Some New Observations on Caffeine-Induced Rhythmic Hyperpolarization in Frog Sympathetic Ganglion Cells

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ABSTRACT — Unstimulated bullfrog sympathetic ganglia were studied in vitro by intracellular and extracellular recording methods. In 80% of the cells impaled with K citrate microelectrodes, caffeine caused initial hyperpolarization (ICH) followed by rhythmic membrane hyperpolarization (RMH). Four different patterns of rhythmicity were observed, the most common being a regular beating pattern. RMH frequency depended on both caffeine and Ca$^{2+}$. Tetraethylammonium reduced RMH amplitude, but did not affect frequency. Caffeine effects on cyclic AMP are not responsible for RMH since neither dibutyryl cyclic AMP nor phosphodiesterase inhibitors elicited RMH. However, the anion in the microelectrode filling solution is critical to both the incidence and amplitude of RMH, the order of effectiveness being: citrate >>> glutamate, acetate and chloride. In cells impaled by electrodes filled with K thiocyanate or K iodide, caffeine also caused large amplitude hyperpolarizing oscillations of membrane potential, suggesting that the effectiveness of citrate is not due to Ca$^{2+}$ chelation. High gain extracellular DC recording revealed no sign of caffeine ICH, RMH or any hyperpolarizing effects. The absence of signs of caffeine hyperpolarization with extracellular recording has several interpretations, and these are discussed.

The relationship between elevated intracellular Ca$^{2+}$ and Ca$^{2+}$-activated K conductance ($g_{KCa}$) has now been demonstrated in many cell types, including erythrocytes (1–3), macrophages (4), hepatocytes (5, 6), aplysia neurons (7), and mammalian sympathetic neurons (8). In frog sympathetic ganglion cells, Kuba and colleagues (9–15) found that caffeine caused rhythmic membrane hyperpolarization (RMH). They concluded that the RMH were oscillatory Ca$^{2+}$-activated increases in $g_K$ secondary to caffeine-induced increases in free intracellular Ca$^{2+}$.

We were interested in RMH as a potential model system for investigating drug action on Ca$^{2+}$-dependent neural functions and therefore attempted to reproduce and validate the caffeine-induced RMH in bullfrog sympathetic ganglion cells. In the present report, we confirm some of the findings of Kuba and Nishi (9) and Kuba (10), and we also present new observations relating to the phenomenon and to possible mechanisms of caffeine-induced RMH.

MATERIALS AND METHODS

The experiments were performed with isolated eighth or ninth paravertebral sympathetic ganglia of the bullfrog (Rana catesbeiana).
The frogs were housed in large plastic cages with continuously running tap water and wet and dry areas. Each shipment of frogs was used for experiments within two weeks from arrival.

After pithing brain and spinal cord, the eighth or ninth ganglia were excised together with the attached preganglionic trunk, rami, and corresponding spinal nerves. The excised tissues were pinned on a Sylgard® (Dow Chemical Co.) floor in a small Petri dish; and connective tissue, including the tightly adherent capsule, was removed under a dissecting microscope. The preganglionic trunk and post-ganglionic ramus was then transected and the isolated ganglion transferred to the intracellular recording chamber.

For intracellular recording, the ganglion was pinned in the flow compartment (80 µl volume) of a Sylgard® chamber. Superfusion of the ganglion was by gravity flow, and the compartment fluid level was maintained constant by suction outflow. The superfusion flow rate was 1–2 µl/sec. Individual ganglion cells were impaled with microelectrodes pulled from borosilicate capillary tubing with ultrathin walls (Frederick C. Haer, Brunswick, ME). The microelectrodes were filled with 0.15 to 1 M K citrate, except in specific instances (described in Results) where KCl, K acetate, K glutamate, K iodide or K thiocyanate were used. Initial tip resistances were generally 20–60 MΩ. The electrodes were connected to a Dagan preamplifier (Model 8500), the output of which was displayed on a storage oscilloscope (Tektronix Model 5115) and recorded on a chart recorder (Gould, Model 2200 or Hewlett Packard, Model 710/B).

High gain (100–200 µV) extracellular DC recordings were made from a platinum-iridium electrode around the ganglion, with the indifferent electrode on the corresponding spinal nerve just caudal to the entrance of the post-ganglionic ramus. In all cases, the tightly adherent connective tissue capsule around the ganglia had been teased off by microdissection. Recordings were made in a tightly sealed moist chamber (3.5 ml volume), containing a 400-µl well of Ringer’s solution, continuously stirred by a magnetic flea. The ganglion was located just beneath the meniscus surface of the Ringer’s solution in the well, and caffeine additions to the well were made from a calibrated micropipette (12 or 24 µl of 100 mM caffeine) to yield final caffeine concentrations of 3 and 6 mM.

Ringer’s solution contained: 112 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES and 1.8 mM NaOH. The pH was 7.2. Caffeine, as the anhydrous base, was prepared as a 100 mM stock solution in distilled water and refrigerated for no longer than one week. For the experiments, appropriate dilutions of the caffeine stock were made with Ringer’s solution. The sources of drugs and chemicals were: Caffeine, physostigmine sulfate, papaverine (base), dibutyryl cyclic AMP, forskolin and K glutamate, from Sigma Chemical Co., St. Louis, MO; triethylcitrate and tetraethylammonium chloride (TEA), from Aldrich Chemical Co., Milwaukee, WI; K iodide, from Fisher Scientific Co., Fair Lawn, NJ; K thiocyanate and K acetate, from Mallinckrodt Chemical Works, St. Louis, MO; and Na fluoride from Wako Chemical Co., Tokyo.

The data were derived from experiments conducted throughout all months of the year, at room temperatures that ranged from 22–28°C.

RESULTS

**Incidence and characteristics of RMH**

In 84% (211/255) of the ganglion cells impaled with K citrate microelectrodes, superfusion with 2 mM caffeine initiated abrupt membrane hyperpolarization (Fig. 1), the amplitude of which varied considerably within this population of cells (range 3 to 30 mV, see Table 1). This initial caffeine hyperpolarization (ICH) subsided slowly (50–60 sec); and several minutes later, repetitive, RMH began (Fig. 1). For most cells, RMH exhibited a regular periodicity and amplitude which was maintained throughout even long durations.
(90–100 min) of caffeine superfusion. There was, therefore, no evidence of tolerance to caffeine. Indeed, RMH could be stopped and re-initiated by switching between normal Ringer and 2 mM caffeine Ringer perfusates (Fig. 1). Table 1 shows the means, standard deviations, and ranges for ICH amplitudes as well as RMH amplitudes and frequencies observed in more than 150 cells during superfusion with 2 mM caffeine.

The regular beating pattern of RMH shown in Fig. 1 was the most common, but not the only pattern observed. Analysis of RMH produced by 2 mM caffeine in 371 cells revealed two principal types of RMH, a bursting pattern and a beating pattern. Each of these patterns could be further subdivided into those with quite regular periodicity and those with an irregular period. These four patterns of RMH are shown in Fig. 2, and their incidence in the population of 371 cells was: regular bursting (5%), irregular bursting (11%), irregular beating (11%), and regular beating (73%). Virtually all of the subsequent work reported in this paper was performed on cells exhibiting the regular beating pattern of RMH.

**Caffeine concentration-effect relationships**

A notable feature of RMH in all cells was its immediate cessation on changeover to su-

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Table 1. Characteristics of initial caffeine hyperpolarization (ICH) and rhythmic membrane hyperpolarization (RMH) elicited by 2 mM caffeine

| Description       | # of Cells | Amplitude (mV) | Frequency (pct/min) |
|-------------------|------------|----------------|--------------------|
| ICH               | 184        | 5.8 ± 5.3*     | —                  |
|                   |            | (Range 3 - 30) |                    |
| RMH               | 170        | 16.0 ± 9.3     | 0.6 ± 0.7          |
|                   |            | (Range 3 - 36) | (Range 0.1 - 10)   |

*Values are x ± S.D.

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Fig. 1. Intracellular recordings of caffeine-induced rhythmic membrane hyperpolarization (RMH) in bullfrog sympathetic neurons. A. Initial caffeine hyperpolarization (ICH) rapidly follows the start (arrow) of the 2-mM caffeine superfusion, and regular RMH develops shortly thereafter. B. A different cell, showing (left) rapid termination of RMH after switching to caffeine-free normal Ringer superfusion. In the same cell 13 min later (right), 2 mM caffeine superfusion again causes ICH and RMH, showing that tolerance to caffeine does not develop.
perfusion with caffeine-free Ringer (Fig. 1). Considering the perfusion flow rates and chamber volume (See Methods) in our system, it is certain that RMH ceased before the medium around the ganglion was caffeine-free. This conclusion is supported by concentration-effect studies for RMH, in which we found the threshold caffeine concentration to be > 0.25 mM and < 0.75 mM (16 cells). Results obtained with caffeine concentrations of 0.75, 2 and 6 mM showed that RMH frequency increased as a function of increasing caffeine concentration, whereas RMH amplitudes varied little or not at all. Figure 3 illustrates the increase in RMH cycle frequency in one cell superfused alternately with 0.75, 2 and 6 mM caffeine. The expanded timebase records in Fig. 3 reveal two other concentration-effect characteristics of RMH: (a) the rate of hyperpolarization was altered only slightly by changing caffeine concentration; (b) the rate of repolarization, i.e., recovery from hyperpolar-
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Fig. 3. Caffeine concentration dependence of rhythmic membrane hyperpolarization (RMH) frequency shown here in a single ganglion cell. Left, caffeine superfusion with, successively, 0.75, 2 and 6 mM progressively increases the frequency of RMH. At right, single cycles of RMH recorded at fast chart speed, showing that the rate of repolarization (recovery from peak hyperpolarization) is accelerated by increasing caffeine concentration. Arrows mark time at which repolarization is 63% complete.

Fig. 4. Log-log plot of RMH frequency and repolarization rate constants versus caffeine concentration.

Role of gK in RMH

Inward constant current pulses (50–100 msec) were used to measure membrane resistance between, and at the peak of, the hyperpolarizing events in RMH. Results in 7 cells during RMH produced by 2 mM caffeine showed a 44 ± 13% (S.D.) decrease in membrane resistance at the peak of hyperpolarization. Further evidence for a selective increase in gK was obtained by altering the extracellular K concentration. As expected, RMH amplitudes increased markedly in K-free Ringer’s solution. Also, the K channel blocker TEA reduced RMH amplitudes in 5 cells by 50 ± 14% (S.D.) at a concentration of 5 mM (Fig. 5). This blocking effect of TEA is particularly relevant to caffeine RMH in view of the finding (12) that gKCa in bullfrog sympathetic ganglion cells is highly sensitive to TEA.

Dependence of RMH on Ca2+

The influence of reduced extracellular Ca2+ on the RMH caused by 2 mM caffeine was tested in 15 different cells. When Ca2+ was decreased from the normal 1.8 mM to 0.9 mM (3 cells), the RMH amplitude was reduced on the average by 19%. Decreasing Ca2+ to 0.6
mM (3 cells) or 0.47 mM (9 cells) caused RMH frequencies and amplitudes to decline progressively, ceasing altogether within 3 to 20 min. During the superfusion with 2 mM caffeine in 0.47 mM Ca\(^{2+}\) Ringer, spontaneous, high-frequency soma spike discharge always appeared. The effects of reduced Ca\(^{2+}\) on the frequency and occurrence of RMH confirm similar findings reported previously (9, 10), which demonstrate the dependence of RMH on Ca\(^{2+}\). We have also investigated equimolar replacement of 1.8 mM Ca\(^{2+}\) with other divalent cations, and we found that caffeine RMH cannot be generated in the presence of Ba\(^{2+}\) or Mg\(^{2+}\). Replacement of Ca\(^{2+}\) by Sr\(^{2+}\), however, uniformly resulted in a bursting pattern of RMH (16).

Effects of phosphodiesterase inhibition and adenylyl cyclase activation

Methylxanthines, including caffeine, are known to inhibit the enzyme phosphodiesterase, thereby causing accumulation of cyclic AMP (17). Kuba and Nishi (9) and Kuba (10) thus considered the possibility that caffeine RMH arose from the accumulation of cyclic AMP and resultant elevation of intracellular Ca\(^{2+}\). They showed, however, that dibutyryl cyclic AMP alone did not elicit RMH. We have also found that dibutyryl cyclic AMP (1–2 mM) was unable to cause RMH. In addi-

Fig. 4. Log-log plot of rhythmic membrane hyperpolarization (RMH) frequency (○) and recovery rate constant (●) (reciprocal of time to 63% recovery, as shown in Fig. 3) versus caffeine concentration. The points at 0.75, 2 and 6 mM caffeine are, respectively, the x ± S.E.M for the following numbers of cells: RMH: 13, 15, 9; recovery rate constant: 11, 12, 6. The lines drawn through the points in each plot represent the calculated regressions.

Fig. 5. Reduction of rhythmic membrane hyperpolarization amplitude by 5 mM tetraethylammonium (TEA). Intracellular recording from a ganglion cell during continuous superfusion with 2 mM caffeine. The TEA superfusion with 5 mM TEA was started and then discontinued at the arrows (↓, ↑).

tion, we examined the effects of two phosphodiesterase inhibitors, papaverine and physostigmine (18), which are chemically distinct from methylxanthines. Neither papaverine (0.2–0.5 mM, 2 cells) nor physostigmine (0.03–0.2 mM, 15 cells) evoked RMH. In the presence of these drugs, generation of RMH by caffeine was not prevented, nor were the qualitative or quantitative characteristics of RMH modified (Fig. 6). Further testing of the cyclic AMP role was made with the adenylate cyclase activators forskolin (19) and NaF (20). We found that forskolin (1–10 μM, 5 cells) and NaF (5–10 μM, 20 cells) were incapable of inducing RMH or modulating caffeine RMH.

The role of microelectrode (or intracellular) anions

In agreement with the report of Morita et al. (11), we have found that caffeine RMH can be produced with the highest regularity (84% of cells) when K citrate filled microelectrodes are used. Small amplitude RMH (3–6 mV) occurred in only 6 of 33 cells (18%) impaled with KCl electrodes and in 3 of 13 (23%) impaled with K acetate electrodes.

The importance of citrate-filled electrodes in the occurrence of caffeine-induced RMH could have several explanations, assuming some intracellular leakage of citrate from the electrode tip. A possible Ca²⁺-chelating action of citrate was considered unlikely, but not proven so by Morita and colleagues (11). To eliminate any chelation factor and simultaneously test the influence of anion polarizability, we investigated electrodes filled with 3 M K iodide or 3 M K thiocyanate. In each of 10 cells impaled with K iodide-filled electrodes, caffeine produced the typical ICH seen with citrate-filled electrodes and a subsequent rhythmic oscillation of membrane potential (Fig. 7). Results with K thiocyanate filled electrodes were similar (4 cells). Although the oscillatory cycles recorded with K iodide-filled electrodes were quasiharmonic compared to the “sawtooth” RMH obtained with citrate-filled electrodes, the cycle intervals and the amplitudes were not remarkably different.

Furthermore, we made a contemporaneous

![Fig. 6. Physostigmine or papaverine do not initiate rhythmic membrane hyperpolarization (RMH), nor do they modify the subsequent appearance of caffeine-induced RMH. Each record is from a different cell in which either physostigmine or papaverine was superfused alone, then discontinued and 2-mM caffeine superfusion begun. The physostigmine concentration is that of the base. Note that caffeine still elicits initial caffeine hyperpolarization and RMH of typical amplitude and frequency after papaverine or physostigmine.](image-url)
comparison of electrodes filled with either 0.15 M K citrate or 0.45 M K glutamate. In 10 cells impaled with citrate-filled electrodes, 3 mM caffeine elicited ICH in all of them and elicited RMH with a mean amplitude of 23 mV in 7 (70%). With the use of glutamate-filled electrodes, however, 3 mM caffeine elicited ICH in 4 of 9 cells (44%) and a 5 mV RMH in only 1 of the 9 cells (11%).

**Extracellular recording**

High gain extracellular DC recordings (see Methods) were made in 20 isolated, unstimulated ganglia before, during and after exposure to 3–6 mM caffeine. In no case did we observe any evidence of either ICH or RMH. In these experiments, we also made numerous variations in the normal extracellular Ringer’s solution in attempts to favor the occurrence of caffeine-induced ICH or RMH. For example, NaI was substituted for NaCl, K-free solutions were tested, and triethyl citrate (1–10 mM) was also added on the premise of achieving some elevation of intracellular citrate that might mimic the conditions present during intracellular recording with K citrate-filled microelectrodes. Despite all these maneuvers, we consistently failed to observe any evidence of ICH, RMH or of any hyperpolarizing response.

We confirmed the suitability of the extracellular DC recording method by immersion of the ganglion in 3 mM acetylcholine. Peak depolarizations reached amplitudes of 600 µV or greater and subsided exponentially over several minutes. The amplitude of the peak acetylcholine depolarization recorded extracellularly is approximately twenty five percent of the amplitude of the extracellularly recorded compound action potential. This agrees well with intracellular recording data (21) showing that peak acetylcholine depolarization ($\bar{x} = 12.1$ mV) is also approximately twenty five percent of the normal resting potential amplitudes (45–50 mV). Consequently, the extracellular recording method can easily detect currents associated with modest DC potential changes in the ganglion.
DISCUSSION

Many aspects of intracellularly recorded caffeine-induced RMH in bullfrog sympathetic ganglion cells have been characterized in the thorough studies by Kuba and his colleagues (9–11, 22). These reports concluded that RMH reflect cyclic increases in gK, presumably activated by cyclic elevations of intracellular Ca$^{2+}$. The present study has confirmed several of the major features of caffeine RMH as described in the above-cited work of Kuba and his colleagues, and adds new observations that may pertain to the still obscure mechanism by which caffeine causes RMH.

We found, for example, as did Kuba and Nishi (9), that dibutyryl cyclic AMP is unable to generate RMH. Furthermore, we have shown (Fig. 6) that two non-xanthine phosphodiesterase inhibitors, papaverine (23) and physostigmine (18), are also incapable of generating RMH. Since the adenylate cyclase activators forskolin and NaF were also ineffective in causing RMH, it is unlikely that cyclic AMP has any significant mechanistic role in caffeine RMH.

We have also confirmed (9, 11) the dependence of caffeine RMH on Ca$^{2+}$ and caffeine. Recently, Akaike and Sadoshima (24) have reported that caffeine-induced currents recorded by patch clamp in the cell-attached bullfrog sympathetic neuron are also dependent upon Ca$^{2+}$ and caffeine. This dual dependency has led to the proposition (22, 24) that caffeine-induced Ca$^{2+}$ influx leads to a release of intracellular Ca$^{2+}$ from subsurface junctional organ storage sites thought to be the equivalent of the endoplasmic reticulum. With regard to this proposition it is of interest that we were unable to induce RMH with forskolin. In rabbit aorta at least, forskolin has been shown to be equivalent to caffeine in inducing Ca$^{2+}$ release from intracellular stores (25).

Our new observation that caffeine-induced RMH occurs in four general pattern groups (Fig. 2) could be a source of clues pertinent to RMH mechanisms. The regular and irregular bursting and beating RMH patterns shown in Fig. 2 bear a close resemblance to the glucose-dependent oscillatory Ca$^{2+}$ spike patterns observed in β cells of pancreatic islets (26). By computer simulation of the β cell oscillatory Ca$^{2+}$ spikes, Chay and Rinzel (27) reproduced the experimentally observed spiking patterns solely by varying the rate of intracellular Ca$^{2+}$ uptake (kCa) within the narrow range from 0.038 to 0.045 msec$^{-1}$. With this small variation in kCa$^{2+}$, they obtained four different oscillatory spike patterns: periodic bursting, chaotic (irregular) bursting, chaotic beating, and periodic beating, which were essentially similar to the patterns of caffeine RMH we have observed. Since oscillations of Ca$^{2+}$ are the key element common to β cell spikes and to activation of gK in RMH, it is of interest that we were unable to induce RMH with forskolin. In rabbit aorta at least, forskolin has been shown to be equivalent to caffeine in inducing Ca$^{2+}$ efflux.

Although RMH depends on both caffeine and Ca$^{2+}$, our results demonstrate that both the occurrence and the amplitudes of RMH involve a third critical determinant, the anion accompanying K$^+$ in the intracellular microelectrode. We have found that citrate (in contrast to chloride, acetate or glutamate) in the electrode filling solution yields a much higher incidence of both ICH and RMH as well as larger amplitudes of hyperpolarization. Thus, we confirm Kuba’s report (11) that the order of favorable anions in electrode filling solutions was citrate > acetate and/or sulfate > chloride, with the result that amplitudes and frequencies of caffeine RMH were greater when cells were impaled by K citrate electrodes.

Leakage of citrate anions from the microelectrode tip must be a critical factor; otherwise, the incidence and characteristics of caf-
feine RMH would be expected to be identical for all K-containing electrode filling solutions. It is especially interesting, therefore, to point out that Akaike and Sadoshima (24) used K⁺ aspartate in the internal solution for their recordings of caffeine-induced currents using whole-cell patch clamp techniques in the bullfrog sympathetic neuron. We are apparently the first to report (Fig. 7) that intracellular electrodes filled with K iodide or thiocyanate enable caffeine to generate oscillatory large-amplitude RMH. This finding makes it unlikely that either citrate or aspartate function simply as Ca²⁺ chelators.

Anions such as citrate could also conceivably affect gK directly. Weis-Fogh (28) demonstrated that citrate, at pH values between 7.1 and 7.9 greatly increases the rate of K⁺ loss from human platelets, by increasing membrane permeability to K⁺. At the higher pH (7.9), citrate additionally inhibited K⁺ pump activity. Thus, the large amplitude caffeine RMH obtained in ganglion cells impaled with K citrate electrodes might reflect increased K⁺ permeability attributable to citrate leakage from the electrode tip. The mechanism of the anion contribution to the phenomenon of intracellularly recorded caffeine RMH remains an unsolved problem.

An important new finding in the present study is the fact that we did not detect any sign of caffeine ICH or RMH using high gain extracellular recording methods. Since these extracellular recording experiments are unable to duplicate the conditions existing with a citrate-filled microelectrode, the negative results are probably best compared to the intracellular data with K chloride-filled microelectrodes. In the latter case, only 18% of the cells had RMH, and the amplitude was low (3–6 mV). If the currents generated among such a small percentage of cells were largely out of phase, there could be insufficient summation of the currents to be detected extracellularly, even with high amplification.

Consequently, the absence of ICH or RMH in extracellular recordings neither proves nor disproves that these phenomena only occur with intracellular microelectrode impalement. Recently, Kuba and colleagues (29, 30), using the Ca²⁺-indicator dye Fura-2, observed that caffeine causes oscillations of intracellular Ca²⁺ in intact, cultured bullfrog ganglion cells. While these elegant results are highly suggestive, they do not demonstrate that the Ca²⁺ oscillations activate gK. In other words, they do not demonstrate RMH in the intact, uninjured cell. Therefore, the question of whether caffeine RMH occurs in intact, uninjured cells still awaits a final answer.

It is pertinent to recall that in intact frog muscle cells, Bianchi (31) showed that caffeine dose-dependently caused virtually equal increases in both influx and efflux of Ca²⁺. Hence in intact cells with a normal intracellular environment, caffeine might cause little or no net variation in intracellular Ca²⁺, and therefore little or no variation in gK. However, the interaction of caffeine and an unusual (unusual in nature and/or concentration) intracellular anion results in a high incidence of large cyclic oscillations in gK, and therefore presumably in intracellular Ca²⁺ as well. In view of Bianchi’s original study (31), it may be that the contribution of intracellularly leaked citrate to caffeine RMH is to alter the magnitude or kinetics of the normally balanced Ca²⁺ influx-efflux process.

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