoxia plus glucopenia deprivation) was more damaging to the nervous tissue of the detrusor muscle than singularly applied anoxia or glucose deprivation (Pessina et al., 1997). The previous study, as well as the present investigation, showed that the response to nerve-mediated stimulation declined rapidly as O_2 and glucose were withdrawn and recovered only to a limited extent when perfusion with O_2 and glucose started again. In the present study, it was shown that VIP improved this recovery by protecting nervous tissues against anoxia/glucopenia damage. To elicit neuroprotection, however, strips were incubated with VIP either during the anoxia/glucopenia phase and the first 30 min of reperfusion. In the last few years, increasing evidence has been accumulating that reactive O_2 species, i.e. hydrogen peroxide (H_2O_2) as well as superoxide anion (O_2^-) and hydroxyl radical ($^{\cdot}OH$) generated in greater amounts after reperfusion of the ischaemic tissue (Bulkley, 1987; Park and Granger, 1988) are important mediators of tissue injury during reperfusion following an ischaemic insult. Reactive O_2 species, including the singlet molecular O_2 ($^1O_2$), by promoting lipid peroxidation can damage cell membranes, causing severe dysfunction with impairment of intracellular Ca^{2+} homeostasis. In turn, increases of intracellular Ca^{2+} concentration may activate a cascade of events, which leads to neuronal cell death.

VIP has been shown to protect myocardium (Kalfin et al., 1994), renal tissue (Uzuner et al., 1995) and skeletal muscle (Tuncel et al., 1997) from severe ischaemia–reperfusion injury and to directly scavenge singlet oxygen as efficiently as beta carotene (Misra and Misra, 1990). In the present study, VIP at 0.3 μM concentration protected effectively urinary bladder strips undergoing anoxia/glucopenia–reperfusion injury. Moreover, our study demonstrated for the first time that VIP exhibits significant antiperoxidant activity, although the molecular mechanism by which it scavenges peroxyl radicals is still unknown. We can hypothesize that its antioxidant activity is important for the neuroprotection afforded on guinea-pig detrusor strips subjected to anoxia/glucopenia and reperfusion. In fact, α-tocopherol and some hindered phenols, like BHA (2-*tert*-butyl,4-hydroxyanisole) and di-*tert*-BHA, have been demonstrated to protect the detrusor nerves from ischaemia-like and reperfusion injury and their neuroprotection has been shown to correlate with their antioxidant activity (manuscript in preparation).

VIP has been shown to relax rabbit (Levin and Wain, 1981), human and pig (Klarskov et al., 1984) detrusor strips, thus suggesting its role as a potential inhibitory transmitter, while Sjogren et al. (1985) have observed only a minimal relaxant effect of VIP on human bladder, in agreement with our observation and that of Callahan and Creed (1986) on guinea-pig urinary bladder strips. The mechanism(s) by which VIP protects the detrusor muscle nerves from anoxia/glucopenia and reperfusion injury is only a matter of speculation, though a loss of VIP among other sensory neuropeptides in the obstructed human bladder has been previously described (Chapple et al., 1992). In conclusion, the pharmacological action of VIP, outlined in the present investigation, possibly mediated also by a specific receptor(s) activation, may represent a new therapeutic option for the control of functional disorders of the bladder.

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