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3-Isobutyl-1-methylxanthine (IBMX) affects potassium permeability in rat sensory neurones via pathways that are sensitive and insensitive to $[\text{Ca}^{2+}]_i$

Received: 13 December 1994/Received after revision: 8 March 1995/Accepted: 9 March 1995

Abstract The effects of externally applied 3-isobutyl-1-methylxanthine (IBMX), in millimolar concentrations, on the membrane currents in dorsal root ganglia (DRG) neurones isolated from newborn rats were investigated using the amphotericin-based “perforated” patch-clamp technique. In some experiments, simultaneous measurements of intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) were performed using fura-2 microfluorimetry. Applications of IBMX induced elevation of $[\text{Ca}^{2+}]_i$ resulting from $\text{Ca}^{2+}$ release from caffeine-ryanodine-sensitive internal stores. In addition to $\text{Ca}^{2+}$ release, IBMX produced a biphasic membrane current response comprised of an inward current transiently interrupted by outward current. The onset of the inward current slightly preceded the onset of the $[\text{Ca}^{2+}]_i$ transient, while the interrupting outward current developed synchronously with the $[\text{Ca}^{2+}]_i$ rise. The development of IBMX-induced outward current ultimately needed the $[\text{Ca}^{2+}]_i$ elevation. After the depletion of $\text{Ca}^{2+}$ stores by IBMX or caffeine exposure, the subsequent IBMX challenge failed to produce both the $[\text{Ca}^{2+}]_i$ transient and outward membrane current, although the inward current remained unchanged. Both components of the IBMX-induced membrane current response had a reversal potential close to the $\text{K}^+$ equilibrium potential and the IBMX-induced membrane current response disappeared while dialysing the cell interior with $\text{K}^+$-free, $\text{Cs}^+$-containing solutions suggesting their association with $\text{K}^+$ channel activity. External administration of 10 mM tetraethylammonium chloride (TEA-Cl) evoked an inward current similar to that observed in response to IBMX; in the presence of TEA-Cl, IBMX application was almost unable to induce additional inward current. IBMX (5 mM) effectively (~50%) inhibited $\text{K}^+$ currents evoked by step depolarizations of membrane potential. We suggest that IBMX affects membrane permeability via activation of $\text{Ca}^{2+}$-regulated $\text{K}^+$ channels and direct inhibition of TEA-sensitive $\text{K}^+$ channels.

Key words Sensory neurones · IBMX · $\text{Ca}^{2+}$ release · $\text{K}^+$ currents · M-currents · $\text{Ca}^{2+}$-dependent $\text{K}^+$ currents

Introduction

Methylxanthines are known to be potent stimulators of the nervous system. This stimulatory action is accomplished via different routes, namely (1) via inhibition of cytoplasmic phosphodiesterases, with subsequent increases of the level of intracellular cyclic nucleotides; (2) liberation of $\text{Ca}^{2+}$ from intracellular stores; and (3) modulation of membrane ionic channels [7]. All of these three mechanisms may be accounted for by methylxanthine-induced changes of membrane permeability in nervous cells. Of the methylxanthines, the actions of caffeine and theophylline on neuronal membrane currents have been studied in detail. Caffeine was reported to initiate an outward $\text{K}^+$ current resulting from the activation of $\text{K}^+$ channels controlled by $\text{Ca}^{2+}$ which was released from the internal pools [14, 18, 22]. Caffeine was also reported to activate an inward current associated with inhibition of the M-current found in bullfrog sympathetic neurones [4, 14, 16] and neuroblastoma × glioma hybrid cells [17]. Theophylline has similar neuronal effects, inducing $\text{Ca}^{2+}$ release from the internal stores and affecting the $\text{K}^+$ permeability of the membrane (see [15] for review). However, the majority of these experiments have been done on bullfrog preparations, and the mechanisms of the action of methylxanthine on membrane channels in mammalian neurones remains
largely unknown. In the present study we have investigated the effects of another representative of the methylxanthine family, 3-isobutyl-1-methylxanthine (IBMX), on both intracellular Ca\(^{2+}\) homeostasis and the membrane permeability of freshly dissociated rat dorsal root ganglia (DRG) neurones. To prevent damage of the intracellular machinery caused by intracellular dialysis, the amphotericin-based perforated patch-clamp recording technique [11] was used.

### Materials and methods

#### Cell preparation

Experiments were performed on neurones acutely isolated from thoracic and lumbar DRG of newborn (2- to 7-day-old) rats. After dissection, ganglia were enzymatically treated in a Tyrode-based isolation medium supplemented by 1 mg/ml protease (Sigma, St. Louis, Mo., USA, Type XIV). Tissue was incubated in isolation medium at 35°C for 25–30 min. After the end of the enzymatic treatment, cells were isolated by gentle pipetting in Tyrode solution. The cell suspension was plated on sterile glass coverslips; 1 h after plating, the cells adhered to the glass so that experiments could be performed.

Measurements of intracellular free Ca\(^{2+}\) concentration, [Ca\(^{2+}\)\(_{\text{in}}\)]

For [Ca\(^{2+}\)\(_{\text{in}}\)] measurements, glass coverslips with adhered cells were placed in an experimental chamber mounted on the stage of an inverted fluorescent microscope equipped with epifluorescence. [Ca\(^{2+}\)\(_{\text{in}}\)] was measured with the Ca\(^{2+}\)-sensitive fluorescent dye fura-2, as described previously [20, 24]. Neurones were loaded with fura-2 acetoxymethylester (fura-2/AM) by cell incubation in normal physiological Tyrode solution supplemented with 5 µM fura-2/AM (in dimethylsulphoxide (DMSO)) and 0.02% Pluronic F-127 detergent for 30 min at 22°C.

For fura-2 fluorescence measurements, cells were alternately illuminated at 360 ± 5 nm and 390 ± 5 nm from a 150-W xenon lamp. The excitation wavelengths were chosen by a filter changing device, driven by a step motor, which allowed rapid switching between different excitation wavelengths. The light was passed through the microscope epifluorescence illuminator and focused through a 40 x phase-contrast objective (numerical aperture – 0.65). The emission fluorescence was recorded in the region of 480–520 nm, which was selected by appropriate barrier filters. The emission signal was collected and amplified by the photomultiplier. The [Ca\(^{2+}\)]\(_{\text{in}}\) values were calculated on the basis of the equation of Grynkiewicz et al. [9]; the \(K_{\text{B}}\), \(R_{\text{min}}\), and \(R_{\text{max}}\) values obtained utilizing the intracellular calibration procedure were 2800 nM, 0.6 and 9.1 respectively.

Analogue signals were fed into an IBM-compatible personal computer via a Labmaster T1-1 interface (Axon Instruments, USA). Data acquisition and analysis were controlled by pClamp software, version 5.5 (Axon Instruments).

The experimental chamber was continuously superfused with Tyrode solution at a rate of 5 ml/min, and a fast application system for changing external solutions [24] allowed a complete change of solution surrounding the cell in < 100 ms. All experiments were performed at 22–24°C. All data are given as the mean ± S.D.

#### Single-cell electrophysiology

In order to prevent damage of the intracellular enzymatic machinery, the “perforated” modification [11] of the whole-cell patch-clamp technique in voltage-clamp mode [10] was used for monitoring transmembrane ionic currents. Amphotericin B was used for accessing the cell interior. Recording pipettes were fabricated from borosilicate capillaries (Hilgenrein, Malsfeld, Germany) and had resistances of between 3 MΩ and 5 MΩ when filled with intrappetite solution. The access resistance was < 15 MΩ and was further compensated using standard features of the EPC-7 amplifier (30% compensation). Voltage signals were amplified using conventional electronics (EPC-7 amplifier, List Electronics, Germany), filtered at 3 kHz and sampled at 5–10 kHz by a Labmaster T1-1 interface connected to an AT-compatible computer system, which also served as a stimulus generator. The experiments were controlled by pClamp software version 5.5.

#### Solution and reagents

All solutions were freshly prepared from refrigerated stock solutions, and filtered through 0.22-µm filters (Schleicher and Schuell, Germany) before use. The Tyrode solution contained (in mM): NaCl 140, KCl 4, CaCl\(_2\) 2, MgCl\(_2\) 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH 5, glucose 10, pH = 7.4. In Ca\(^{2+}\)-free solution, CaCl\(_2\) was omitted, MgCl\(_2\) was increased to 4 mM and 0.2 mM ethylenebis(oxonitrilo)tetraacetate (EGTA) was added, yielding an estimated Ca\(^{2+}\) concentration of about 30 nM. The intrappetite solution contained (in mM): KCl 55, K\(_2\)SO\(_4\) 70, MgCl\(_2\) 7, HEPES/KOH 10, amphotericin 240 µg/ml, pH = 7.4. In K\(^{+}\)-free intrappetite solution, K\(^{+}\) ions were substituted by Cs\(^{+}\). Fura-2/AM and Pluronic F-127 were obtained from Molecular Probes, Eugene, Ore., USA and all other chemicals were from Sigma.

### Results

**IBMX induces Ca\(^{2+}\) release from caffeine- or ryanodine-sensitive internal Ca\(^{2+}\) stores**

While monitoring the [Ca\(^{2+}\)\(_{\text{in}}\)] in rat DRG neurones, we found that external application of 0.5–5 mM IBMX induced a fast transient elevation of [Ca\(^{2+}\)\(_{\text{in}}\)] from the resting level of 88 ± 12 nM (n = 32) to 200–400 nM (Fig. 1). The IBMX-triggered [Ca\(^{2+}\)\(_{\text{in}}\)] transients persisted in Ca\(^{2+}\)-free extracellular solutions, thus indicating their origin from internal stores. Exposure of DRG neurones to caffeine, which induces intracellular Ca\(^{2+}\) liberation due to activation of a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism [8, 24], with subsequent depletion of releasable Ca\(^{2+}\) stores, prevented [Ca\(^{2+}\)\(_{\text{in}}\)] responses to successive IBMX application. Furthermore, ryanodine and procaine – known pharmacological modulators of CICR [13] – effectively blocked the IBMX-induced [Ca\(^{2+}\)\(_{\text{in}}\)] elevation, suggesting that IBMX, as well as other methylxanthines, interacts with Ca\(^{2+}\)-gated Ca\(^{2+}\)-release channels of neuronal endoplasmic reticulum Ca\(^{2+}\) stores (see also [23]).

Similarly to caffeine, IBMX applications deplete internal Ca\(^{2+}\) stores of releasable Ca\(^{2+}\) which can be observed as a progressive decay of the amplitudes of [Ca\(^{2+}\)\(_{\text{in}}\)] transients in response to subsequent IBMX applications (Fig. 1B). Once depleted, Ca\(^{2+}\) stores could be spontaneously refilled by releasable Ca\(^{2+}\); the