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Short communication

Subsensitivity of P2X but not vanilloid 1 receptors in dorsal root ganglia of rats caused by cyclophosphamide cystitis

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Abstract

The application of cyclophosphamide to rats was used to induce interstitial cystitis. Behavioural studies indicated a strong pain reaction that developed within 2 h and levelled off thereafter causing a constant pain during the following 18 h. Neurons prepared from L6/S1 dorsal root ganglia innervating the urinary bladder responded to the application of capsaicin or α,β-methylene ATP (α,β-meATP) with an increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ responses to capsaicin were identical in the dorsal root ganglion cells of cyclophosphamide- and saline-treated rats, whereas α,β-meATP induced less increase in $[Ca^{2+}]_i$ in the cyclophosphamide-treated animals than in their saline-treated counterparts. Hence, α,β-meATP-sensitive $P2X_3$ and/or $P2X_{2/3}$ receptors of L6/S1 dorsal root ganglion neurons were functionally downregulated during subacute pain caused by experimental cystitis. In contrast, capsaicin-sensitive vanilloid 1 receptors did not react to the same procedure. Thoracal dorsal root ganglia, not innervating the urinary bladder, were also unaltered in their responsiveness to α,β-meATP by cyclophosphamide treatment.

Keywords: Dorsal root ganglion; P2X receptor; Vanilloid 1 receptor; Cyclophosphamide-induced cystitis

1. Introduction

Dorsal root ganglia are endowed with co-localized populations (Vulchanova et al., 1998) of cationic channels gated either by ATP ($P2X_3$ receptors; Chizh and Illes, 2001) or by pungent compounds such as capsaicin, but also by noxious heat and acidic pH (vanilloid 1 receptors; Szallasi and Blumberg, 1999). In a neuropathic pain model, a differential regulation of the two receptor profiles has been observed, i.e. the $P2X_3$ receptor density did not change, although vanilloid 1 receptors were markedly upregulated leading to thermal hyperalgesia (Fukuoka et al., 2002). By contrast, chronic inflammatory pain caused an upregulation of $P2X_3$ receptors in dorsal root ganglia that in turn could account for neuronal hypersensitivity (Xu and Huang, 2002).

$P2X_3$ receptor-deficient mice exhibit a marked urinary bladder hyperreflexia, characterized by decreased voiding frequency and increased bladder capacity, but normal bladder pressures (Cockayne et al., 2000). Moreover, cyclophosphamide-induced cystitis causes a reduced sensitivity of $P2X_1$ receptors at smooth muscle strips of the urinary bladder towards the agonistic β,γ-methylene ATP (Mok et al., 2000). Hence, the present experiments were aimed at clarifying the question, whether during cyclophosphamide cystitis L6/S1 dorsal root ganglia innervating the urinary bladder (Applebaum et al., 1980) also exhibit a functional downregulation of P2X receptors with or without an accompanying change in vanilloid 1 receptor function. An increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was used as a measure of receptor activation.

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2. Materials and methods

2.1. Behavioural study

Rats (own strain WIST/Lei) weighing 180–220 g were used. All procedures conformed to the ethical guidelines regarding the care and use of animals and were approved by the Committee on Animal Care and Use of the relevant local governmental body (permission, TVV 28/01). Cystitis was induced by intraperitoneal application of cyclophosphamide (200 mg/kg; Sigma), dissolved in 0.9% NaCl; control rats received the solvent only. The behavioural parameters associated with cystitis-induced pain were scored by a procedure modified after Lanteri-Minet et al. (1995). Pain scores were obtained for 15-min periods throughout the observation time of 4 h immediately after injection and for 30 min 20 h after injection. The severity of pain behaviour was classified according to a scale as follows: 0 = alertness, normal behaviour and mobility; 1 = sedation, open eyes; 2 = decreased breathing rate, half-closed eyes or sleeping behaviour without decreased breathing rate; 3 = decreased breathing rate, flat position, limpness, nearly closed eyes, apathy; 5 = like score 4 but brief "crises" (backward walking, abdominal stretching, twitches). When the animal changed its behaviour between different stages within the 15-min observation period, the mean value of two scores was given. In addition, the breathing frequency (number per minute) was counted in each 15-min period.

2.2. Isolation and culture of rat dorsal root ganglion cells

Twenty hours after the injection of cyclophosphamide or saline, rats were killed with CO_2 and decapitated in order to obtain cell cultures. L6/S1 dorsal root ganglia innervating the urinary bladder or thoracal dorsal root ganglia were prepared. The isolation and culturing procedures have been described in detail previously (Himmel et al., 2002).

2.3. Intracellular Ca^{2+} measurements

Dorsal root ganglion cell cultures from days 2 to 4 were loaded for 50–60 min at 37 °C in the dark with the cell permeant acetoxymethyl ester of the fluorescent Ca^{2+} indicator Fura-2 (1 μM, Molecular Probes). Simultaneously, cells were exposed to the fluorescein isothiocyanate (FITC)-labelled *Bandeiraea simplicifolia* isolectin IB_4 (0.25 μg/ml, Sigma). To remove excess extracellular Fura-2 and IB_4, glass cover slips were washed several times with extracellular medium (in mM: NaCl, 140; KCl, 4.5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH) and kept at room temperature protected from light. Ca^{2+} imaging experiments were performed as previously described by an inverted microscope (IX-70; Olympus) and a Peltier cooled charge-coupled device camera (Imago; Till Photonic) (Himmel et al., 2002). Intracellular Fura-2 was alternately excited at 340 and 380 nm; the emitted light was measured at a wavelength of 510 nm above the cell bodies of IB_4-positive dorsal root ganglion neurons. Drugs (capsaicin, α,β-methylene ATP [α,β-meATP] lithium salt; Sigma) and the washout solution were applied via the flush valves of a fast pressurized local application system (DAD-12; Adams and List Associates). When the KCl content of the extracellular medium was increased from 4.5 to 50 mM, the NaCl concentration was correspondingly decreased to prevent a change in osmolarity.

2.4. Evaluation and statistics

Drug (capsaicin and α,β-meATP)-induced increases in $[Ca^{2+}]_i$ over basal levels were compared with the effect of 50 mM K^+ in each experiment and were expressed as a percentage of this value. The concentration–response curve of α,β-meATP was fitted using the software Origin (Microcal™ Software). Means ± S.E.M. of n determinations are shown. Statistical comparisons were made by the parametric Student's t-test or the nonparametric Mann–Whitney rank sum test, as appropriate. A probability level of 0.05 or less was considered to be statistically significant.

3. Results

The pain score of the cyclophosphamide (200 mg/kg i.p.)-treated rats gradually increased from 0 to 3.7 ± 0.1 after 2 h and did not change after additional 2 (4 h, 3.8 ± 0.1) or 18 h (20 h, 3.4 ± 0.1; $n = 12$ each). These changes were statistically significant when compared with the respective scores of the saline-treated animals (2 h, 0.9 ± 0.2; 4 h, 1.2 ± 0.2; 20 h, 0.4 ± 0.1) ($n = 10$ and $P < 0.05$ each). The breathing frequency of the rats decreased from 89.3 ± 2.6 to 50.8 ± 2.0 per min 30 min after cyclophosphamide injection and gradually recovered to steady-state values after 4 h in total (71.0 ± 0.9 per min; $n = 12$ each). The corresponding breathing frequencies of the saline-treated animals were 87.6 ± 1.9, 94.6 ± 3.3 and 87.6 ± 1.7 per min, respectively ($n = 10$ and $P < 0.05$ each). There was no complete recovery of the breathing frequency even 20 h after treatment with cyclophosphamide.

In the following experiments, a 50 mM K^+-containing extracellular medium was applied for 2 s (Fig. 1) onto L6/S1 dorsal root ganglia known to innervate the urinary bladder. The ensuing depolarisation of the neuronal membrane led to the opening of voltage-dependent Ca^{2+} channels and induced $[Ca^{2+}]_i$ transients. The increase in the fluorescence ratio over the baseline was the same in dorsal root ganglia from saline- (0.87 ± 0.14; $n = 12$) and cyclophosphamide-treated (0.88 ± 0.12; $n = 15$) rats. Neurons were defined by their IB_4-positivity and their ellipsoid-ovoid-spherical shape. After an interval of 15 min, cumulative concentration–response curves were constructed for

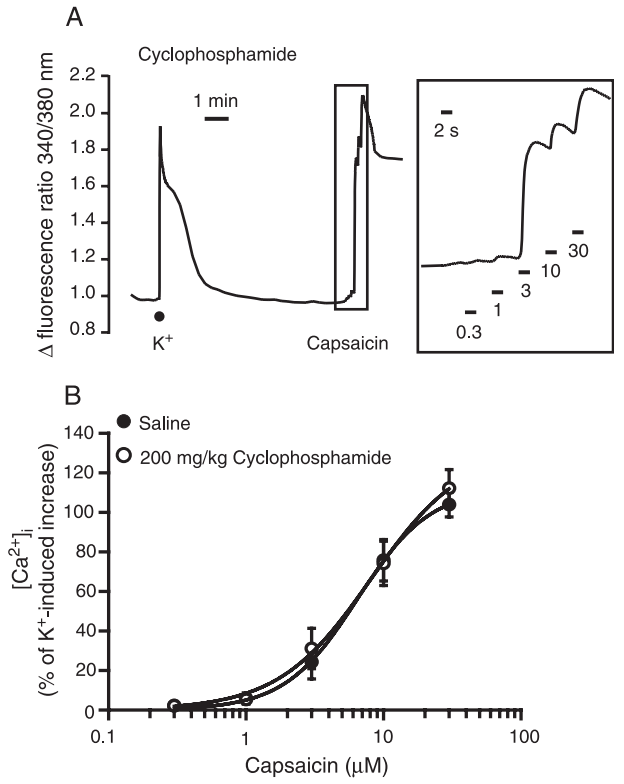

Fig. 1. Effect of a high K^+-containing external medium and capsaicin on $[Ca^{2+}]_i$ in rat dorsal root ganglion neurons in primary culture. K^+ (50 mM) was applied for 2 s, whereas each concentration of capsaicin (0.3–30 μM) was applied for 2 s cumulatively, separated by washout periods of 6 s. (A) Example for a typical recording in a dorsal root ganglion neuron of a rat killed 20 h after the intraperitoneal injection of 200 mg/kg cyclophosphamide. Note that the recording within the frame is reproduced with a larger time-scale a second time. (B) The concentration–response curve of capsaicin was identical in dorsal root ganglion neurons obtained from saline- (●; $n=12$) and cyclophosphamide (○; $n=14$)-treated rats.

capsaicin by applying increasing concentrations (0.3, 1, 3, 10 and 30 μM) of the agonist for 2 s separated by washout periods of 6 s. The treatment of rats with cyclophosphamide did not interfere with the capsaicin-induced increase of $[Ca^{2+}]_i$ (Fig. 1B).

In a separate series of experiments, L6/S1 dorsal root ganglia of saline-treated rats were exposed to one or two individual concentrations of α,β-meATP (0.003, 0.01, 0.03, 0.1, 0.3, 3 and 10 μM) for 10 s each, spaced apart by 15-min intervals (Fig. 2). The resulting concentration–response curve had a maximum at 0.3 μM α,β-meATP and a calculated EC_{50} value of 33.9 nM (Fig. 2B). The dorsal root ganglia were also exposed to the usual 50 mM K^+-containing extracellular medium. Treatment with cyclophosphamide markedly decreased the $[Ca^{2+}]_i$ transients caused by α,β-meATP (0.03 and 0.3 μM). It is noteworthy that the increase in the fluorescence ratios caused by 50 mM K^+ in the saline- (0.85 ± 0.08; $n=27$) and cyclophosphamide-treated dorsal root ganglia (0.95 ± 0.07; $n=24$) were indistinguishable, thereby excluding a possible nonselective effect of cyclophosphamide.

Finally, thoracal dorsal root ganglia that do not innervate the urinary bladder were also prepared both from saline- and cyclophosphamide-treated rats. The dorsal root ganglia were superfused for 2 s with 50 mM K^+ medium and 15 min later with α,β-meATP (30 μM) for 10 s. Pretreatment of rats with cyclophosphamide failed to alter the increase of $[Ca^{2+}]_i$ by 50 mM K^+ (1.16 ± 0.12; $n=9$) when compared with dorsal root ganglia of saline-treated rats (0.95 ± 0.14; $n=9$). The effect of α,β-meATP (30 μM) expressed as a percentage of the 50 mM K^+-induced $[Ca^{2+}]_i$ transient was also identical in the two groups of animals (saline, $39.9 \pm 3.6\%$; cyclophosphamide, $39.2 \pm 1.0\%$; $n=9$ each). A submaximal concentration of α,β-meATP (30 μM) was chosen for these experiments, based on the application of 3, 30 or 300 μM α,β-meATP ($19.9 \pm 3.8\%$, $n=18$; $23.6 \pm 3.6\%$, $n=15$; $37.8 \pm 8.6\%$, $n=16$). It is noteworthy that thoracal dorsal root ganglia had a considerably lower sensitivity to α,β-meATP than L6/S1 dorsal root ganglia (compared with Fig. 2).

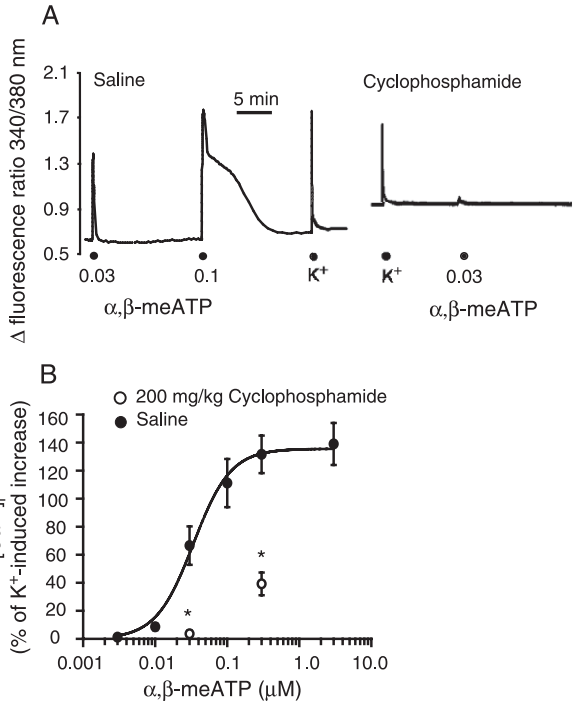

Fig. 2. Effect of a high K^+-containing external medium and α,β-meATP on $[Ca^{2+}]_i$ in rat dorsal root ganglion neurons in primary culture. K^+ (50 mM) and α,β-meATP (0.003–3 μM) were applied for 2 and 10 s, respectively; maximally two α,β-meATP concentrations, spaced 15 min apart, were ejected onto every single dorsal root ganglion neuron. (A) Examples for typical recordings in dorsal root ganglion neurons of rats killed 20 h after the intraperitoneal injection of 200 mg/kg cyclophosphamide (right panel, $n=10$) or an equivalent amount of saline (left panel, $n=6$). (B) Concentration–response curve for α,β-meATP (●; $n=6–11$) in dorsal root ganglion neurons obtained from saline-treated rats. α,β-meATP (○; $n=10–14$) was less effective in dorsal root ganglion neurons obtained from cyclophosphamide-treated rats than in their saline-treated counterparts (*$P<0.05$).

4. Discussion

The present results show that 20 h after the injection to rats of cyclophosphamide that causes a persistent and painful interstitial cystitis (Lanteri-Minet et al., 1995), in lumbosacral dorsal root ganglion neurons kept for 2–4 days in culture, a pronounced subsensitivity of P2X receptors develops, without any change in vanilloid 1 receptor function. Dorsal root ganglion neurons are endowed with either homomeric rapidly desensitising $P2X_3$ or heteromeric slowly desensitising $P2X_{2/3}$ receptors both involved in the sensation of pain (Chizh and Illes, 2001). The activation of these receptors leads to the entry of external Ca^{2+} either via the receptor channel itself or via subsequently opening voltage-sensitive Ca^{2+} channels; both Ca^{2+} entry pathways appear to equally contribute to $[Ca^{2+}]_i$ responses of P2X receptor-expressing cells (Koshimizu et al., 2000). In consequence, ATP-induced $[Ca^{2+}]_i$ transients are of much slower time-course than the underlying membrane currents and therefore do not allow to differentiate between responses to $P2X_3$ or $P2X_{2/3}$ receptor activation (Ueno et al., 1998). Although, in tissue culture, nine types of rat dorsal root ganglion cells were described, based on their current signatures and immunocytochemical properties, all IB_4-positive neurons were sensitive to capsaicin and ATP; in addition, they exhibited completely desensitising responses to ATP within the exposure time of 10 s used in this study (Petruska et al., 2000).

It is noteworthy that α,β-meATP is an agonist at $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors, but at no other type of P2X receptor-channels (Chizh and Illes, 2001). In the present Ca^{2+} imaging study, α,β-meATP had a considerably higher potency to stimulate $P2X_3$ and/or $P2X_{2/3}$ receptors of L6/S1 dorsal root ganglion cells of saline-treated rats than in previous experiments utilizing patch-clamp recordings (Chizh and Illes, 2001). This appears to be due to the selection of the L6/S1 dorsal root ganglion population for these measurements, in contrast to thoracal dorsal root ganglia that exhibited a lower sensitivity to α,β-meATP. In accordance with the reported decrease of $P2X_1$ receptor-mediated mechanical responses to β,γ-methylene ATP in the urinary bladder smooth muscle (Mok et al., 2000), $P2X_3$ receptors of L6/S1 dorsal root ganglion neurons also became less sensitive to α,β-meATP as a consequence of cyclophosphamide-induced cystitis. The most likely reason is that a massive release of ATP occurs from the inflamed bladder tissue causing downregulation of both $P2X_1$ and $P2X_3$ receptors, the latter situated at the peripheral terminals of dorsal root ganglion cells; as a consequence, $P2X_3$ receptors may be downregulated at the cell bodies of these neurons as well. At the same time, neither the depolarising responses to a high potassium-containing external medium nor the effect of vanilloid 1 receptor activation by capsaicin was altered by cyclophosphamide. Moreover, the magnitude of the $[Ca^{2+}]_i$ transients caused by α,β-meATP in thoracal dorsal root ganglia that do not innervate the urinary bladder also remained similar in the saline- and cyclophosphamide-treated animals.

In support of the functional data, a strong decrease of $P2X_3$- but not vanilloid 1-immunoreactivity in the L6/S1 dorsal root ganglion neurons of cyclophosphamide-treated rats was reported when compared with their saline-treated counterparts; these results were generated by immunohistochemical measurements coupled with confocal laser-scanning microscopy (C. Allgaier, H. Sobottka and P. Illes, unpublished).

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