

NSL 08540

Influence of Na/Ca exchange and mobilization of intracellular calcium on the time course of the slow afterhyperpolarization current (I_{AHP}) in bullfrog sympathetic ganglion neurons

Joanne W. Goh^a, Maria V. Sanches-Vives^b and Peter S. Pennefather^c

^aDepartment of Pharmacology and Toxicology, Queen's University, Kingston, Ont. (Canada), ^bInstituto de Neurociencias, Universidad de Alicante, Alicante (Spain) and ^cMRC Nerve Cell and Synapse Group, Faculty of Pharmacy, University of Toronto, Toronto, Ont. (Canada)

(Received 18 September 1991; Revised version received 15 January 1992; Accepted 20 January 1992)

Key words: Afterhyperpolarization current; Bullfrog ganglion neuron; Intracellular calcium store; Na/Ca exchange; Ryanodine; Calcium-activated potassium channel

I_{AHP} is a calcium dependent potassium current that underlies slow afterhyperpolarizations following action potentials in bullfrog sympathetic ganglion neurons. The decay rate of I_{AHP} increases with increasing calcium loads. This effect was found not to be due to mobilization on intracellular calcium from ryanodine and caffeine sensitive stores. The relation is not affected by ryanodine at concentrations that block mobilization in the presence of caffeine, a drug that enhances mobilization of those stores. Nor does the relation seem to be due to a reduction of the driving force of the Na/Ca exchange process. The relation between decay rate and calcium load persists when Na^+ is replaced by Li^+ . Our results suggest that Na/Ca exchange and mobilization of intracellular calcium normally have little influence in determining the time course of I_{AHP} in these neurons.

Action potentials (APs) in B neurons of bullfrog sympathetic ganglia are followed by a slow afterhyperpolarization (AHP) that is voltage insensitive and calcium dependent. The physiological role of the slow AHP in neurons is to modulate excitability in those neurons, in particular, to reduce their propensity for generation of multiple action potentials. The slow AHP is a major contributor to the process of spike frequency adaptation [1]. The current underlying AHP's in bullfrog sympathetic neurons, I_{AHP} , is generated by a distinct class of channels that are uniquely sensitive to pharmacological blockade by the bee venom peptide apamin, d-tubocurarine [1] as well as by a minor component of a scorpion venom, leiurotoxin I [13].

These pharmacological properties suggest that I_{AHP} is generated by a small conductance channel. This channel is known to be activated by calcium in the 100–300 nM range [6]. Because of their lack of voltage sensitivity and their ability to respond to calcium levels only slightly above rest, the AHP channels can act as a sensitive indicator of the time course of calcium signals evoked by action potentials in sympathetic neurons. We have shown that this time course is prolonged by increasing

the calcium load [1, 11, 12], so that AHPs that follow trains of APs are longer than those which follow single APs. Two possible explanations for this observation are examined here.

The first possibility is that with large calcium loads, intracellular calcium can reach levels sufficiently high to mobilize calcium from intracellular stores. In the presence of caffeine, calcium mobilization is sensitized to an extent that the stores can discharge spontaneously and give rise to spontaneous AHP currents with decay times similar to the prolonged I_{AHP} s that follow trains of APs (see below). Moreover, Kawai and Watanabe [4, 5] have reported that ryanodine, an alkaloid which interferes with calcium release from the caffeine sensitive pool of intracellular calcium, can shorten AHPs recorded in rat sympathetic ganglia. The second possibility examined is that Na/Ca exchange is involved in clearing calcium from the vicinity of AHP channels and that with increasing calcium loads, Na/Ca exchange become less effective due to saturation of the exchanger. We conclude however, that normally, neither Na/Ca exchange nor mobilization of intracellular calcium are major determinants of the time course I_{AHP} or of the relation between the magnitude of calcium loads and the duration of I_{AHP} .

The preparation used was intact paravertebral sympathetic ganglia from adult bullfrogs (*Rana ca-*

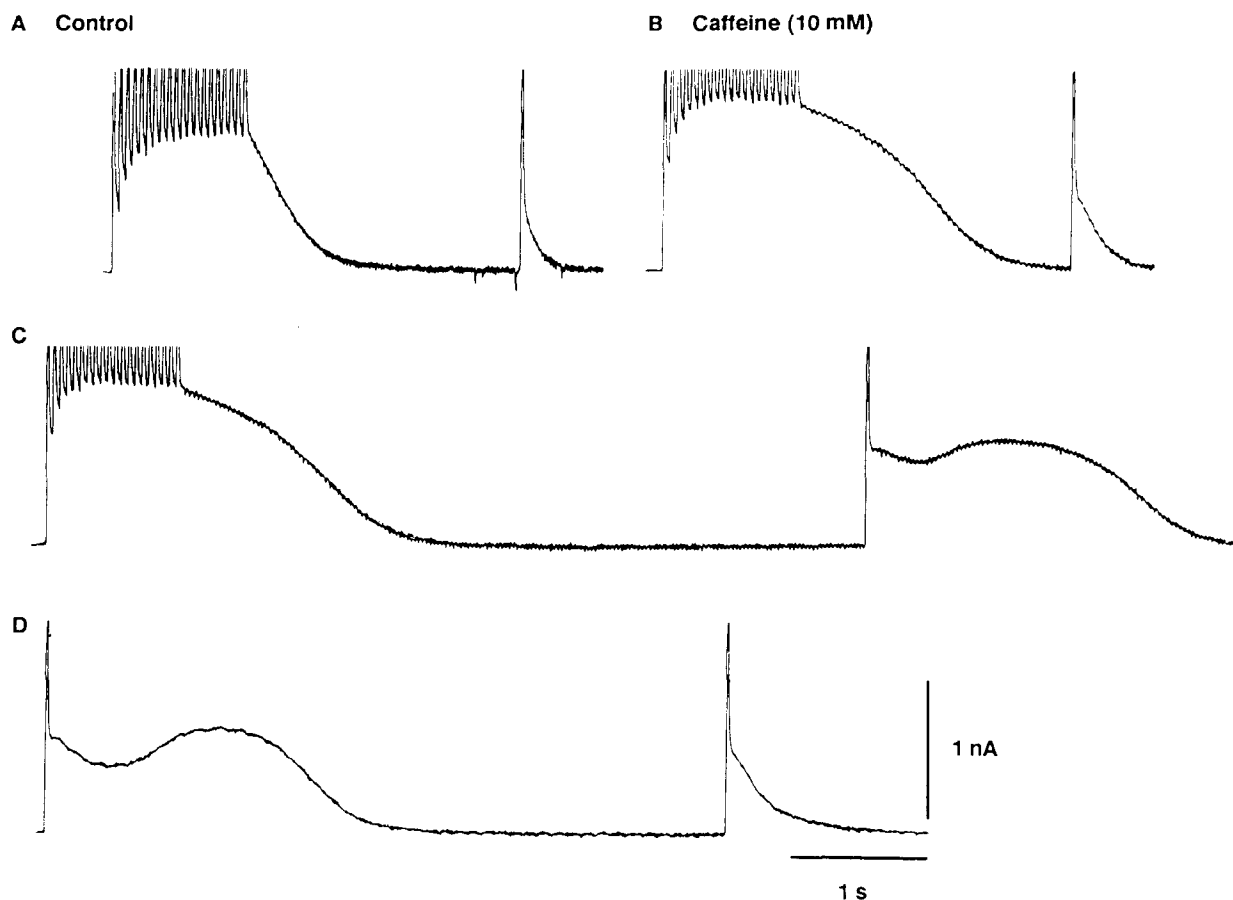


Fig. 1. Effect of caffeine on I_{AHP} evoked by single brief depolarizations or by 1 s trains of 20 such depolarizations. In all traces the cell was held at -60 mV using a single electrode discontinuous voltage clamp. The depolarizations were reproduced by changing the holding potential to 0 mV for 10 ms. A: control I_{AHP} following a train and following a single depolarization 2 s later. Note, the I_{AHP} following a single depolarization decays at a faster rate than following a train. Both I_{AHP} s decay at a fairly constant rate, (i.e. in an exponential manner). B: same protocol, only in the presence of 10 mM caffeine. Note, decay rate of I_{AHP} following the train now increases with time but there is still a large difference in mean decay rates between I_{AHP} following a train and following a single depolarization. C: in the presence of caffeine, when the interval between the beginning of the train and the single depolarization is increased to 6 s single depolarization now is capable of evoking a slowly rising I_{AHP} . The slow component reflects the action of calcium mobilized from intracellular stores and has the same time course as spontaneous outward currents recorded in this cell after the addition of caffeine. The early component reflects AHP channels evoked by calcium that enters the neuron during the depolarization. The non-exponential nature of the decay of I_{AHP} following the train also reflects summation of calcium from intracellular and extracellular sources. D: also in the presence of 10 mM caffeine, the trace shows that 5 s is insufficient for repletion of intracellular stores to a point that the second pulse can trigger their discharge. Note that when intracellular stores are mobilized the decay of I_{AHP} is the same regardless of the calcium load from extracellular sources (compare with C). Scales are the same in all sections of the figure. Scale bars in D are vertical: 1 nA and horizontal: 1 s. All traces in the figure were recorded from a single representative cell.

tesbeiana). Following stunning, decapitation and pithing, ganglia were isolated from the animal and excess connective tissue was dissected away. The ganglia were pinned onto a Sylgard (Dow-Corning) base in a petridish and treated with 1% trypsin (Sigma, Type I) for 8 – 10 min to facilitate removal of remaining connective tissue. The tissue was submerged and constantly perfused with Ringer solution (115 mM NaCl, 2.5 mM KCl, 4 mM CaCl_2 , 1 mM MgCl and 2.5 mM Tris; pH 7.2). Experiments were conducted at room temperature, approximately 24°C . Caffeine and ryanodine were prepared as stock solutions and added to perfusing Ringer solution in the appropriate amounts. In some experiments Na⁺

free medium was prepared by completely substituting NaCl with equimolar quantities of LiCl. Ryanodine was purchased from Research Biochemicals Inc. All other chemicals were purchased from Sigma.

Recording microelectrodes were filled with 3 M KCl and had resistances between 20 and 30 M Ω . Cycle rate for discontinuous current and voltage clamp was 6 – 12 kHz and duty cycle was $1:2$. Capacity compensation was continuously monitored and adjusted to ensure proper headstage settling. The slow afterhyperpolarization triggered by either a single spike or one or more trains of APs was recorded in current-clamp mode. The current underlying this slow potential, I_{AHP} , was recorded using

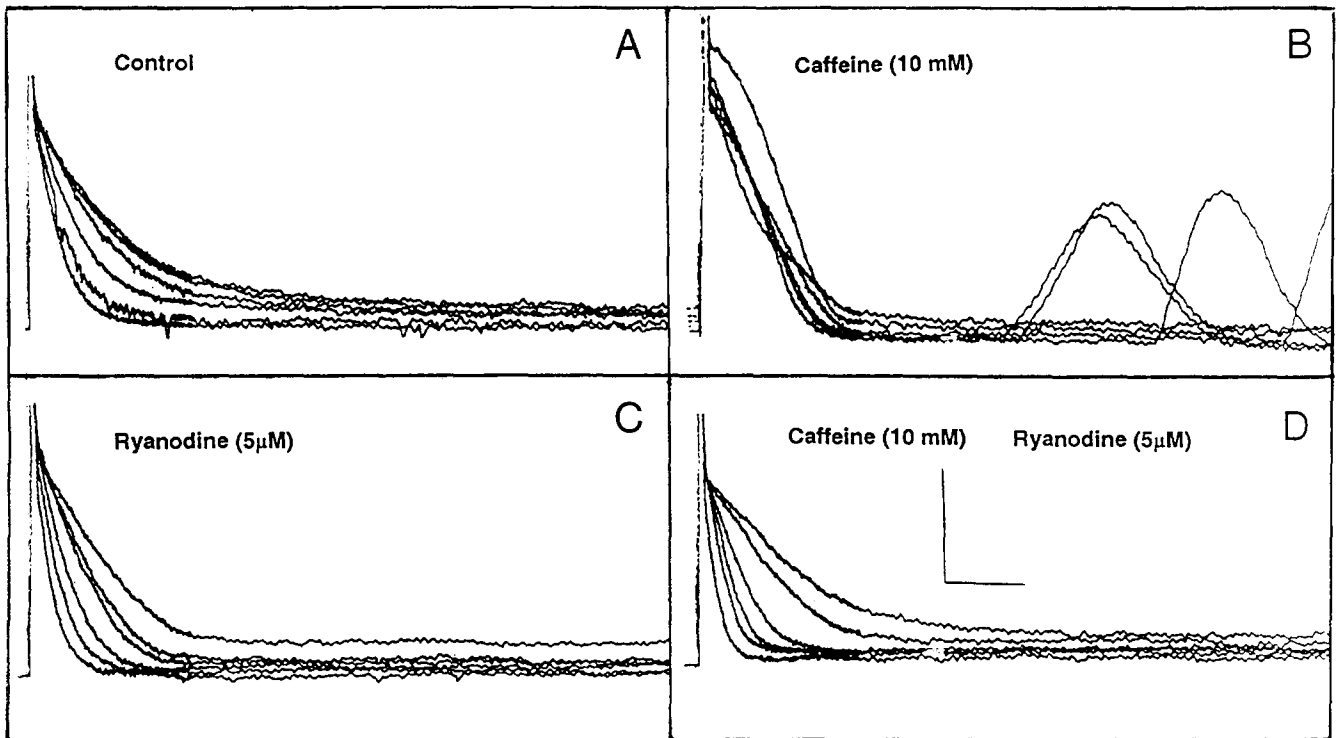


Fig. 2. Effect of caffeine and ryanodine on the relation between decay rate of I_{AHP} and calcium load. Each box shows superimposed records of I_{AHP} , recorded using the hybrid clamp technique, following trains of increasing durations of bursts of action potentials. Bursts, of 2–4 action potentials were evoked by a 50 ms current clamp pulse from -60 mV. Trains (10 Hz) of 1, 2, 4, 8, 16 and 24 such burst preceded the initiation of a 10 s period of voltage clamp to -60 mV. Trains were separated by at least 30 s of recovery time. A: control. B: in the presence of 10 mM caffeine. C: in the presence of 5 μM ryanodine. Scales are the same for A, B, and C. Scale bars are vertical: 0.5 nA and horizontal: 1 s.

the hybrid clamp protocol where current clamp was switched to voltage clamp immediately upon repolarization of the last AP [1]. Single APs were evoked by brief, suprathreshold intracellular current pulses (2 ms duration) and single or multiple trains of 2–4 APs per train were triggered by applying current clamp commands (1–2 nA) for 50 ms with an interstimulus interval of 50 ms. In some experiments the cell was continuously voltage clamped and calcium influx was evoked by 10 ms depolarizations to 0 mV. During each experiment, responses were simultaneously displayed on a storage oscilloscope captured for later analysis using pClamp software (Axon Instruments) and were plotted on a Gould 2400 strip chart recorder.

As observed by others [3, 4], we found that 10 mM caffeine induced the occurrence of spontaneous hyperpolarizing potentials. After 5–10 min exposure, these spontaneous potentials became much less frequent. However, they could still be evoked by brief depolarizations (see below). In Fig. 1, a ganglion neuron was held in the single electrode voltage clamp mode at -60 mV, and 10 ms pulses to 0 mV, either in the form of individual pulses or 1 s, 20 Hz trains, were used to evoke calcium influx and generate I_{AHP} . The slowly decaying outward

current that follows these stimuli is almost entirely I_{AHP} [1]. Notice that the decay rate of I_{AHP} following the train was slower than that following a single pulse. Addition of 10 mM caffeine to the bathing medium prolonged I_{AHP} that followed either the train or single pulse. Normally, I_{AHP} decayed monotonically after the end of an AP. In the presence of caffeine however, a single pulse could initiate an AHP current that first decayed then began to rise slowly before decaying at a slow rate (Fig. 1B,C). Since the magnitude of the I_{AHP} reflects intracellular calcium levels [1] and influx of calcium is minimal at -60 mV, the slow rise of I_{AHP} under voltage clamp conditions probably reflects a slow discharge of intracellular stores triggered either by the depolarization per se, the calcium that comes in during the pulse, or a combination of these two influences. In zero calcium medium a slow response sometimes could still be evoked but, only once or twice before the cell became refractory and intracellular stores presumably were depleted (data not shown). The latter observation suggests that depolarization alone can be a sufficient trigger of the slow response in the presence of caffeine.

Even when calcium is present in the bathing medium, it takes some time for the cell to recover its ability to

TABLE 1
EFFECT OF Na⁺-FREE MEDIUM (Li⁺-SUBSTITUTED) ON HALF-DECAY TIME OF I_{AHP}

*Each train of 2–4 action potentials was evoked by a 50 ms long current-clamp command. Interval between trains was 50 ms.

	I_{AHP} half-decay time (ms)			
	1 Spike	5 Trains*	10 Trains*	20 Trains*
Control	113 ± 7	267 ± 41	470 ± 66	640 ± 123
Li ⁺	120 ± 12	380 ± 76	630 ± 104	780 ± 131
% Increase	6 ± 6%	40 ± 7% [†]	34 ± 9% [†]	25 ± 16% [†]
<i>n</i>	3	3	4	4

[†] Significant increase ($P < 0.05$). All values reported are mean ± S.E.M.

generate the slow AHP current evoked by a brief depolarization. Hence, the second pulse in Fig. 1B did not evoke a slow response while the second pulse in Fig. 1C did. When trains of pulses were followed by a single pulse at varying intervals, the mean refractory period was found to be 6.7 ± 0.6 s ($n=6$) from the beginning of the train. Recovery of the slow response tended to be all or none [see also 7]. The convex, as opposed to exponential, appearance of the AHP current following a train of pulses in the presence of caffeine, most likely results from the I_{AHP} response due to mobilization of intracellular calcium being superimposed on I_{AHP} generated by calcium that entered the neuron during the train. It is of note, that at a time when calcium mobilization is refractory, the I_{AHP} following a single pulse still decays more slowly in the presence of caffeine than in its absence (Fig. 1B,D). This may arise because caffeine can cause a tonic increase in intracellular calcium levels [8] thereby mimicking the effect of increasing calcium levels through action potentials.

Fig. 2 shows superimposed records of I_{AHP} recorded using the hybrid clamp technique following trains, of increasing durations, of bursts of action potentials. Bursts of 2–4 action potentials were evoked by 50 ms current clamp pulses from -60 mV. Trains (10 Hz) of 1, 2, 4, 8, 16 and 24 such bursts preceded the initiation of a 10 second period of voltage clamp to -60 mV. Trains were separated by at least 30 s of recovery time. In Fig. 2A the longest I_{AHP} record followed the longest train. I_{AHP} duration increased as the number of trains used to evoke I_{AHP} becomes larger. The slow component that follows long trains is associated with potentiation by calcium of another current, I_{M} [see 8] as well as a slow tail of I_{AHP} . In the presence of 10 mM caffeine (Fig. 2B) exactly the same set of trains of action potentials all evoked I_{AHP} 's with similar prolonged duration regardless of train size. The slowly rising and slowly falling outward currents

seen towards the end of some of the traces in Fig. 2B were caused by AHP channels activated by spontaneous release of calcium from intracellular stores. The ability to trigger the release of calcium from these stores by depolarization was not consistently observed. In the experiment depicted in Fig. 2B the delayed activation of I_{AHP} , characteristic of the slow discharge of calcium from intracellular stores, was only observed following one of the trains. In that case, the slowly rising AHP current may have resulted from the coincidence of a spontaneous discharge of intracellular stores and the end of a train. In all cases tested ($n=6$) ryanodine, $5 \mu\text{M}$ (Fig. 2C) prevented the oscillations induced by caffeine and the prolongation of I_{AHP} but did not influence the relation between numbers of APs used to evoke an I_{AHP} and the duration of I_{AHP} . A lack of effect on I_{AHP} was also observed with $300 \mu\text{M}$ ryanodine, a concentration that will directly block the calcium release channel [10]. Note that the slow component of outward current following long trains of APs (Fig. 2A,C) was not observed in the presence of caffeine (Fig. 2B). The suppression of the slow component was associated with an inhibition of I_{M} suggesting that, in this cell, caffeine had caused a tonic elevation of calcium that was sufficiently high to suppress I_{M} and prevent the potentiation of I_{M} seen with smaller elevations of calcium (see ref. 8). The larger elevation of intracellular calcium might also explain why, in this cell, depolarization could not trigger the release of calcium stores (see Fig. 1). The effectiveness of APs in loading the cell with calcium apparently varied from cell to cell as there was no correlation between number of spikes per burst and the increment of I_{AHP} time constant with increasing time duration.

The role of Na/Ca exchange in determining the time course of I_{AHP} was examined by replacing Na⁺ with Li⁺. It is known that Li⁺ will not support Na/Ca exchange [9] but will be handled as well as Na⁺ by tetrodotoxin (TTX) and voltage-sensitive Na⁺ channels. As expected, replacement of Na⁺ by Li⁺ had no effect on the shape of action APs or on AP threshold. Although Na/Ca exchange was presumably blocked, there was only a small but significant prolongation of I_{AHP} . Moreover, this prolongation (30–40%) was only observed with I_{AHP} that followed trains of APs (Table 1). The duration of I_{AHP} that followed single AP was not prolonged by blocking Na/Ca exchange.

We have shown in the past that the duration of I_{AHP} can be prolonged by increasing the amount of calcium that enters the neuron during activation of I_{AHP} [1, 11, 12]. In this paper, the action of calcium is shown not to be due to modulation of the Na/Ca exchange process or the process whereby calcium is mobilized from caffeine and ryanodine sensitive intracellular stores. The effect of

increasing calcium loads on the decay rate of I_{AHP} was unchanged when Na/Ca exchange was blocked by replacing Na^+ with Li^+ or when calcium mobilization was blocked by ryanodine. It is nevertheless of interest to consider further to what extent calcium mobilization and Na/Ca exchange could influence the time course of I_{AHP} .

The action of ryanodine to shorten the duration of AHPs recorded in rat sympathetic ganglia [4], in contrast to the present results in bullfrog neurons, suggests that in those neurons calcium from intracellular stores can summate with action potential calcium in generating I_{AHP} s even in the absence of sensitizing drugs like caffeine. This additional calcium from intracellular stores could either be released tonically, leading to higher resting levels of calcium, or be mobilized during the action potential. Either mechanism could lead to the ryanodine sensitive prolongation of AHPs. However, the observation that a major portion of that prolongation in rat neurons is inhibited by evoking APs at a frequency of 1 Hz [5] suggests that most of the additional calcium is mobilized phasically from stores by APs. Presumably, at 1 Hz, there is insufficient time between APs for those stores to be replenished. Bullfrog neurons contain a ryanodine sensitive pool of intracellular calcium whose mobilization is sensitized by caffeine and possibly citrate [see 3]. The calcium release channel for this pool is modulated by a variety of allosteric influences, including membrane potential, adenine nucleotides and divalent cations [14]. Differences in the levels of these modulators between rat and frog neurons may account for the lack of ryanodine sensitivity of normal I_{AHP} in the frog. It is of note that in guinea pig vagal neurons that also exhibit fast and slow components of I_{AHP} the slow component also is sensitive to ryanodine [15].

With regards to the role of Na/Ca exchange in determining the rate of clearance of action potential calcium from the compartment adjacent the inner surface of the plasma membrane, our observations are similar to those of Korn and Horn [2]. Those authors examined the influence of Na/Ca exchange on the duration of calcium dependent chloride currents that follow calcium currents evoked by brief depolarizations in pituitary cells. The decay rate of the calcium dependent current increased with the calcium load. Blockade of Na/Ca exchange had no effect on the decay rate of the currents that followed small calcium loads but reduced the decay rate of currents following larger loads by 20–30%. As in our study, the relation between decay rate and calcium load was steepened by blocking Na/Ca exchange but, only slightly. It is likely that most of the immediate clearance of calcium from the vicinity of AHP channels which de-

termines the decay rate I_{AHP} is due to diffusion and uptake by buffers [11, 12, 16].

This study was financially supported by the Medical Research Council of Canada. P.S.P. is a Career Scientist of the Ontario Ministry of Health. J.W.G. is a Medical Research Council of Canada Scholar. M.V.S.-V. was supported by the Spanish Ministry of Science and Education (F.P.I. Program).

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