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Inositol trisphosphate-induced hyperpolarization in rat dorsal root ganglion neurons

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Inositol 1,4,5-trisphosphate (1,4,5-$InsP_3$) was perfused into rat dorsal root ganglion (DRG) neurons by whole-cell patch-clamp electrodes, while measuring the membrane potential. This operation evoked a transient (2–3 min) membrane hyperpolarization of about −15 mV (from −42 mV) followed by a depolarization. The membrane hyperpolarization was abolished when 30 mM EGTA was perfused together with 1,4,5-$InsP_3$ or when 0.2 mM quinine was added to the bath solution. The hyperpolarizing response was enhanced when a low-Ca^{2+} EGTA-free intracellular solution was used. Two $InsP_3$ isomers induced a different response. Our results suggest that the hyperpolarization is due to 1,4,5-$InsP_3$-induced Ca^{2+} release which may trigger Ca-sensitive K^+ channels to open. Present results show that cultured DRG neurons are able to respond to 1,4,5-$InsP_3$ perfusion in the whole-cell configuration.

DRG; 1,4,5-$InsP_3$; Hyperpolarization; Ca^{2+}-dependent K^+ channel

1. INTRODUCTION

Dorsal root ganglion (DRG) neurons are widely used to study neuron regeneration. A recently developed dorsal root ganglion culture model has been shown to be particularly useful for the study of growth cone behaviour, cell migration and the interactions between the different DRG cell types (neurons, satellite cells, Schwann cells and fibroblasts) during cell growth and nerve fibre myelination [1].

Electrophysiological measurements showed that the neurons in these cultures in a chemically defined medium are excitable up to 35 days of culture [1,2], consistent with the presence of both inward currents and outward currents measured under voltage clamp [1]. The morphological and electrophysiological results obtained so far, provide a basis for a more detailed study on the interaction between ion transporters and second messengers and their role in neurite outgrowth and neuron regeneration.

Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate (1,4,5-$InsP_3$) are important second messengers in many different cell types [3], including neurons [4]. In addition, the second-messenger cAMP influences DRG neurite outgrowth by a lowering of the growth cone Ca^{2+} level [5,6]. We were interested in a possible role of 1,4,5-$InsP_3$ and 1,4,5-$InsP_3$-related processes in neurite outgrowth, ion transporter control and neuron regeneration. In the present work we have investigated the effects of 1,4,5-$InsP_3$ on some electrophysiological properties of rat DRG neurons. Our results suggest that 1,4,5-$InsP_3$ is able to modulate $[Ca^{2+}]_i$ in cultured DRG neurons. Perfusion of these cells with 1,4,5-$InsP_3$ in the whole-cell patch-clamp configuration elicited a transient membrane hyperpolarization. This hyperpolarization is likely to be due to the opening of Ca^{2+}-dependent K^+-channels.

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2. MATERIALS AND METHODS

2.1. *Cell culture conditions*

The dorsal root ganglion (DRG) cells were mechanically isolated from neonatal rats [7] and cultured in the chemically defined R12 serum-free culture medium [1] as described before. Only 1-day cultured neurons were used for experiments.

2.2. *Membrane potential measurement and cell perfusion*

For membrane potential measurements and simultaneous cell perfusion, the whole-cell configuration of the patch-clamp technique [8] was used with an EPC-7 patch-clamp amplifier (List Electronic, Germany). Glass coverslips with attached neurons were mounted to an open bottom Teflon culture dish [9] which was placed on the stage of an inverted phase-contrast microscope. The membrane potential was recorded with a personal computer using the software package pClamp (version 5.1, Axon Instruments, Burlingame, CA) and by a pen recorder. The membrane resistance was measured from the stationary potential responses upon small hyperpolarizing current-step pulses (steps ≤ 20 pA, see Fig. 1A, inset).

2.3. *Composition of solutions*

For control experiments, the DRG neurons were bathed in an extracellular solution (ECS) composed of (mM) 140 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 6.0 glucose and 10 HEPES/NaOH (pH 7.2). The solution was refreshed every 10–20 min. The patch pipette was filled

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with an intracellular solution (ICS) containing (mM) 140 KCl, 10 NaCl, 1.0 $CaCl_2$, 2.0 $MgCl_2$, 10 EGTA, and 10 HEPES/KOH (pH 7.2). For inositol phosphate experiments, the patch pipette was filled with different intracellular solutions together with different 1,4,5-$InsP_3$ or $InsP_2$ concentrations. 1,4,5-$InsP_3$- and $InsP_2$-containing solutions were freshly made before use. 1,4,5-$InsP_3$ was obtained from Sigma Chem. Co. (St. Louis, USA). 1,4-$InsP_2$ and 4,5-$InsP_2$ were from Boehringer Mannheim (Germany).

The high-EGTA intracellular solution was ICS with (mM) 30 EGTA, 2.99 $CaCl_2$ and 3.47 $MgCl_2$, resulting in the same free calcium and magnesium concentration as in ICS. The Ca^{2+}-free intracellular solution consisted of (mM) 145 KCl, 10 NaCl, 2.0 $MgCl_2$, 10 HEPES/KOH (pH 7.2). The quinine (Sigma) containing solutions were freshly made before use.

Data are presented as mean values ± standard deviations (M ± S.D.), with n = the number of cells measured. Paired Student's t-tests were used for comparing the membrane potential at 0 time (immediately after the whole-cell configuration was obtained) and after 1 min. Student's t-test was used to compare mean membrane potentials obtained in different experimental conditions.

3. RESULTS

3.1. *Resting membrane potential and resistance*

The resting membrane potential of DRG neurons was measured immediately after formation of the whole-cell configuration [1,2]. Under the control condition (pipettes filled only with ICS), the resting membrane potential of neurons was −48.1 ± 11.3 mV (n = 18) (see Table I) and in most cases, neurons were excitable (having action potentials), which is in agreement with our previous results [1,2]. Resting membrane potentials were in most cases stable for more than 30 min. For the experiments, only neurons with stable resting membrane potentials more negative than −20 mV were used. Neurons that showed a depolarization of the membrane potential to values close to 0 mV within 2 min after achieving the whole-cell configuration were also excluded.

The membrane resistance (R_m), in most cases, strongly depended on the membrane potential when hold at different potential values by current injection (Fig. 1A). R_m ranged from 100 to 400 MΩ at membrane potentials more positive than −50 mV. At membrane potentials more negative than −50 to −60 mV R_m increased remarkably (range 400–2000 MΩ) with more negative potential values. This membrane resistance increase is likely to be due to the closure of voltage-dependent ion channels at membrane potentials more negative than −50 mV.

3.2. *Effects of inositol phosphates on the membrane potential*

The effect of 1,4,5-$InsP_3$ on the membrane potential was investigated by using patch-pipettes filled with ICS supplemented with 100 μM 1,4,5-$InsP_3$. This allowed us to perfuse the cells with 1,4,5-$InsP_3$-containing solutions while the membrane potential was measured. Immediately after obtaining the whole-cell configuration the mean measured membrane potential was −42 ± 15.9 mV (n = 16), which is not significantly different from the membrane potential measured in control experiments ($P > 0.05$) (Table I). From 9 tested neurons, 8 neurons had action potentials which is similar as in control neurons. In contrast to the control experiments the membrane potential measured with 100 μM 1,4,5-$InsP_3$ in the pipette was not constant but showed a significant ($P < 0.01$) transient hyperpolarization (Fig. 2). This hyperpolarization reached its most negative value at about 1–2 min after the whole-cell configuration was achieved. Usually, the transient hyperpolarization was followed by a slower depolarization towards 0 mV (Fig. 2). The hyperpolarization was accompanied only by a slight (or no) increase in membrane resistance (Fig. 1B), which was much smaller than the membrane resistance increase upon hyperpolarization of the control cells (by means of current injection) (cf. Fig. 1A).

The magnitude of the hyperpolarization was only slightly 1,4,5-$InsP_3$ dose-dependent in a concentration range between 25 and 100 μM. However, the number of responding cells depended strongly on the applied 1,4,5-$InsP_3$ concentration in this concentration range (Fig. 3). The mean membrane potential change (ΔE_m) of responding cells was −14.8 ± 11.9 mV (n = 12) for 100 μM 1,4,5-$InsP_3$ and −10.8 ± 6.2 mV (n = 4) for 25 μM 1,4,5-$InsP_3$. At a concentration of 25 μM about 50% of

Table I

The membrane potential (E_m) after 1 s and at about 1 min after formation of whole-cell configuration under various conditions

Extracellular solution	Intracellular solution	E_m (1 s) (mV)	E_m (1 min) (mV)
ECS	ICS	−48.1 ± 11.3 (n = 18)	−46.3 ± 14.4 (n = 18)
ECS	ICS + 100 μM 1,4,5-$InsP_3$	−42.6 ± 15.6 (n = 16)	−53.4 ± 19.0 (n = 16)
ECS	high-EGTA[1]	−46.8 ± 4.2 (n = 5)	−48.0 ± 4.5 (n = 5)
ECS	high-EGTA + 100 μM 1,4,5-$InsP_3$	−45.0 ± 10.0 (n = 7)	−43.4 ± 9.2 (n = 7)
ECS	Ca^{2+}-free[2]	−38.9 ± 11.5 (n = 8)	−32.0 ± 18.5 (n = 8)
ECS	Ca^{2+}-free + 100 μM 1,4,5-$InsP_3$	−33.0 ± 12.0 (n = 5)	−50.0 ± 14.1 (n = 5)
ECS + 0.2 mM quinine	ICS + 100 μM 1,4,5-$InsP_3$	−36.7 ± 17.8 (n = 6)	−24.7 ± 22.3 (n = 6)
ECS	ICS + 100 μM 4,5-IP_2	−38.3 ± 9.4 (n = 4)	−26.3 ± 13.1 (n = 4)
ECS	ICS + 100 μM 1,4-IP_2	−30.0 ± 12.2 (n = 5)	−14.0 ± 11.9 (n = 5)

[1] ICS with high EGTA (30 mM), pH 7.2
[2] See section 2.3, pH 7.2

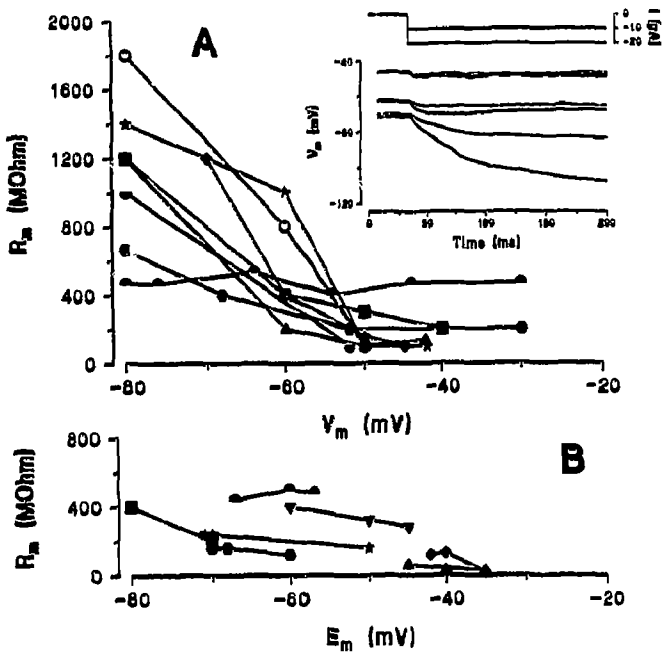

Fig. 1. (A) The increase in the membrane resistance of DRG neurons upon hyperpolarization of the membrane to potentials < −50 mV using a holding current. Data from 8 experiments are shown. The inset shows how the membrane resistance was measured in one neuron. The membrane potentials were held at values shown. Two current pulses (in steps of −40 pA) were applied. The membrane resistances were calculated by dividing the stationary ΔV_m by the applied ΔI. (B) The changes in the DRG neuron membrane resistance during 100 μM 1,4,5-$InsP_3$- induced hyperpolarizations. Data from 7 experiments.

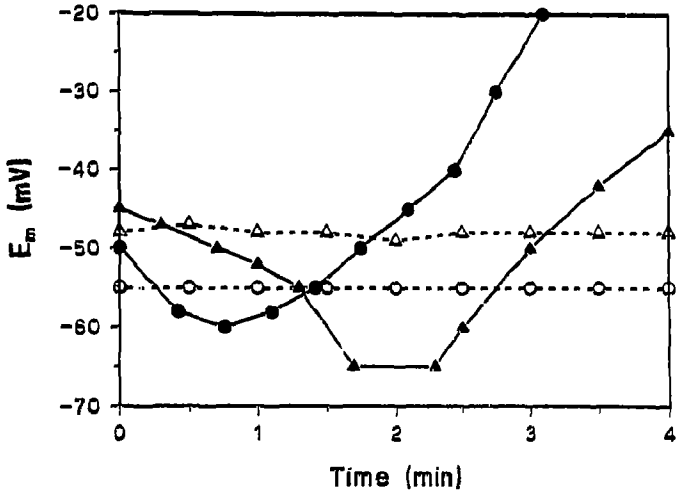

Fig. 2. Responses of the DRG neuron membrane potential upon stimulation with 100 μM 1,4,5-$InsP_3$ (filled symbols) and of controls (without 100μM 1,4,5-$InsP_3$, open symbols). Responses from 4 experiments are shown. The two 1,4,5-$InsP_3$ curves selected illustrate the variability in the exact time course of the hyperpolarization.

the cells responded with a membrane hyperpolarization (Fig. 3).

To examine whether the hyperpolarization was directly due to a response to 1,4,5-$InsP_3$ or due to a response to a 1,4,5-$InsP_3$ breakdown product, we performed experiments with two different $InsP_2$ isomers. Perfusion of cells with ICS + 100 μM 1,4-$InsP_2$ did not cause a membrane hyperpolarization response in DRG neurons (Table I). These cells started to depolarize rapidly and subsequently died. However, the response to the other isomer, 100 μM 4,5-$InsP_2$, was different (Table I). After a first depolarization the membrane potential repolarized again (maximum value reached at about 2 min after the whole-cell configuration was obtained) to the initial membrane potential value (n = 4). This repolarization was accompanied by a membrane resistance decrease. Since the membrane potential responses to both 1,4-$InsP_2$ and 4,5-$InsP_2$ were much different from the response to 1,4,5-$InsP_3$ we conclude that the 1,4,5-$InsP_3$-induced membrane hyperpolarization is not due to the formation of the 1,4,5-$InsP_3$ breakdown products 1,4- and 4,5-$InsP_2$.

3.3. *The mechanism of the 1,4,5-$InsP_3$-induced hyperpolarization*

In order to investigate the 1,4,5-$InsP_3$ mechanism of action, a number of different experiments were performed. Since 1,4,5-$InsP_3$ is known to release calcium from intracellular stores we tested the possible involvement of $[Ca^{2+}]_i$ in the 1,4,5-$InsP_3$-induced membrane hyperpolarization. The role of changes in $[Ca^{2+}]_i$ was investigated by using different calcium buffering solutions supplemented with 100 μM 1,4,5-$InsP_3$ in the pipette.

The 1,4,5-$InsP_3$-induced hyperpolarization disappeared when the patch-clamp electrodes were filled with a high EGTA (30 mM) intracellular solution together with 100 μM 1,4,5-$InsP_3$ (Table I). On the other hand, 1,4,5-$InsP_3$ in a non-calcium buffered Ca^{2+}-free (no EGTA) intracellular solution induced a membrane hyperpolarization (Table I). These results suggest that the 1,4,5-$InsP_3$-induced hyperpolarization is mediated by a (local) 1,4,5-$InsP_3$-induced $[Ca^{2+}]_i$ elevation. Increases in $[Ca^{2+}]_i$ lead to the activation of calcium-dependent potassium channels. Calcium-dependent potassium channels can be blocked by quinine [10]. Addition of 0.2 mM quinine to the extracellular medium completely abolished the 1,4,5-$InsP_3$-induced hyperpolarization (Table I). Our experiments, therefore, suggest that the 1,4,5-$InsP_3$-induced membrane hyperpolarization in cultured DRG neurons is due to the activation of calcium-dependent potassium channels via an 1,4,5-$InsP_3$-induced elevation of the cytoplasmic calcium level.

4. DISCUSSION

The study of the role of second messengers in cell function, morphology and development requires independent measures to show that the second messengers used can be introduced functionally into the studied cells. Our experiments demonstrate that DRG neurons

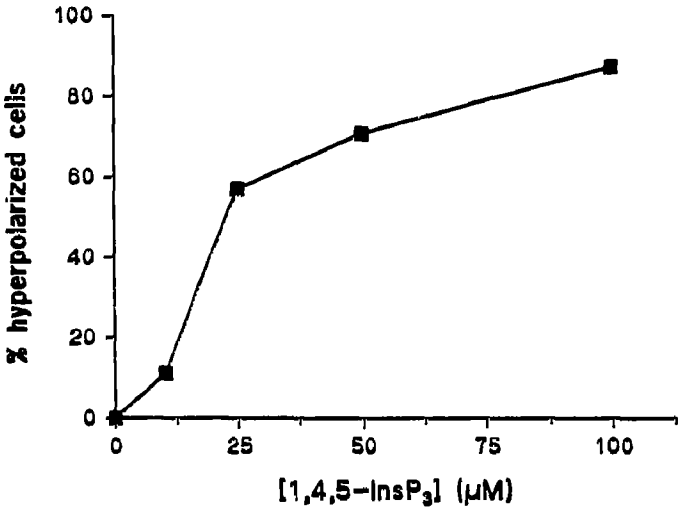

Fig. 3. Percentage of the DRG neurons which responded to perfusion with 1,4,5-$InsP_3$ as a function of the used 1,4,5-$InsP_3$ concentration.

from cultures in a chemically defined medium show electrophysiological responses consistent with intracellular calcium release upon perfusion of the cell with 1,4,5-$InsP_3$.

It is well known that $[Ca^{2+}]_i$ and modulation of $[Ca^{2+}]_i$ plays an important role in neuron regeneration, excitability and neurotransmitter release [4]. 1,4,5-$InsP_3$ is a second messenger in signal transduction, which mobilizes intracellular calcium, with an important function in controlling various cellular processes (e.g. [3,4,6,11]). 1,4,5-$InsP_3$ injection induces a hyperpolarization in various types of neurons [4,12]. In our experiments, we used DRG neurons which do contain caffeine- and ryanodine-sensitive intracellular Ca^{2+} stores [13]. Intracellular 1,4,5-$InsP_3$ perfusion was found to evoke a transient hyperpolarization followed by a depolarization (Fig. 2). This hyperpolarization is dependent on calcium-buffering (Table I). Therefore, the hyperpolarization is likely associated with an 1,4,5-$InsP_3$-induced Ca^{2+} release.

DRG neurons in cultures show a large morphological and physiological heterogeneity [1,2,14–16]. This variability is reflected, as well, in the electrophysiological parameters and probably also in the 1,4,5-$InsP_3$-induced responses of these cells. A broad range was observed in membrane potentials and membrane resistances (Fig. 1A, Table I). In most cells 1,4,5-$InsP_3$ evoked large hyperpolarizations (mean ΔE_m > 10 mV). However, in some cells only small hyperpolarizations (ΔE_m < 5 mV) or no effect could be observed. We tried to minimize the heterogeneity by using only 1-day cultured cells which had stable membrane potentials more negative than −20 mV. A further analysis of the 1,4,5-$InsP_3$-induced hyperpolarization showed that this hyperpolarization is accompanied by only a slight or no increase in membrane resistance (Fig. 1B). This increase is much smaller than the membrane-resistance increase observed upon a similar (induced by current injection) hyperpolarization in control cells (cf. Fig. 1A). From this we conclude that the slight 1,4,5-$InsP_3$-induced membrane resistance increase is likely composed of a membrane-resistance increase due to hyperpolarization of the plasma membrane (as in current-injected control cells), and a membrane-resistance decrease due to the opening of plasma membrane ion channels. The fact that the potassium-channel blocker quinine completely inhibits the 1,4,5-$InsP_3$-induced hyperpolarization (Table I) confirms this conclusion. Hence, this suggests that the 1,4,5-$InsP_3$-induced hyperpolarization is at least due to the opening of potassium channels. However, the absence of a net decrease in R_m implies that besides channel opening, other than K^+ channels must close. A similar hyperpolarization accompanied membrane resistance increase, due to closure of chloride channels, was observed in the acetylcholine-induced response in sympathetic neurons and the phorbol-12,13-dibutyrate-induced response in hippocampal pyramidal cells [17,18]. Such chloride channels may be present in DRG cells as well.

The dependency on the 1,4,5-$InsP_3$ concentration is mainly reflected in the number of responding cells (Fig. 3), and not in the magnitude of the hyperpolarization. This may suggest the presence of an 'all-or-nothing' mechanism consisting of positive feedback regulation as found in many calcium oscillating systems [3,19]. $[Ca^{2+}]_i$ measurements during $InsP_3$ perfusion may reveal more about the presence of such a mechanism in DRG cells. In conclusion, the present work provides a first step towards the establishment of the functional role of 1,4,5-$InsP_3$ in DRG neurons.

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