Turkish Journal of Medical Sciences

Volume 31 | Number 1 Article 4

1-1-2001

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AYAR, AHMET and SCOTT, RODERICK H. (2001) "Difficulties Associated with the Study of Action Potential After-potentials in Rat Cultured Sensory Neurones," *Turkish Journal of Medical Sciences*: Vol. 31: No. 1, Article 4. Available at: https://journals.tubitak.gov.tr/medical/vol31/iss1/4

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Turk J Med Sci
31(2001) 29-34
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Difficulties Associated with the Study of Action Potential After-potentials in Rat Cultured Sensory Neurones

Received: June 07, 2000

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Abstract: We investigated the properties of action potential after-potentials in cultured dorsal root ganglion neurones from neonatal rats using the whole cell patch clamp technique. The aims of this study were to characterize after-potentials and investigate the possible influences of Ca^{2+}-induced Ca^{2+} release (CICR) on neuronal excitability. The reversal potentials of action potential after-depolarizations and after-hyperpolarizations were estimated. Although evidence for both Ca^{2+}-activated chloride and potassium conductances was obtained, the data indicated that the after-potentials were contaminatied by a variety of distinct ionic events. Apamin was used to attenuate potassium conductances and appeared to enhance the after-depolarizations due to Ca^{2+}-activated chloride conductances. Ba^{2+} substitution for Ca^{2+} significantly broadened the duration of evoked action potentials, which were followed by large-amplitude after-depolarizations. The chloride channel blocker niflumic acid was used to identify Ca^{2+}-activated chloride conductances. Studies were also carried out with ryanodine to investigate divalent cation-induced -Ca^{2+} release. However, ryanodine attenuated both the prolonged Ba^{2+}-action potentials and after-depolarizations. We conclude that cultured DRG neurones express a variety of channels, which can contribute to action potential after-potentials and thus provide distinct and variable influences on neuronal excitability.

Key Words: Ryanodine, after-potentials, cultured sensory neurones, apamin and niflumic acid.

Introduction

Calcium is one of the most diverse and widespread of intracellular second messengers in cells. In neuronal preparations, Ca^{2+} plays a key role in a variety of cellular physiological processes, including vesicular neurotransmitter release, altered gene expression, enzyme activation and changes in neuronal excitability through the modulation of Ca^{2+}-sensitive ion channels (1). Increases in intracellular Ca^{2+} can be achieved by the entry of Ca^{2+} from extracellular space through voltage and ligand-gated membrane channels, and Ca^{2+} can be released from intracellular stores. Two distinct types of intracellular Ca^{2+} stores have been identified, one sensitive to inositol 1,4,5-trisphosphate ($InsP_3$) (2, 3), and the other sensitive to caffeine, ryanodine, cADP-ribose and dihydrosphingosine (4-10). Despite their pharmacological differences, both types of intracellular stores are sensitive to Ca^{2+} itself, and the phenomenon of CICR provides mechanisms for the amplification of local $[Ca^{2+}]_i$ signals with Ca^{2+} release from internal stores (5, 11-13).

We have previously shown that CICR may occur in cultured rat DRG neurones following a single action potential and under voltage clamp conditions. Evidence for CICR was in part provided by the antagonistic actions of the plant alkaloid ryanodine (9). In the present study, we tried using a pharmacological approach to address the difficulties associated with contamination of Ca^{2+}-activated chloride conductances with Ca^{2+}-activated potassium conductances, within action potential after-potentials.

Replacement of extracellular Ca^{2+} with equimolar Ba^{2+} was used in part of the study to determine whether Ba^{2+} passing through voltage-activated Ca^{2+} channels would support the activation of after-depolarizations. These experiments were done because Ba^{2+} action potentials may offer another approach to the study of divalent cation-induced Ca^{2+} release from stores. Additionally, Ba^{2+} may remove the problems encountered with chloride conductances being masked or contaminated with potassium conductances.

Material and Method

Cell Culture

Dorsal-root ganglion (DRG) neurones were grown in primary culture as previously described (7). One-to two-day-old Wistar rats were decapitated and the ganglia from the spinal columns were dissected out, and incubated at 37°C in collagenase and trypsin (Sigma, 0.125%, 13 min, 0.25%, 6 min respectively). Ganglia were dissociated into single cells by trituration through a flame-constricted Pasteur pipette. Cells were plated on poly-L-ornithine/laminin-coated glass coverslips (16 mm, round). Cells were grown in Ham's F14 (supplemented with glutamine, Imperial Laboratories, Andover, Hampshire UK) with 10% heat-inactivated Horse Serum (GIBCO, Grand Island, NY), penicillin/streptomycin (ICN, 5.000 IU/ml and 5.000 μg/ml respectively) and nerve growth factor (NGF) (Sigma, 10 μg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and used between 2 days and 1 month after plating.

Electrophysiology

Experiments were conducted at room temperature (18-20°C). The whole cell variant of the patch clamp technique (14) was used to record from cultured neonatal rat DRG neurones. The patch pipettes were made from Pyrex borosilicate glass tubing (1.4/1.6mm outer diameter, 0.8/1.0mm bore with 0.15mm fibre attached to the inside wall, Plowden and Thompson Ltd, Dial Glass Works) using a two-stage vertical microelectrode puller (David Kopf Instruments, Tujunca, USA, Model 730), giving a resistance of 3-7 MΩ when filled with patch pipette solution. An Axoclamp 2A switching amplifier (Axon Instruments) was used. Recordings were made from cells following the formation of GΩ seal resistances prior to entering the whole-cell recording configuration.

Solutions and Drugs

For the current clamp recordings, the patch pipette was filled with solution (in mM): KCl, 140; $CaCl_2$, 0.1; $MgCl_2$, 2.0; ATP, 2.0; HEPES, 10.0; EGTA, 1.1. The pH and osmolarity were adjusted to 7.4 and 310 mOsm with Tris and sucrose, respectively. The extracellular recording medium contained (in mM): NaCl, 130; KCl, 3.0; $MgCl_2$, 0.6; $CaCl_2$ or $BaCl_2$, 2.0; $NaHCO_3$, 1.0; HEPES, 10.0; glucose, 5.0. The pH was adjusted with NaOH to 7.4 and the osmolarity to 310-320 mOsm with sucrose.

The drugs used in this study were ryanodine (Research Biochemicals International), apamin (Research Biochemicals International) and niflumic acid (Sigma). All drugs were applied by low-pressure ejection from a blunt pipette positioned about 100 μ away from the cell being recorded.

Data analysis

Action potentials and accompanying after-potentials were recorded onto digital audio tape using a digital audio tape recorder (Biologic). Data analysis was either carried out directly using an oscilloscope or following acquisition using Cambridge Electronic Design computer software. Data are presented as mean values ± standard error of means (S.E.M.). Student's *t* test was used for statistical comparison of paired and unpaired data (Microcal Origin) as appropriate. P values <0.05 were considered statistically significantly different from control values.

Results

Action potentials were evoked by 5ms depolarizing current commands from various holding potentials and the reversal potentials of the after-potentials estimated. DRG neurones which showed a predominant after-depolarization had a mean reversal potential of -20 ± 2 mV (n=4, Fig. 1A). This estimated value was 21 mV negative to the predicted equilibrium potential for chloride. When mixed after-potentials (9) were clearly observed (i.e., after-hyperpolarization followed by after-depolarization) the discrepancy was greater, with a mean value of -47 ± 2 mV (n=6) for the estimated reversal potential. The after-depolarizations can have clear excitatory influences on action potential firing (9), and this effect was particularly apparent when the after-depolarizations followed an anode break spike (Fig. 1B).

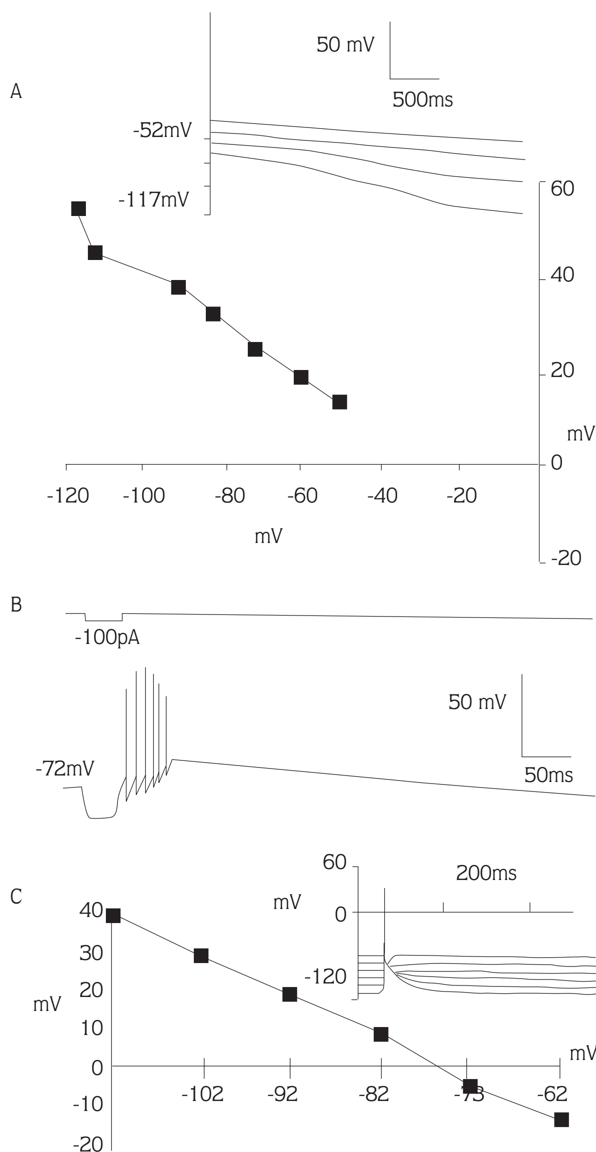

Figure 1. Action potential after potentials. **A.** Graph showing the effect of membrane potential (-52 to -117 mV) on the amplitude of action potential after-depolarizations. The estimated reversal potential for the after-potential recorded from this neurones is -27 mV. The inset record shows the action potentials and after-depolarizations evoked in a cultured DRG neurone. **B.** Record shows the hyperpolarizing current command applied to activate an anode break spike from a resting membrane potential of -72 mV. The anode break spike is followed by an after-depolarization which drives a burst of five action potentials. **C.** Graph showing the effect of membrane potential (-62 to -112 mV) on the amplitude of action potential after-hyperpolarizations. The estimated reversal potential for the after-potential recorded from these neurones is -76 mV. The inset record shows the action potentials and after-potentials evoked.

Anomalies between reversal potential and the predicted equilibrium potential were also seen for DRG neurones which showed predominant after-hyperpolarizations (Fig. 1C). These after-potentials might be expected to reverse close to the potassium equilibrium potential, approximately -97mV; however, the mean reversal potential for these events was -75 ± 2 mV (n=11).

To address the problem of chloride and potassium conductances contaminating one another, we firstly used the "small" conductance Ca^{2+}-activated potassium channel blocker apamin. At a concentration of 0.5 μM, apamin attenuated the repolarizing phase of the action potentials and caused significant prolongation of action potentials and after-depolarizations (n=6; Fig. 2A). These data were difficult to intrepret because the larger Ca^{2+} influx during prolonged action potentials would inevitably increase Ca^{2+}-dependent after-depolarizations. However, at a lower concentration, of 0.1 μM apamin was found to increase the amplitude of the after-depolarization without altering the characteristics of the action potential (n=3, Fig. 2B).

Using Ba^{2+} as the charge carrying divalent cation attenuated potassium conductances, including the delayed rectifier and Ca^{2+}-activated potassium conductances. The mean peak amplitude of the Ba^{2+} action potential was 105 ± 7 mV (n=7). The large amplitude of these events probably reflects potassium channel inhibition and the inability of Ba^{2+} to support divalent cation-induced Ca^{2+} channel inactivation. Barium action potential durations were not consistent even when repeatedly recorded from the same cell. The approximate mean value for the duration of the prolonged action potentials was 4200 ±2520 msec (n=7). Niflumic acid (10μM) applied for 3 to 5 minutes significantly reduced Ba^{2+} action potential after-depolarization from 24 ± 8 mV to 6 ± 6 mV (n=7 $p<0.05$). The effect was not due to variability in the duration of the prolonged action potentials. Figure 2C shows a record from a cell where the duration of the action potential was increased, resulting in a larger influx of divalent cation, but niflumic acid still reduced the amplitude of the after-depolarization.

To determine the possible role of Ba^{2+}-induced Ca^{2+} release (15), ryanodine was used to try to block mobilization of Ca^{2+} from stores. However, ryanodine (10μM) application for 3 to 5 minutes reduced both the duration of Ba^{2+} action potentials and the after-

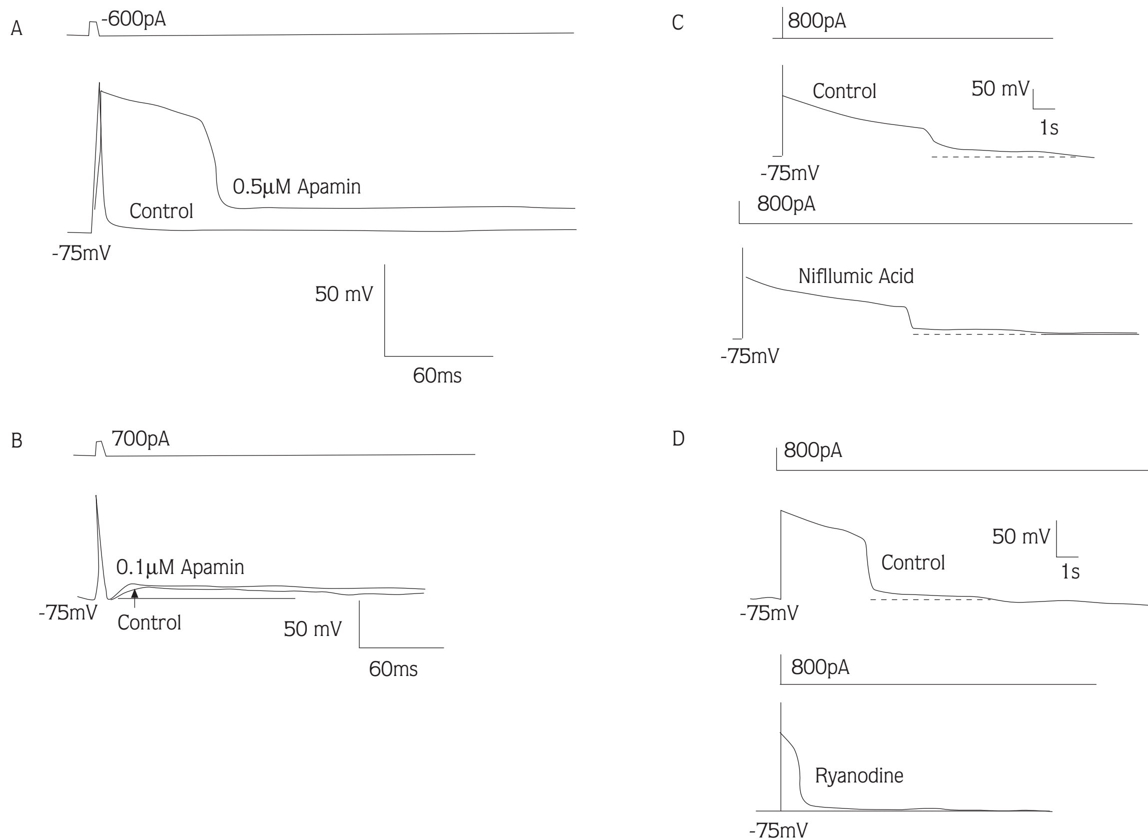

Figure 2. The effects of apamin and Ba^{2+} on after-depolarizations. Current clamp records showing action potentials with after-depolarizations evoked by brief (5ms) current commands from holding potentials of -75mV. **A.** Record showing the prolongation of an evoked action potential by 0.5 μM apamin. **B.** From a holding potential of -75 mV, 3 minutes application of apamin (0.1 μM) evoked an apparent enhancement of the after-depolarization without altering the properties of the action potential. **C.** Niflumic acid (10 μM) blocks the Ba^{2+} action potential after-depolarization. **D.** Ryanodine (10 μM) attenuated both Ba^{2+} action potentials and after-depolarizations.

depolarization amplitudes (Fig. 2D). In this study, the inhibitory actions of ryanodine did not reverse 5 minutes after removal of the pressure ejection pipette containing the drug.

Discussion

The problem of contamination of Ca^{2+}-activated chloride conductances with Ca^{2+}- activated potassium conductances can be eliminated under voltage clamp conditions with a combination of CsCl-based patch pipette solution and TEA in the external recording medium (9, 16- 18). Difficulties remain in studying after-potentials and using these events to investigate CICR following single-action potentials (9).

Apamin provided a useful tool for attenuating potassium conductances which partially mask the depolarizing effects of the Ca^{2+}-activated chloride conductance. However, 0.1 μM apamin did not completely abolish the after-hyperpolarization, and it is likely that contamination by apamin-resistant potassium conductances still takes place. The use of Ba^{2+} as a charge carrier in place of Ca^{2+} was also problematic. Ba^{2+} was found to increase the duration of action potentials in this study by inhibiting delayed voltage-dependent K^{+} conductances and therefore action potential repolarization (16). Ba^{2+} increased the duration of action potentials by blocking a number of K^{+} channels (15) and thus promoted the influx of divalent cations through voltage-activated Ca^{2+} channels (19). In rat sensory neurones, Ba^{2+} appears to also indirectly activate Ca^{2+}-

activated chloride currents (16-18) by triggering Ca^{2+} release from caffeine-sensitive intracellular stores. Barium-activation of chloride currents was found to be sensitive to caffeine, which depleted Ca^{2+} stores and abolished the Cl^- tail currents (17). Substitution of Ca^{2+} for Ba^{2+} under current clamp recording conditions still enabled the activation of action potential after-depolarizations which were not contaminated with K^+ conductances, and suggested that Ba^{2+} -induced Ca^{2+} release occurred. The sensitivity of the Ba^{2+}-activated after-depolarizations to the Cl^- channel blocker niflumic acid suggested that these potentials were due to Ca^{2+}-activated chloride channels.

Our data strongly supports the contention that divalent cation-induced Ca^{2+} release occurs under our recording conditions. However, Shmigol et al. (20) failed to find evidence for CICR following single action potentials in these neurones when they measured $[Ca^{2+}]_i$ concentration using indo-1. Nevertheless, in this same study CICR was detected following trains of action potentials. This difference could be due to the use of different methods, as Shmigol et al. (20) measured the global activation of CICR, which may need relatively large Ca^{2+} entry with longer depolarizations. In our studies, the detection of CICR following a single action potential in some neurones may result from local $[Ca^{2+}]_i$ increase (9), as it has been shown in neuronal preparations that increases in $[Ca^{2+}]_i$ can be confined to small intracellular compartments (21). In rat DRG neurones no information is yet available on the localization of Ca^{2+} release channels, although it has but consistently been found that $[Ca^{2+}]_i$ fluctuations are sensitive to ryanodine (20). It is clear from our studies that neuronal excitability is influenced by Ca^{2+}-activated conductances in variable ways which may reflect the heterogeneous characteristics of sensory neurones both in culture and in vivo. We also suggest that, in addition to the influence of Ca^{2+} entry through voltage-activated ion channels, CICR can influence the excitability of cultured DRG neurones.

Acknowledgements

The authors wish to thank Fırat University and the Turkish government for supporting this study.

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