

4-AMINOPYRIDINE-INDUCED CONTRACTURE IN FROG VENTRICLE IS DUE TO CALCIUM RELEASED FROM INTRACELLULAR STORES

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Abstract : The aim of the study is to demonstrate the presence of intracellular calcium store in frog ventricle based on contractures induced by 4-aminopyridine in calcium-free media. Frog-ventricular strips were subjected to field stimulation at 0.2 Hz and the force of contraction was recorded after stabilization. The preparation was then kept quiescent for some time in solutions with different sodium concentrations, containing 0 or 1 mmol/L calcium. Caffeine, 4-aminopyridine (4-AP), or tetraethylammonium chloride was then added. Frog skeletal muscle preparations were used as positive controls for the caffeine experiments. Frog ventricular preparations did not develop contractures (sustained contractions) in the presence of caffeine (25 mmol/L), while frog skeletal muscle preparations developed caffeine-induced contractures. However, 4-AP (16 mmol/L) was able to induce contractures in quiescent frog ventricular preparations, even when they were superfused with calcium-free solution. 4-AP contractures in frog ventricle were seen in the presence of nifedipine also. Amplitude of 4-AP evoked contractures in frog ventricle were much larger in low sodium (30 mmol/L) and sodium-free (sodium substituted by lithium) solutions than in normal sodium solution, suggesting that the route of extrusion of the cytosolic calcium (released from intracellular stores by 4-AP) is the sodium calcium exchanger, which gets reversed in low sodium solutions. Tetraethylammonium chloride (TEA) was not able to induce contractures in frog ventricle suggesting that the contracture evoked by 4-AP is not due to its potassium channel blocking effect. In quiescent frog skeletal muscle preparations, caffeine as well as 4-AP induced contractures in calcium-free solutions. We therefore conclude that there is a caffeine-insensitive, 4-AP sensitive intracellular calcium store in the frog ventricle.

Key words : aminopyridine calcium store caffeine contracture
frog ventricle TEA

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INTRODUCTION

The aim of this study is to demonstrate that there are functional intracellular calcium stores in the frog-ventricle based on contractures induced by 4-aminopyridine in calcium-free media. The excitation-contraction (E-C) coupling in mammalian myocardium is brought about by a process called calcium-induced calcium release. Depolarization of cardiomyocyte T-tubule causes influx of Ca^{2+} through the L-type Ca^{2+} channels. This Ca^{2+} influx causes release of Ca^{2+} from the sarcoplasmic reticulum (SR) through the ryanodine receptors (RyRs) (1). Frog ventricular myocardium on the other hand is thought to depend exclusively on extra-cellular Ca^{2+} for E-C coupling (2). Radioactive ryanodine binding assays, immunohistochemistry and electron microscopy have shown a near total absence of RyRs in the frog ventricle, but electron microscopy of the frog ventricular myocardium has shown a network of relatively scarce, but well organized SR (3).

Caffeine (5–30 mmol/L) produces contractures in quiescent mammalian and frog skeletal muscle preparations and in the mammalian myocardium bathed in normal extracellular solution. The contracture is due to emptying of the SR Ca^{2+} into the cytosol (4) and the amplitude of contracture is in fact used as a measure of SR Ca^{2+} content (5). Chapman and colleagues (4, 6) reported caffeine-contractures in frog atrial preparations in calcium-free medium. However caffeine-contractures do not develop in frog ventricular preparations in calcium-free solutions, and this seems to suggest the absence of functional intracellular Ca^{2+} stores in frog ventricle.

Nevertheless, there is some evidence supporting the presence of functional intracellular Ca^{2+} stores in the frog ventricle. Anderson et al. (7) have shown that the first post-pause beat in the frog ventricle is diminished in amplitude as compared to control, but treatment of strips with ouabain enhanced the amplitude of the first post-pause beat suggesting that in the absence of ouabain, significant extrusion of cellular Ca^{2+} occurred during the pause. According to them, because resting tension did not increase during the pause in ouabain-treated strips, the non-extruded Ca^{2+} must have been sequestered into a compartment such as SR. Similar reports from our laboratory demonstrating positive force-frequency relationship and rest-induced decay in contraction amplitude using frog ventricular strips also favour the presence of an intracellular Ca^{2+} store in frog ventricle (8, 9).

The study presented here provides evidence in support of functional intracellular Ca^{2+} stores in the frog ventricle. It is shown that this store is sensitive to 4-AP (a K^+ channel blocker known to mobilize Ca^{2+} from intracellular Ca^{2+} stores (10, 11)) but not caffeine. It is also shown that the major route of extrusion of Ca^{2+} that enters the cytosol from intracellular stores due to action of 4-AP, is the sodium-calcium exchanger on the plasma membrane.

MATERIAL AND METHODS

Frogs of both sexes, weighing between 70 and 110 g, belonging to the species *Rana hexadactyla* were used. The frogs were anaesthetized with ether prior to pithing. The procedures were approved by the

institutional animal ethics committee.

Frog ventricular strip: Hearts were isolated from pithed frogs and placed in cold, well-oxygenated, normal sodium physiological solution. Circular rings were made from the ventricle after excising the atrial tissue and the tip of the ventricle. The ring was cut open and the resulting strip which was about 1 to 1.5 cm in length and 2 mm or less in thickness, was mounted in a temperature-controlled bath (25–28°C) and superfused with the same solution as above. The solution was continuously oxygenated. The free end of the strip was connected to a force-transducer and the force of contraction was recorded on a chart-recorder (for caffeine experiments) or acquired into a computer (4-AP and TEA experiments). The strip was paced with field stimulation by silver electrodes at 0.2 Hz with square pulses of 5 Volt amplitude and 4.5 millisecond duration.

Frog skeletal muscle preparations, to serve as positive controls for caffeine contractures, were made from the sartorius muscle.

Sources of drugs and chemicals: 4-AP and TEA were purchased from SIGMA and caffeine was purchased from Loba Chemie. EGTA was bought from SISCO research laboratories; Nifedipine was a gift from Torrent pharmaceuticals.

Experimental protocol for caffeine experiments :

Frog ventricular strips were paced at 0.2 Hz in either normal or low sodium solutions (40 mmol/L sodium in this set of experiments) with 1 mmol/L Ca^{2+} . After

stabilization, the stimulus was switched off and after about 3 minutes of quiescence, caffeine was added to the superfusate at a final concentration of 25 mmol/L. Where frog skeletal muscle preparations (sartorius) were used as positive control, the preparation was stabilized with field stimulation in calcium-free normal sodium solution and 25 mmol/L caffeine was added after 2 to 3 minutes of quiescence.

Experimental protocol for 4-AP and TEA :

After mounting the ventricular strip, it was paced at 0.2 Hz for a minimum of 30 minutes for stabilization in the normal sodium solution with 1 mmol/L Ca^{2+} . Once the force stabilized, stimulation was switched off and the solution was changed to calcium-free solution with 0.2 mmol/L EGTA and any one of the three sodium concentrations (normal, low or zero sodium). After 10 minutes, 16 mmol/L 4-AP or TEA was added to the bathing solution and the effect recorded. Effect of 4-AP on frog sartorius muscle preparations was also tested using the same protocol.

Superfusion solutions of the following composition (in mmol/L) were used in the study :

Normal sodium solution – NaCl 117, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, NaH_2PO_4 0.2, Na_2HPO_4 0.8, glucose 10, pH adjusted to 7.4 with 1 molar NaOH.

Low sodium solution – NaCl 30, sucrose 174, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, NaH_2PO_4 0.2, Na_2HPO_4 0.8, glucose 10, pH adjusted to 7.4 with 1 molar NaOH. In the caffeine experiments, this solution contained 40 mmol/L sodium.

Sodium-free solution – LiCl 117, KCl 3, CaCl₂ 1, MgCl₂ 1, HEPES 10, glucose 10, pH adjusted to 7.4 with 1 molar LiOH. (sodium chloride replaced with lithium chloride).

EGTA: The concentration of EGTA used in the calcium-free solutions was 0.2 mmol/L. Required volume of EGTA stock solution (200 mmol/L) was added to the solution to get the final concentration. The stock solution of EGTA was prepared by adding the required amount to distilled water and titrating with 1 molar NaOH till all the EGTA dissolved. To make up 10 ml of the solution, we used 8 ml of distilled water and about 2 ml of 1 molar NaOH.

Nifedipine stock: 1 millimolar stock was made in ethanol and was diluted in the final solution to get a concentration of 10 µmol/L. Since nifedipine is photosensitive, the nifedipine experiments were done in a dark environment.

Statistical analysis: The 4-AP-evoked contracture amplitude was normalized as a percentage of the stimulus-induced contraction amplitude (recorded prior to 4-AP application) for comparison between the three groups with the different sodium concentrations. Results are expressed as median (interquartile range). Contractures induced by 4-AP in normal, low sodium and sodium-free solutions were compared using Kruskal-Wallis test as the data was not normally distributed. Multiple comparisons between the three groups were done using Bonferroni adjustment. The overall alpha (0.05) was divided by three and a P value of <0.0166 was considered significant.

RESULTS

After a period of quiescence in the normal sodium solution, when the frog ventricular preparation was paced at 0.2 Hz, the initial amplitude of contraction was very low and it increased gradually to a steady-state (positive staircase, Fig. 1A). This positive staircase phenomenon was seen in all the experiments.

Effects of caffeine

In quiescent frog skeletal muscle preparations (n=4) bathed in calcium-free solution, addition of 25 mmol/L caffeine, caused a contracture where the tension began to rise within 10 seconds of application of caffeine (Fig. 1B). Caffeine contractures did not develop in quiescent frog ventricular preparations in either normal sodium (n=3, Fig. 1C) or in low sodium solution (40 mmol/L sodium) (n=5, Fig. 1D), even when there was 1 mmol/L Ca²⁺ in the superfusate.

Effects of 4-Aminopyridine:

In quiescent frog ventricular strips bathed in a calcium-free normal sodium solution (with 0.2 mmol/L EGTA) for 10 minutes, addition of 4-AP (16 mmol/L) produced contractures (Fig. 2 A, n=6). The effect was almost instantaneous and was reversible. Superfusion with normal sodium solution (with 1 mmol/L Ca²⁺) after the contracture (to wash 4-AP) helped in quick restitution of tension to baseline. When the preparation was stimulated subsequently, contractions reappeared. 4-AP-evoked contractures were much larger when the frog ventricular strips were bathed in a calcium-

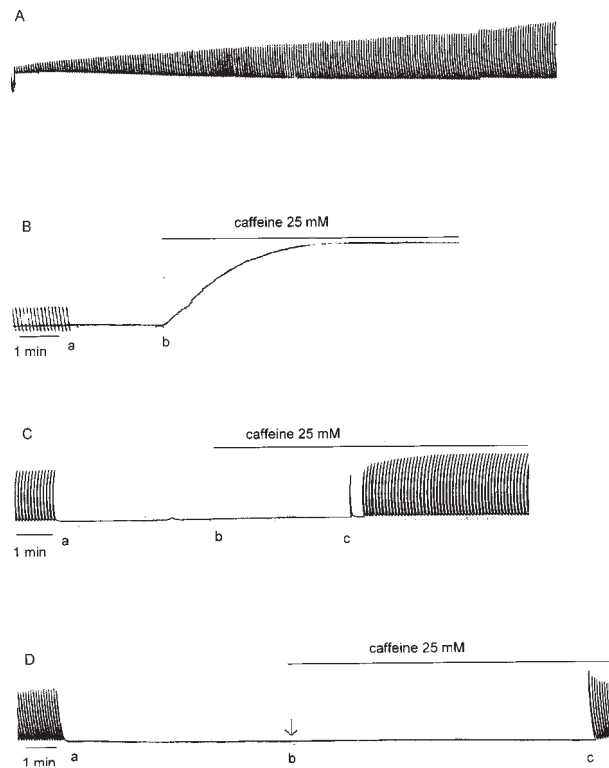


Fig. 1 : (A) Gradual increase in contractile amplitude in a frog ventricular strip, which has been through a period of quiescence (while dissecting and mounting on the experimental set up), when subjected to field stimulation at 0.2 Hz; (B) Caffeine contracture in a frog sartorius muscle preparation bathed in calcium-free solution; (C) Caffeine had no effect in a quiescent frog ventricle preparation when the perfusate had normal sodium and calcium concentrations. (D) Caffeine had no effect on frog ventricle even when the perfusate had low sodium (40 mmol/L). In B, C and D, at a, pacing was stopped, and at b, 25 mmol/L caffeine was added. Stimulation was re-started at c to demonstrate that the tissue was able to contract even though caffeine did not induce contractures.

free solution with low sodium (30 mmol/L) (Fig. 2B, $n=6$) or zero sodium (sodium chloride replaced with 117 mmol/L lithium chloride) (Fig. 2C, $n=6$). Even though the contractures seen with calcium-free low sodium solutions were larger in comparison to those seen with calcium-free normal

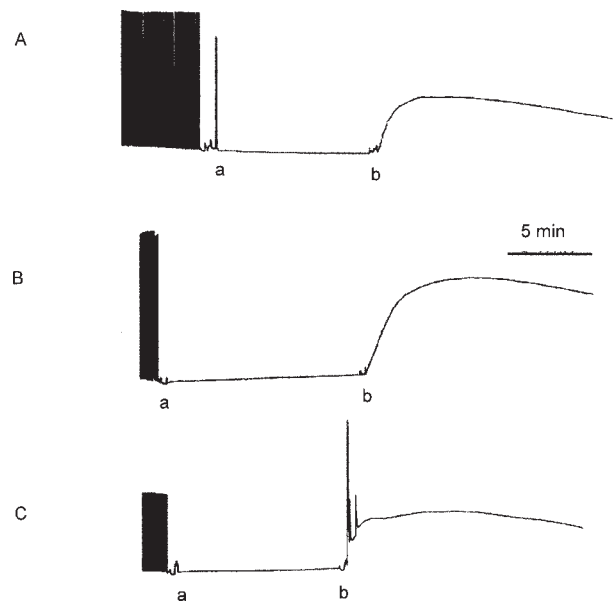


Fig. 2 : 4-Aminopyridine contractures in frog ventricular strips bathed in test solutions containing zero calcium with (A) normal sodium ($n=6$), (B) low sodium (30 mmol/L, $n=6$) and (C) zero sodium (sodium substituted with lithium, $n=6$). At a, stimulation was stopped and solution changed from normal solution to test solution with zero calcium and 0.2 mmol/L EGTA and kept quiescent for about 10 minutes and at b, 16 mmol/L 4-AP was added to the perfusate.

sodium solutions, the difference was not statistically significant. Amplitude of 4-AP induced contractures in sodium-free solutions was significantly higher than that seen with normal sodium (Fig. 3, $n=6$, $P=0.006$, Kruskal-Wallis test with Bonferroni adjustment).

4-AP was able to induce contractures in calcium-free solutions even in the presence of L-type Ca^{2+} channel blockers like nifedipine (Fig. 4A, $n=4$). Since nifedipine did not have an effect on 4-AP-induced contractures, the effect of ethanol was not tested. TEA (16 mmol/L), another K^+ channel blocker, was not able to induce contracture-

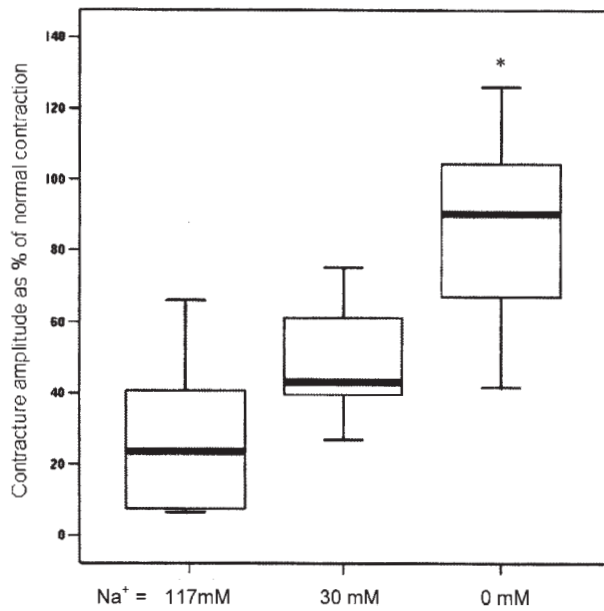


Fig. 3: Box plot showing the comparison of 4-aminopyridine contracture amplitude between the three groups. 4-AP contracture is larger in sodium-free solution (sodium substituted with lithium) than in normal sodium and low sodium solutions.

*sodium-free solution vs normal sodium solution, $P < 0.01$ with Kruskal-Wallis test followed by Bonferroni adjustment, ($n = 6$ in each group).

in the frog ventricular strip (Fig. 4B, $n=4$) under similar experimental conditions. Subsequent addition of 4-AP however produced a contracture in the same preparations.

4-AP induced contractures in frog skeletal muscle as well (Fig. 4C, $n=4$).

DISCUSSION

When the ventricular preparation is stimulated after a period of quiescence, the force picks up gradually (positive staircase, Fig. 1A). Given that the possible sources of contractile calcium are the extracellular fluid

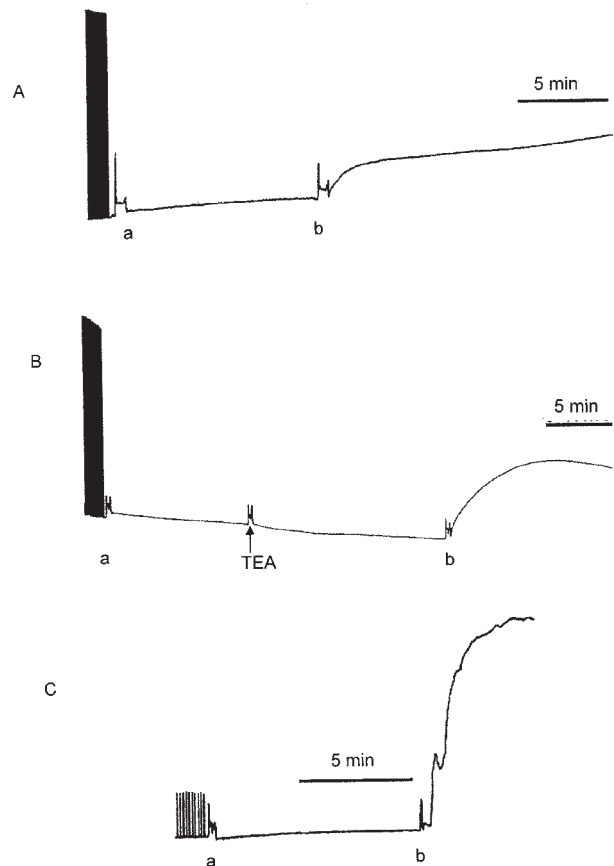


Fig. 4: 4-aminopyridine induces contracture *in frog ventricle* even in the presence of nifedipine; (B) TEA did not produce contractures in *frog-ventricle* while 4-AP produced contractures in the same tissue after TEA; (C) 4-AP induced contractures in *frog sartorius muscle* preparation. In all three tracings A, B and C, at *a*, stimulation was stopped and solution changed from normal solution to normal sodium solution with zero calcium, 0.2 mmol/L EGTA (and 10 $\mu\text{mol/L}$ nifedipine in the case of A alone) and kept quiescent for sometime and at *b*, 16 mmol/L 4-AP was added to the perfusate.

and the intracellular store, calcium from one of these two sources must increase gradually with repeated stimuli to account for the gradual build up of force. If the prevailing view that, in the frog myocardium, during an action potential, most if not all of the Ca^{2+} for contraction enters the cell from the

extracellular fluid via the; L-type Ca^{2+} channels (2, 3, 12, 13) is essentially true, then the positive stair case that is shown in Fig. 1A must mean that L type Ca^{2+} channels are facilitated with repeated stimuli. On the other hand, L-type calcium channels are known to inactivate with repeated stimuli or even during the course of a voltage pulse due to voltage and calcium-dependent mechanisms (14, 15). Evidence for facilitation of L-type calcium current with repeated stimulation does exist, but such facilitation is shown to be short-lived, for a few seconds only (17, 18). In our experiments, the duration for which the positive stair-case was built-up and maintained being very long, (up to 30 minutes in most cases) it is unlikely that this could be due to facilitation of L-type calcium channels. The positive stair-case that we observed in all preparations paced at 0.2 Hz for more than 30 minutes under control conditions, suggests the existence of a source of calcium (other than L-type calcium channels) which increases with repeated stimuli. The most obvious inference is an intracellular store which gets gradually repleted with calcium which enters the cell with every pulse. Such a phenomenon of store repletion during contractions is stated as the cause for the positive staircase in the mammalian cardiac muscle which begins to contract after a period of quiescence (19). The same phenomenon should account for the positive staircase in the frog ventricle also. The issue however with the frog ventricle is the fact that contractures do not develop with conventional agents like caffeine which release calcium from the store; it is important therefore to find an agent that releases calcium from the putative store to demonstrate the presence of such a store.

In our experiments we found that 4-AP, a known potassium channel blocker, is able to evoke contractures in the frog ventricle even when bathed in calcium-free medium. 4-AP induced contractures were seen in frog ventricular strips bathed in calcium-free solutions even in the presence of an L-type Ca^{2+} channel blocker, nifedipine. Therefore, external Ca^{2+} influx plays no role in 4-AP induced contractures. The calcium for the 4-AP evoked contractures must come from an intracellular store. Inability of another potassium channel blocker, TEA, to evoke contractures in the frog ventricle indicates that the Ca^{2+} mobilizing property of 4-AP is independent of K^+ channel blockade.

Effects of 4-AP on Ca^{2+} homeostasis have been reported earlier (11). 4-AP-induced contracture in frog ventricular strips bathed in calcium-free solutions has also been reported earlier (20). As for mechanism of action, it has been suggested that 4-AP releases Ca^{2+} from intracellular Ca^{2+} stores through elevation of inositol triphosphate (IP_3). Inhibition of sarcoendoplasmic reticulum Ca^{2+} ATPase activity by 4-AP has also been suggested in skeletal muscle (10).

Hence the cause of 4-AP contracture in the frog ventricle could be either IP_3 elevation or inhibition of the SR calcium ATPase (analogous to contractures induced in skeletal muscle with thapsigargin or cyclopiazonic acid). Inhibition of SR calcium ATPase is a possibility since electron microscopy of the frog ventricle does suggest the presence of Ca^{2+} ATPase in free SR (3).

The absence of caffeine-contractures in frog ventricle in our studies, argues for

caffeine-insensitivity of the Ca^{2+} release mechanism from the intracellular store in this tissue, rather than absence of the store per se. Caffeine-contractions were elicited in the frog skeletal muscle as positive control. Since it has been reported that in frog atria, caffeine-contractions though not seen in normal sodium media, develop when external sodium is reduced (6), we applied caffeine in low sodium and sodium-free media to the frog-ventricle; even then contractions were not seen.

Calcium released in to the cytosol during a 4-AP contraction must be getting extruded through the sodium-calcium exchanger on the plasma membrane, because the 4-AP contractions were much larger when the preparation was bathed in low sodium or sodium-free solutions, essentially conditions inhibiting the Ca^{2+} extrusive mode of the sodium calcium exchanger (9).

In conclusion our results suggest a functional intracellular store for Ca^{2+} in frog ventricle. This store, which is sensitive to 4-aminopyridine, is however, pharmacologically different from the conventional SR because the conventional modulator of SR calcium store, caffeine, does not have any effect on the frog-ventricle.

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Conflict of interest: The authors have no conflict of interest.

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