Effect of illuminated nifedipine, a potent antioxidant, on intestinal and vascular smooth muscles

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1 The effects of nifedipine (Nif) and its illuminated nitroso product nitrosopine (NTP) were investigated on lipid peroxidation, KCl elevated smooth muscle tension, and ionic currents of single smooth muscle cells.

2 Illumination of Nif at 400–700 nm within 24–48 h changed it completely to a potent antioxidant, NTP.

3 Nif relaxed the KCl-induced contractions of guinea-pig taenia caeci and rat aorta and reduced the amplitude of the evoked inward Ca^{2+} current of taenia caeci cells in a concentration-dependent manner. NTP (up to 100 μM) was ineffective in this respect. Pretreatment by NTP (10 μM) did not affect the actions of Nif.

4 The evidence suggests that NTP, generated by day-light illumination from Nif, exerts antioxidant activity but is devoid of voltage-dependent Ca^{2+} channel (VDC) blocking property and does not interfere with the action of Nif on the smooth muscle cell membrane VDC.

Keywords: Illuminated nifedipine; smooth muscle; calcium current; antioxidant

Introduction

Ca^{2+} channel blockers (CCB) are widely used in treatment of smooth muscle disorders (e.g. ischaemic heart disease, Follath, 1989). Their beneficial effect is ascribed to inhibition of Ca^{2+} influx into the smooth muscle cells (Godfraind *et al.*, 1986; Speralakis *et al.*, 1992). It has been shown that some of the CCB also possess a free-radical scavenging or antioxidant activity (Mak & Weglicki, 1990). Nifedipine (Nif; 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) exposed to daylight converts to the nitroso-compound nitrosopine (NTP; 2,6-dimethyl-4-(2-nitroso-phenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, Majeed *et al.*, 1987).

NTP was recently described as a potent free radical scavenger with the ability to inhibit lipid peroxidation (Mišik *et al.*, 1991). The aim of this study was therefore to prepare a constantly illuminated and chemically pure NTP and compare its action on smooth muscle tension, membrane currents and lipid peroxidation with that of Nif.

Methods

Guinea-pigs (weighing 400–600 g) and rats (weighing 200–300 g) were killed by cervical dislocation and exsanguination. Taenia of the guinea-pig caecum and rat aortae were isolated. Longitudinal muscle strips of the taenia caeci, 20 mm in length (Kadlec & Bauer, 1974) and rings of the aorta, 3 mm wide (Biamino & Knuckenberg, 1969) were used for isometric recording of the muscle tension. After 60 min stabilization, the experiments were carried out under an initial tension of 10 mN and at 37°C.

For membrane current recording, the smooth muscle cells of the taenia caeci were isolated enzymatically according to Soloviev *et al.* (1991) using a mixture of 2 mg collagenase and 3 mg bovine serum albumin in 1 ml of nominally Ca^{2+} -free external solution. Otherwise the procedure was similar to that

described earlier (Bauer & Ito, 1991; Bauer *et al.*, 1994). The ionic currents were recorded with glass capillary microelectrodes (resistance of 3–5 M Ω) at room temperature using a patch clamp amplifier (Axopatch-1D) under whole-cell voltage clamp configuration (Hamill *et al.*, 1981).

To study the effects of the drugs on lipid peroxidation (LPO), egg phosphatidyl choline (PC) liposomes were used; 20 μl of egg yolk PC (10 mg ml^{-1} ethanol) was evaporated under a stream of N_2 followed by 30 min evaporation in a vacuum ($\leq 10^{-2}$ Pa). The dried samples were hydrated with 240 μl of the buffer (in mM): NaCl 150, Tris 5, pH 7.4 (HCl). The LPO was elicited by addition of 5 μl of 2 mM FeSO_4 and 5 μl of 1 mM H_2O_2 to these samples. The relative LPO was assessed by measuring the formation of 2-thiobarbituric acid reactive (TBA) products, mainly of the TBA-malondialdehyde complex according to a slightly modified (Ondriaš *et al.*, 1989) method of Haenen & Bast (1983). After 60 min incubation of the samples at 37°C, 150 μl of butylated hydroxytoluene (BHT) (75 mg BHT 50 ml^{-1} ethanol) was added to prevent further LPO. Then 1.5 ml of TBA solution (2.1 g TBA, 84 g trichloroacetic acid, 3.57 ml of 37% HCl diluted with H_2O to 500 ml) was added and the samples were incubated for 20 min at 80°C. The samples were then cooled at 10°C in water bath and centrifuged at 1600 g 5 min. The supernatant was analyzed spectroscopically, where the absorption at [534 nm – (500 nm – 576 nm)/2] was taken as a relative value of LPO.

Solutions

The bathing solutions were (in mM): NaCl 117, KCl 5, CaCl_2 2.5, MgSO_4 1.2, NaH_2PO_4 1.2, NaHCO_3 25, glucose 11, pH 7.4, for mechanical recording and NaCl 135, KCl 6, CaCl_2 2.5, MgCl_2 1.2, NaH_2PO_4 1.0, NaHCO_3 10, HEPES 10, glucose 5.4, pH 7.4 (NaOH) for patch clamp recording. The bathing solutions were gassed with 95% O_2 and 5% CO_2 . The storage solution for isolated cells contained only 0.3 mM CaCl_2 . The pipette solution for measuring the Ca^{2+} current contained (in mM): CsCl 140, MgCl_2 2.5, HEPES 10, ATP 2, EGTA 1, pH 7.2 (NaOH). In potassium depolarizing solution 100 mM KCl was substituted for NaCl.

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Chemicals

NTP was prepared from dissolved Nif (10 mM) in ethanol (u.v.-spectroscopy purity, Lachema) by daylight illumination in a glass tube (wall thickness of 1 mm) using a fluorescent lamp at a wave length corresponding to daylight (400–700 nm) at a distance of 10 cm for different periods of time.

The conversion of Nif (Bayer) to NTP and its purity were determined by gas chromatography mass spectrometry (GC-MS) using the HP 5890A gas chromatograph of Hewlett-Packard, equipped with an HP 5791A mass selective detector and HP 7673A autosampler. Quantification of Nif and NTP in the samples was made by means of the internal standard (darodipine, Sandoz) as well as by addition of known amounts of Nif or NTP to the samples. The lowest amounts detectable by the method used were 10 pg Nif 100 μl^{-1} injection and 50 pg NTP 100 μl^{-1} injection. In control experiments an appropriate amount of the solvent was added to the bathing solution. TBA was purchased from Fluka and BHT from Sigma.

Statistics

Results are expressed as means $\pm$ s.e.mean. Data were analyzed according to Student's *t* test. Probability levels of less than 0.05 were taken as indicating significant differences between means. The pD_2 ($-\log \text{EC}_{50}$) values were calculated according to Arunlakshana & Schild (1959). Acquisition and calculation of patch clamp data were performed by pCLAMP software (Axon Instruments).

Results

Determination of purity by GC-MS showed that the original batches of Nif contained less than 0.05% of NTP. Daylight illumination lasting 24 h resulted in an almost complete conversion of Nif to NTP. To reach full conversion (reduction of Nif concentration by at least 10^5 times) illumination lasting for 48 h was needed. After 48 h there were no traces of Nif in the NTP spectra at maximal gain (10 pg 100 μl^{-1}). The resulting NTP remained unchanged during the following 8 days (Figure 1). Such NTP was used in further experiments.

The lipid peroxidation measured as MDA production under the control conditions was 0.033 ± 0.004 . Nif (25 μM) did not affect (reduction was to 0.028 ± 0.005) significantly ($P < 0.26$) this MDA production while NTP (25 μM) significantly ($P < 0.0001$) reduced it to 0.0034 ± 0.0003 .

In contrast to Nif which relaxed the KCl (100 mM) pre-contracted guinea-pig taenia caeci ($\text{pD}_2 = 8.64 \pm 0.5$) and rat aorta ($\text{pD}_2 = 8.53 \pm 0.2$) with a threshold in the nanomolar concentration range, NTP in concentrations up to 10 μM (when the applied samples contained less than picomolar concentrations of Nif) did not affect the smooth muscle tension elevated by KCl in the guinea-pig taenia caeci ($n = 9$) and rat aorta ($n = 7$). Pretreatment with NTP (10 μM) for 10 min and in the continued presence of NTP in the bathing fluid did not change significantly the concentration-response curve of Nif. The pD_2 values of Nif in these conditions were 8.97 ± 0.6 (taenia caeci, $n = 7$) and 8.74 ± 0.3 (aorta, $n = 5$).

Depolarization of the membrane of isolated single smooth muscle cells of the taenia caeci under whole cell configuration

Figure 1 Gas chromatograms and mass spectra of nifedipine (Nif) before illumination (a) and nitrozopine (NTP) after 24 (b) and 192 h (c) illumination of Nif. Darodipine (Dar) was the internal standard (d).

Figure 2 Effect of nifedipine (Nif, 0.1 μ M) and its nitroso-product nitroso-nifedipine (NTP, 10 μ M) on inward calcium current (I_{Ca}) in smooth muscle cells isolated from guinea-pig taenia caeci. Depolarization steps from -60 mV to 0 mV of 50 ms duration were applied. The pipette solution contained 1 mM EGTA. ($\square$) Control response; ($\blacksquare$) response in the presence of Nif (a); or NTP and Nif (b); ($\circ$) response in the presence of NTP. Concentration-dependent effect of Nif ($\bullet$) and NTP ($\square$) on peak I_{Ca} is shown in (c). Each point represents the mean with s.e.mean of at least six determinations.

from the holding potential (HP) of -60 mV to 0 mV for 50 ms, induced an inward current followed by an outward one. When CsCl replaced KCl in the pipette solution, only inward current was recorded. Ethanol, used as a solvent in our experiments, even at its highest final concentration (0.5 mM) in the bathing fluid did not alter the spontaneous and evoked mechanical and membrane responses ($n=4$).

Neither Nif nor NTP, in the concentration-range from 0.1 nM to 10 μ M, influenced the holding membrane current ($n=5$). Nif (0.1 nM– 10 μ M) decreased the evoked inward calcium current induced by membrane depolarization (Figure 2a) in a concentration-dependent manner with $pD_2 = 7.82 \pm 0.11$ (Figure 2c). In contrast, NTP did not affect significantly the inward Ca^{2+} current up to a concentration of 0.1 mM (Figure 2b,c). Pretreatment with NTP for 10 min and its presence in the bathing fluid at a concentration of 10 μ M, which did not alter the inward calcium current significantly ($103.9 \pm 2.3\%$ in its presence, $n=7$) had no significant effect on the action of Nif (0.1 μ M) (Figure 2b). Nif reduced the inward calcium current to $18.9 \pm 1.8\%$ ($n=7$) in the absence and to $23.1 \pm 1.9\%$ ($n=7$) of the control values in the presence of NTP.

Discussion

In agreement with the results of Majeed *et al.* (1987) the nitro-group on the aromatic ring of Nif is converted to nitroso-group forming NTP under the illumination at $\lambda=400$ –

700 nm. To reach full conversion (reduction of Nif concentration by at least 10^5 times) under our conditions, illumination lasting from 24 to 48 h was needed. The resulting NTP possesses roughly 10 times higher antioxidative activity than unchanged Nif.

Nif as described earlier (Sperelakis *et al.*, 1992) decreased the muscle tension and the inward Ca^{2+} current. In contrast to the increased antioxidative activity, illumination of Nif led to gradual loss of its ability to relax the precontracted smooth muscles (guinea-pig taenia caeci, rat aorta) and to reduce the inward Ca^{2+} current of isolated smooth muscle cells. Since after pretreatment with NTP and even in its presence in high concentration in the bathing fluid, NTP did not influence the action of Nif on muscle strips and isolated cells, we might conclude that NTP does not bind to the binding site for Nif in the calcium channel of the smooth muscle membrane. Thus conversion of a part of the administered dose of Nif to NTP does not interfere with the action of its unaltered molecules.

Reactive oxygen species contribute to the tissue damage caused by atherogenesis and ischaemia/reperfusion. The possible role of an antiperoxidative action of calcium channel blockers in suppression of the atherogenesis has recently been suggested (Henry, 1991). Thus the molecules of Nif converted into NTP might also have been involved in such effect.

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