Inhibition of P2X receptor-mediated inward current by protein kinase C in small-diameter dorsal root ganglion neurons of adult rats

Bi-Hua BIE¹, Yi-Hong ZHANG², Zhi-Qi ZHAO

Institute of Neurobiology, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200032, China

Abstract: Objective The aim of the present study is to verify the ATP-induced varied responses in isolated dorsal root ganglion (DRG) neurons of the adult rat, and investigate the modulatory effects of specific P2X receptor agonist β,γ -me-ATP and protein kinase C (PKC) on P2X receptor-mediated inward current in DRG neurons. **Methods** Whole cell patch-clamp was employed to record the currents on acutely isolated DRG neurons in the adult rats. **Results** β,γ -me-ATP, similar as ATP, evoked 2 distinct subtypes of P2X receptor-mediated inward currents in a dose-dependent manner in DRG neurons. Activation of PKC by phorbol 12,13-dibutyrate (PDBu) significantly inhibited both subtypes of inward currents mediated by P2X receptors in a dose-dependent manner. **Conclusion** Activation of PKC negatively modulated the P2X receptor-mediated currents in rat DRG neurons, which may be of benefit to preventing the over-excitation of nociceptor under inflammatory or neuropathic conditions.

Keywords: dorsal root ganglion; P2X; ATP; β,γ -me-ATP; protein kinase C

1 Introduction

Since the first proposition of purinergic neurotransmission over 30 years ago^[1], ATP and P2 receptors, including ionotropic P2X receptors and metabotropic P2Y receptors, have been widely implicated in purinergic transmission in both the peripheral and the central nervous systems. Until now, 7 subtypes of P2X-purinoceptor family and 7 subtypes of P2Y-purinoceptor family have been recognized^[2]. Based

on the distinct desensitization kinetics, P2X receptors are divided into 2 broad groups according to whether they desensitize rapidly, within 100 to 300 ms, or desensitize slowly and even not at all. Generally, all the rapid desensitizing P2X receptors, including recombinant and native P2X1 and P2X3 receptors, are activated by ATP and its analogue α,β -me-ATP, while the slow desensitizing P2X receptors, including P2X2/3, P2X2, P2X4, P2X5 and P2X6 receptors, can be further subdivided into 2 groups: those are sensitive to α,β -me-ATP, and those are less sensitive or insensitive at all to α,β -me-ATP^[3]. Notably, another agonist β,γ -me-ATP can activate P2X but not P2Y receptors at all, although the subtypes remained uncertain and contradictory^[4,5].

The rat dorsal root ganglion (DRG) neurons express mRNA for 6 of the total 7 P2X receptors^[6,7]. The actions of ATP and α,β -me-ATP in the isolated DRG neurons have been well documented, and 2 types of inward currents with rapid or slow desensitization kinetics are evoked^[8,9]. However, the

Corresponding author: Zhi-Qi ZHAO
Tel: 86-21-54237634; Fax: 86-21-54237647
E-mail: zqzhao@fudan.edu.cn
Article ID: 1673-7067(2009)04-0179-08
CLC number: Q189
Document code: A

Received date: 2009-06-11

Present address: ¹Department of Anesthesiology, The University of Texas-MD Anderson Cancer Center, Houston, TX. bbihua@yahoo.com. ²Department of Physiology & Pharmacology, University of Bristol, University walk, Bristol BS8 1TD U.K. yh.zhang@bristol.ac.uk

proposition that DRG neurons are sensitive to β,γ -me-ATP, a selective P2X agonist, is ambiguous and lacks direct electrophysiological proof. So, the present study was the first to testify the effect of β,γ -me-ATP in adult rat DRG neurons and analyze the subtypes of β,γ -me-ATP-evoked inward currents.

ATP-gated channels unique to small sensory neurons^[6,7] and ATP-induced hyperexcitation in C fibers of the injured nerve^[10] strongly imply that the ion channel gated by ATP may be involved in mediating nociceptive transmission^[2]. In fact, extracellular ATP could elicit non-selective cation currents (Na^+ , K^+ , Ca^{2+}) across the cell membrane through P2X receptors, resulting in an increase in Ca^{2+} influx and cell depolarization^[11,12], thereby increasing the excitability of nociceptor. Furthermore, the direct influx of extracellular Ca^{2+} causes a significant increase in intracellular Ca^{2+} concentration, and triggers a cascade of activations of intracellular messengers including PKC^[13]. PKC activation would modulate the activities of P2X receptors, contributing to nociceptor transduction. Therefore, the second aim of the present study is to investigate the modulatory effects of PDBu (a selective agonist for PKC) on P2X receptor-mediated inward currents in small DRG neurons.

2 Materials and Methods

2.1 Enzymatic isolation of single DRG neuron of rats The enzymatic isolation of single DRG neuron was carried out as described previously^[14]. Briefly, adult male Sprague-Dawley rats (weighing 150-200 g) were decapitated, and dorsal root ganglia of L4-L6 were rapidly dissected, minced and dissociated by incubation in Dulbecco's Modified Eagle's Medium (Gibco, New York, USA) containing trypsin III (0.75 mg/mL, Sigma), collagenase 1A (1.25 mg/mL, Sigma) and DNase IV (0.125 mg/mL, Sigma) at 36 °C for 60 min, after which normal extracellular solution was used to wash out the enzymatic digest. Isolated neurons were obtained by triturating the ganglia through a series of Pasteur pipettes with decreasing tip diameters. Thereafter, the isolated neurons were placed into culture dishes (35 mm) and kept for at least 1 h before electrophysiological recording.

2.2 Electrophysiological recording Whole cell patch-clamp recording was performed on the isolated rat DRG

neurons. The resistance of microelectrode was 3-5 M Ω when filled with pipette solution [containing 140 mmol/L KCl, 2 mmol/L MgCl_2 , 10 mmol/L HEPES, 11 mmol/L ethyleneglycolbis (aminoethyl ether) tetra-acetate and 4 mmol/L $\text{Na}_2\text{-ATP}$, pH 7.4]. The recording chamber was perfused with normal extracellular solution (containing 150 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 10 mmol/L HEPES and 10 mmol/L glucose, pH 7.4).

Whole cell configuration was held at membrane potential of -60 mV. The cells were discarded if their resting membrane potentials were more positive than -40 mV, or they did not exhibit an action potential overshoot, or the seal resistance was less than 1 G Ω during the experimental procedure. Data were recorded using a CEZ2300 whole cell patch-clamp amplifier (Nihon Kohden, Japan), filtered at 2 kHz and sampled at a frequency of 20 kHz using an IBM-compatible computer with a Digidata 1200 interface, pCLAMP 6.0 acquisition and analysis software (Axon Instruments, USA). All electrophysiological experiments were performed at room temperature (20-25 °C).

The DRG cells were superfused with control or test solution, if any, by means of a gravity-driven puffer system placed at a distance of 0.2 mm from the cell. The internal diameter of the final tube near the cell was 0.2 mm. The superfusion system changed the extracellular solution in less than 0.5 s. Dose-effect relations were fitted with the following equation: $y/(a-d)=1/[1+(x/c)^b]$ (a : the asymptotic maximum value, b : Hill coefficient, c : EC50 or IC50, d : the asymptotic minimum value).

2.3 Drugs In this study, the P2X agonists Adenosine 5'-triphosphate Disodium (ATP-Na_2 , Sigma, USA), α,β -methylene-ATP (α,β -me-ATP) and β,γ -methylene-ATP (α,γ -me-ATP), and antagonists trinitrophenyl-ATP (TNP-ATP) (all from RBI, USA) were all freshly dissolved and diluted with normal extracellular solution before the experiment. PDBu and Bisindolylmaleimide (Bis) (both from Sigma, USA) were prepared as stock solution with the concentration of 1 mmol/L, and freshly diluted with extracellular solution before applications.

2.4 Statistical analysis Data were expressed as mean $\pm$

SEM (n = number of observations) and analyzed with paired student's test or ANOVA with post hoc analysis. $P < 0.05$ was considered statistically significant.

3 Results

The cells in the 35 mm dish were observed at $250\times$ magnification with an inverted microscope, and the DRG neurons were scattered in the dish. The cells that had round cell bodies ($12\text{--}30\text{ }\mu\text{m}$) with small axonal projections and distinct nuclei with smooth and clean appearances, were selected for recording. In the present study, a total number of 104 small-diameter DRG neurons were recorded in whole cell patch-clamp configuration.

3.1 P2X receptor-mediated inward current in isolated adult rat DRG neurons Consistent with the previous report^[7], 2 kinds of inward currents were evoked by extracellular application of 1 mmol/L ATP in DRG neurons. The peak value of the current with rapid desensitization kinetics was $0.52\pm 0.08\text{ nA}$ ($n = 6$) and that with slow desensitization kinetics was $0.38\pm 0.09\text{ nA}$ ($n = 7$). Both subtypes of ATP-induced inward currents were almost completely inhibited by co-application of TNP-ATP ($10\text{ }\mu\text{mol/L}$, $n = 5$ in each type), a selec-

tive P2X receptor antagonist.

To find out the subtype of P2X receptor which specifically responses to the selective agonist β,γ -me-ATP, $100\text{ }\mu\text{mol/L}$ β,γ -me-ATP was applied by extracellular perfusion on DRG neurons. As shown in Fig. 1, extracellular application of β,γ -me-ATP ($100\text{ }\mu\text{mol/L}$) significantly evoked 2 distinct types of inward currents with rapid or slow desensitization kinetics, and both of them could be almost completely inhibited by TNP-ATP ($1\text{ }\mu\text{mol/L}$) in DRG neurons (Fig. 1), suggesting the involvement of P2X receptors in the 2 inward currents. Moreover, β,γ -me-ATP acted in a dose-dependent manner (Fig. 2A, B). As shown in Fig. 2C, the rapid desensitization current exhibited EC_{50} of $12.72\text{ }\mu\text{mol/L}$ and Hill coefficient of 1.08 ($n = 6$), while the slow desensitization current showed EC_{50} of $27.96\text{ }\mu\text{mol/L}$ and Hill coefficient of 0.98 ($n = 7$).

3.2 Inhibitory effect of PDBu on P2X-mediated current in isolated DRG neurons To investigate the effect of PKC activation on ATP-induced inward current, PDBu ($1\text{ }\mu\text{mol/L}$), a selective agonist of PKC, was perfused on DRG neurons for 1 min. Activation of PKC significantly inhibited the ATP-induced inward current with rapid desensitization by $49.6\pm 7.1\%$ ($0.42\pm 0.10\text{ nA}$ vs $0.19\pm 0.05\text{ nA}$, $n = 11$, $P < 0.01$, Fig. 3A)

Fig. 1 Extracellular β,γ -me-ATP ($100\text{ }\mu\text{mol/L}$) evoked 2 distinct subtypes of inward currents with (A) rapid desensitization kinetics or (B) slow desensitization kinetics in adult rat DRG neurons, both of which could be almost completely blocked by TNP-ATP ($1\text{ }\mu\text{mol/L}$), suggesting that both currents were mediated by P2X receptors.

or with slow desensitization by $47.4 \pm 7.2\%$ (0.30 ± 0.05 nA vs 0.13 ± 0.02 nA, $n = 21$, $P < 0.01$, Fig. 3C). However, both types of currents were recovered to baseline after 5-min wash-out with normal extracellular solution. In addition, PDBu-induced inhibition was abolished by co-application of Bis ($1 \mu\text{mol/L}$), an inhibitor of PKC (Fig. 3B, D). Furthermore, with the serial

increases in PDBu concentration (0.1 , 0.3 , 1 and $3 \mu\text{mol/L}$), the 2 types of ATP-evoked inward currents were inhibited in a dose-dependent manner, with different IC_{50} values ($0.22 \mu\text{mol/L}$ for rapid-sensitization current, $n = 6$; $0.29 \mu\text{mol/L}$ for slow-sensitization current, $n = 7$, Fig. 4). Similarly, PKC activation by PDBu ($1 \mu\text{mol/L}$) reversibly inhibited the inward currents

Fig. 2 β,γ -me-ATP evoked 2 distinct subtypes of inward currents in a dose-dependent manner. A: the inward currents with rapid desensitization kinetics in response to β,γ -me-ATP with serial concentrations (1 , 3 , 10 , 30 and $100 \mu\text{mol/L}$); B: the inward currents with slow desensitization kinetics in response to β,γ -me-ATP with serial concentrations (1 , 3 , 10 , 30 and $100 \mu\text{mol/L}$); C: concentration-response curves for the 2 subtypes of β,γ -me-ATP-evoked inward currents were fitted with Hill equation: $y/(a-d)=1/[1+(x/c)^b]$. The rapid desensitization current exhibited EC_{50} of $12.72 \mu\text{mol/L}$ and Hill coefficient of 1.08 ($n = 6$), while the slow desensitization current showed EC_{50} of $27.96 \mu\text{mol/L}$ and Hill coefficient of 0.98 ($n = 7$). $*P < 0.05$, comparison between the 2 dose-curves.

Fig. 3 The 2 distinct subtypes of ATP-evoked currents were inhibited by treatment of PDBu, the selective agonist for PKC. A: extracellular ATP-evoked inward currents with rapid desensitization kinetics could be inhibited by pre-application of PDBu (1 $\mu\text{mol/L}$); B: PDBu-induced inhibition could be cancelled by co-application of Bis (1 $\mu\text{mol/L}$); C: extracellular ATP-evoked inward currents with slow desensitization kinetics could be inhibited by pre-application of PDBu (1 $\mu\text{mol/L}$); D: this inhibition could also be cancelled by co-application of Bis (1 $\mu\text{mol/L}$).

Fig. 4 PDBu inhibited both subtypes of ATP-evoked inward currents in a dose-dependent manner. The inward currents with (A) rapid or (B) slow desensitization kinetics evoked by ATP (1 $\mu\text{mol/L}$) were inhibited by PDBu (0.1, 0.3, 1 and 3 $\mu\text{mol/L}$). C: concentration-effect curves for the inhibitory effect of PDBu on ATP-evoked 2 subtypes of inward currents were fitted with Hill equation: $y/(a-d)=1/[1+(x/c)^b]$. IC_{50} for rapid-sensitization current was 0.22 $\mu\text{mol/L}$ ($n = 6$), and IC_{50} for slow-sensitization current was 0.29 $\mu\text{mol/L}$ ($n = 7$).

evoked by the selective P2X receptor agonists, including 100 $\mu\text{mol/L}$ β,γ -me-ATP (0.17 ± 0.05 nA vs 0.09 ± 0.02 nA, $n = 11$, $P < 0.05$) and 40 mmol/L α,β -me-ATP (0.43 ± 0.12 nA vs 0.22 ± 0.06 nA, $n = 3$, $P < 0.05$). However, both inhibition could be cancelled by co-application of PKC inhibitor, Bis (1 $\mu\text{mol/L}$).

4 Discussion

In our knowledge, the present study demonstrated for the first time that β,γ -me-ATP, a selective P2X agonist, evoked 2 subtypes of inward currents with rapid or slow desensitization kinetics in isolated adult rat DRG neurons, suggesting that this agonist may act on 2 distinct subtypes of P2X receptors, although the action of β,γ -me-ATP was not detected in the DRG neurons from the neonatal rats^[4]. In general, P2X may mediate the rapid desensitization responses in rat DRG neurons through P2X3 receptor, whereas it mediates the slow desensitization responses through P2X2/3 receptor activation^[8]. Also, β,γ -me-ATP acted on the 2 subtypes of P2X receptors with similar Hill Coefficients (1.08 and 0.98, respectively), suggesting that there was one binding site for β,γ -me-ATP on each P2X receptor in rat DRG neurons. In addition, β,γ -me-ATP was more sensitive to rapid desensitizing P2X receptors, as evidenced by the smaller EC_{50} for this subtype current than that for slow desensitizing current (12.72 $\mu\text{mol/L}$ vs 27.96 $\mu\text{mol/L}$).

The second finding in the present study was that both subtypes of P2X receptor-mediated inward currents were negatively modulated by the activation of PKC in rat DRG neurons, which was consistent with the observation that PKC activation by PMA could significantly inhibit the increase of $[\text{Ca}^{2+}]_i$ elicited by ATP and P2X agonist in human neutrophils^[15]. DRG neurons recorded in the present study were small in diameter, commonly considered as nociceptors. Immunohistochemical studies have revealed that P2X receptor is highly expressed in nociceptors^[6,7,16]. Intraplantar injection of P2X receptor agonist, such as α,β -me-ATP, could induce a nociceptive response in rat^[10,17,18]. Activations of presynaptic P2X receptors on DRG terminals, similarly as in brain stem slices^[19], can increase glutamatergic transmission in DRG-dorsal horn synapses in culture^[20]. These data suggest that ATP may function as a transmitter or a modulator of

nociception, although the role of each receptor subtype in this pathway remains unknown. Therefore, the modulations of P2X receptors in DRG neurons may influence the transmission of nociceptive signal. Combined with the possible role of P2X receptors in DRG neurons, activated PKC could negatively modulate the activity of P2X receptors, which may be beneficial to preventing the overexcitation of nociceptor under inflammatory or neuropathic conditions.

In conclusion, our studies reveal that in isolated adult rat DRG neurons, the P2X agonist β,γ -me-ATP, as well as ATP, could evoke 2 subtypes of inward currents with rapid or slow desensitization kinetics in a dose-dependent manner with differential EC_{50} values. Furthermore, activation of PKC by its selective agonist PDBu could inhibit the 2 subtypes of P2X-mediated currents in a dose-dependent manner, which may be beneficial to preventing the overexcitation of nociceptor under inflammatory or neuropathic conditions.

Acknowledgement: This work was supported by grants from the National Basic Research Development Program of China (No. 2006CB500807) and the National Natural Science Foundation of China (No. 30600178).

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蛋白激酶 C 抑制 P2X 受体介导的大鼠小直径背根节神经元内向电流

别毕华, 张一红, 赵志奇

复旦大学神经生物学研究所, 脑科学研究院, 国家医学神经生物学重点实验室, 上海 200032

摘要: 目的 在成年大鼠背根节神经元, 观察 ATP 诱导的多样性反应, 研究 P2X 激动剂 β, γ -me-ATP 以及蛋白激酶 C 对 P2X 受体介导的内向电流的调制作用。方法 应用全细胞膜片钳记录急性分离的大鼠背根节神经元电活动。结果 与 ATP 诱导的反应相同, P2X 激动剂 β, γ -me-ATP 也引起 P2X 受体介导的两种类型的内向电流, 并呈剂量依赖性。蛋白激酶 C 激动剂 phorbol 12,13- dibutyrate (PDBu) 能显著抑制 P2X 受体介导的两类内向电流, 并呈剂量依赖关系。结论 蛋白激酶 C 负向性调制大鼠背根节神经元的 P2X 受体介导的内向电流, 提示蛋白激酶 C 的激动剂可用于防止炎症和神经病理条件下伤害性感受器的过度兴奋。

关键词: 背根节; P2X 受体; ATP; β, γ -me-ATP; 蛋白激酶 C