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Original Research

The role of PLC-IP_3 cascade on 4-aminopyridine (4-AP) contracture in electrically-driven rat atrial and diaphragmatic strips: new evidence by neomycin and heparin

Ozlem Kucukhuseyin[1*], Sumbul Khalid[2], Uteuliyev Yerzhan Sabitaliyevich[3], Cihat Kucukhuseyin[4]

[1] Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, 34093 Capa, Istanbul, Turkey
[2] Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad, Pakistan
[3] Kazakhstan Medical University KSPH, Almaty, Kazakhstan
[4] Department of Medical Pharmacology, Istanbul University Cerrahpasa Medical Faculty, 34098 Cerrahpasa, Istanbul, Turkey

Correspondence to: ozlemkh@istanbul.edu.tr, ozlem.kh@gmail.com

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Abstract: Induction of cardiac contractures by 4-AP in Ca^{2+}-free medium implied the involvement of SR and PLC-IP_3 cascade. Thus, the role of PLC-IP_3 cascade against contractile actions of 4-AP in electrically-driven rat atrial and diaphragmatic strips were studied both in the presence, and absence of Ca^{2+} using neomycin, a PLC inhibitor, and heparin, an IP_3-R antagonist. 4-AP was applied cumulatively in logarithmically increasing concentrations in the range of 1-16µg/ml, and the preparations were treated with neomycin (400µM) or heparin (400µg/ml) for 3min prior to 4-AP injection. Post-rest potentiation in atrial strips was obtained by interruption of stimulation for 30min. 4-AP caused biphasic alteration in twitch amplitudes, as initially increased up to 16mM and then depressed due to contracture development, which were not affected significantly by neomycin and heparin. Both atrial and denervated diaphragmatic strips challenged to 4-AP in the presence and absence of Ca^{2+} developed dose dependent contractures which were significantly antagonized both by neomycin and heparin ($p<0.05$). Post-rest first contractions in controls were found to be reduced by 2min exposure to 4mM 4-AP and augmented by 3min exposure to heparin alone. 4-AP responses in the presence of neomycin and heparin were significantly higher than with those only treated with 4-AP alone and lesser than controls. Because of the fact that 4-AP inducing contracture in Ca^{2+}-free medium, Ca^{2+} causing contracture should be of SR in origin. Depending on these results, it was concluded that activation of PLC-IP_3 cascade by 4-AP is involved in the mediation of contracture and contractile actions of this molecule.

Key words: 4-AP; Contracture; PLC; IP_3; SR; Neomycin; Heparin.

Introduction

Rapidly increasing breakthroughs in cellular physiology have helped us to improve our understanding related to circuitry for calcium signaling. Increasingly it is being realized that 4-aminopyridine (4-AP) interacts widely with calcium (Ca^{2+}) in lots of excitable tissues. Such interactions with Ca^{2+} might be mediated by inhibition of K^+ channels (1,2), promotion of Ca^{2+}-influx through Ca^{2+} channels into the effector cells (3,4) or by interaction with intracellular Ca^{2+} depots (5-7). It has been well documented that the biggest part of Ca^{2+} responsible excitation-contraction coupling takes its origin from the Ca^{2+} release through Ryn/Ca^{2+} channels triggered by the influx of extracellular Ca^{2+}, a phenomenon known as "Ca^{2+}-induced Ca^{2+}-release" (8). Therefore, the process of excitation-contraction is rather complex and apparently involve both extra- and intracellular components.

In 2000, the induction of contracture by 4-AP in Ca2+-free media on spontaneously beating frog ventricular strips was shown by our study team for the first time (6). More recently, an identical study by Bhaskar et al., 2008 supports our finding that when the normal medium was replaced by a Ca^{2+}-free solution, spontaneous contractions were fully diminished in accompaniment with a gradually developing dose-dependent contracture (8). Since extracellular Ca^{2+} is null in these experiments, the main source of Ca^{2+} mediating contracture was thought to be Ca^{2+} stored in the sarcoplasmic reticulum (SR) and to lesser extent Ca^{2+} bound to the phosphatidylserine at the inner surface of plasma membrane. Indeed, the latter view was supported by the fact that $^{45}Ca^{2+}$-binding to phosphatidylserine monolayer in vitro was shown to be inhibited by 4-AP in a dose-dependent manner (5). Furthermore, interruption of electrical stimulation for 30 sec in cardiac muscle causes the first post-rest contraction to be more powerful than the amplitude of pre-rest contraction, a phenomenon known as post-rest potentiation which is thought to be due to accumulation of Ca^{2+} in SR during resting period which causes more Ca^{2+} to be released through Ryn/Ca^{2+} channels and thus augments first post-rest contraction (9,10). Inhibition of Ca^{2+}-ATPase in SR by 4-AP may also play a role in such a potentiation and contracture development by this agent (11). Although the effects of 4-AP on the plasmalemmal K^+ ve Ca^{2+} currents were adequately studied, its effects on intracellular Ca^{2+} movements as well as on Ca^{2+} release from the SR were not fully elucidated. Therefore, it is important to know the detailed properties of the cellular Ca^{2+} stores and Ca^{2+} release mechanisms.

The main part of Ca^{2+} responsible for excitation-contraction coupling mainly relates to the Ca^{2+} release from SR triggered by extracellular Ca^{2+} entering the cell dur-

ing an action potential. On the other hand, intracellular accumulation of Ca^{2+} in many cells has been reported to be mediated by inositol 1,4,5-triphosphate (IP_3) cascade (12). The activation of some receptors in various cells induces the conversion of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), to IP_3 and diacylglycerol (DAG) which are the activator of sarcoplasmic Ca^{2+} channels and protein kinase C (PKC), respectively. Two different kinds of Ca^{2+} release mechanisms are defined in the sarcoplasmic membrane of smooth muscle cells: ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release channel (Ryn/ Ca^{2+} channels) (13), and IP_3 induced Ca^{2+}release channel (14). Similarly, Jeanne et al. (2001) have also shown the presence of two separate Ca^{2+} release systems in cultured striated muscle cells. Therefore, it is quite logical to consider that phospholipase C (PLC), a membranous enzyme which gives rise to the generation of IP_3 and DAG from PIP_2 as well as IP_3/Ca^{2+} channels and Ryn/ Ca^{2+} channels in SR might also be potential targets for 4-AP in addition to sarcoplasmic Ca^{2+}/ATPase (15).

Freeman (1979) suggested the involvement of PLC-IP_3 cascade activation in 4-AP-induced contracture which might be prevented by neomycin, a PLC inhibitor (16). On the other hand, if PLC-IP_3 cascade is involved in the mediation of 4-AP contracture, it would be prevented by a PLC inhibitor as said or by an IP3-R antagonist, as heparin (17,18). Therefore, the aim of this study was to explore the role of PLC-IP_3 cascade, if any, in the induction of contracture by 4-AP, and to get more insights into its underlying mechanisms. This study has been designed specifically to unravel interaction of 4-AP with PLC-IP_3 cascade.

Materials and Methods

Drugs and Chemicals

Heparin purchased from Sigma-Aldrich Ltd. (Budapest, Hungary), 4-aminopyridine and KCl from Merck Ltd. (Budapest, Hungary) and neomycin sulfate is a gift of Pfizer Inc. (NY, USA). All these chemicals were dissolved in deionized distilled water.

Animals

Experiments were conducted on both electrically-driven atrial and diaphragmatic muscle strips from adult Wistar-Albino rats of both sexes 200-250 g in weight. All experimental animals were fed ad libitum with standard rat bait at ambient temperature with free access to tap water.

Experimental set-up

After stunning and exsanguination of test animals, the heart and diaphragmatic muscle excised rapidly from the chest cavity and put into a dish filled with oxygenated Tyrode solution kept at ambient temperature. Strips prepared from both atria and denervated diaphragmatic muscles (about 3 x 6mm in dimension) placed vertically between two silver electrodes and then delivered into a 10ml organ bath filled with Tyrode solution oxygenated continuously by a mixture of 95% O_2 + 5% CO_2. The composition of Tyrode solution was (g/L): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, NaH_2PO_4 0.05, $NaHCO_3$ 1.0, glucose 1.0. The bath temperature was kept constant at 30°C by a thermostatic circulation pump (Braun Melsungen). One end of the strip attached to the hook of the electrode and the threads tied to the other end to an isometric force-displacement transducer (Grass model FT03) under a resting tension of 0.5 g for atrial strips and 1.0 g for diaphragmatic strips. Atrial strips were contracted electrically with impulses of 2 x threshold voltage and 2 msec duration generated by a Grass Model S88 double-channel stimulator at a rate of 2 Hz and diaphragmatic strips with impulses of 2 x threshold voltage and 5msec duration at a rate of 0.2 Hz, respectively. All contractions were displayed on Grass Model 79 polygraph. The preparations were allowed to equilibrate at least for 30 minutes during which the bathing medium was washed with fresh solution every 10 minutes.

Experimental procedure

Experiments on denervated diaphragmatic muscle strips prepared from rat diaphragmatic muscles kept 2-3 hours in oxygenating Tyrode solution at room temperature were conducted in a medium with normal Ca^{2+} concentration. 4-AP responses alone were obtained by applying the drug cumulatively in logarithmically increasing concentrations in the range of 1-16 mM with 2 min interval. 4-AP responses in the presence of 400 µM neomycin (19) and to 400µg/ml heparin (20)) were obtained after incubating the strips with these agents for 3 min.

Experiments on atrial strips were performed either in the normal Tyrode solution containing Ca^{2+} or in the Ca^{2+}-free solution prepared by subtracting Ca^{2+} and treating it with 0.5 mM/L EGTA. Control 4-AP responses were obtained in the same fashion as in the diaphragmatic strips by applying the drug cumulatively in logarithmically increasing concentrations in the range of 4-64 mM with 2 min interval and 4-AP responses in the presence of 400 µM neomycin, and to 400 µg/ml heparin after pre-incubating the strips with these agents again for 3 min.

When electrical stimulation was interrupted for 30 sec, the first contraction after re-initiation of stimulation following the resting period was shown to be higher than the amplitude of control twitch contractions at the pre-resting period. This phenomenon is known as post-rest potentiation which is thought to be mediated by the profuse release of Ca^{2+} from the SR. After control responses were obtained in drug-free medium, post-rest responses after 2 min pre-incubation with 4 mM 4-AP or 3 min pre-incubation with neomycin or heparin plus 2 min pre-incubation with 4 mM 4-AP were recorded.

Following replacement of bathing medium with the Ca^{2+}-free solution, twitch contractions gradually declined to zero within a time course of 5 min. Following twitch contractions nullified, 4-AP was applied cumulatively with 2 min interval to get control contracture responses or contracture responses in the presence of 400 µM neomycin and 400 µg/ml heparin added to the bathing medium 3 min before 4-AP.

Statistical Analysis

For the uniformity of evaluations in each experiment, the amplitudes of contractions under the influence of drugs were expressed as percent of contractions in untreated controls. Twitch contractions expressed as

%- control in mean ± S.E.M. were plotted on the ordinate and drug concentrations on the abscissa. Statistical analysis of the data was performed using the Student's t-test for paired experiments with each preparation as its own control. The significance level was set at $p < 0.05$.

Results

Interaction of 4-AP with heparin and neomycin on atrial twitch contractions and contractures in a medium with normal Ca^{2+}

When 4-AP was added to the organ bath in logarithmically increasing concentrations for every two min, twitch amplitude underwent biphasic changes, for example, increased up to 16mM beyond which it is reduced due to development of gradual dose-dependent contracture as illustrated by original tracing. As shown in Figure 1B, the biphasic time-course of twitch responses to 4-AP was not found to be altered by pre-treatment of the atrial strip with 400 µg/ml heparin for three min. However, the contracture becoming manifest at 8 mM 4-AP and getting increased up to 64 mM in a dose-dependent manner significantly depressed (Figure 1C, Table 1). Similar results were obtained when atrial strips were exposed to 400 µM neomycin for three min, where twitch amplitude remained unaltered (Figure 2A) and contracture developed beyond 8 mM 4-AP (Figure 2B, Table 1) depressed significantly ($p< 0.05$). Control %-contractures with 4-AP were in the order of 26.85±4.72, 110.7±17.24 and 188.73±12.92 at 16, 32 and 64 mM 4-AP which were significantly reduced to 1.52±1.19, 13.9±1.05 and128.6±16.85 by pre-treatment with 400 µg/ml heparin for three min as shown in Figure 1 and 2, and in Table 1, respectively ($p< 0.05$). The depression of 4-AP-induced contractures by neomycin and by heparin can be taken as evidence for the interaction of 4-AP with PLC-IP$_3$ cascade known to mediate the effects of several endogenous active substances such as noradrenaline, angiotensin II or endothelin 1 causing the contraction in smooth muscles.

As illustrated in Figure 3A and B, the first contraction following interruption of atrial electrical stimulation for 30 min in untreated controls was elevated in as much as 176.23±15.54 % with respect to the pre-rest amplitude of twitch contractions. The first post-rest contraction reduced significantly from 176.23±15.54 % in controls, however, to 124.46±4.64 % after pre-treatment of preparations with 4 mM 4-AP for 2 min ($p< 0.05$). When the preparations were treated with 400 µg/ml heparin or 400 µM neomycin for 3 min, first post-rest contraction values were 247.6±11.47 % and 178.89±33.23 %, respectively, which were significantly higher than first post-rest contraction values in the presence of 4 mM 4-AP. However, difference of the values was non-significant when compared with controls. These experimental results revealed that both heparin and neomycin interacted with 4-AP in an antagonistic manner on post-rest first contractions.

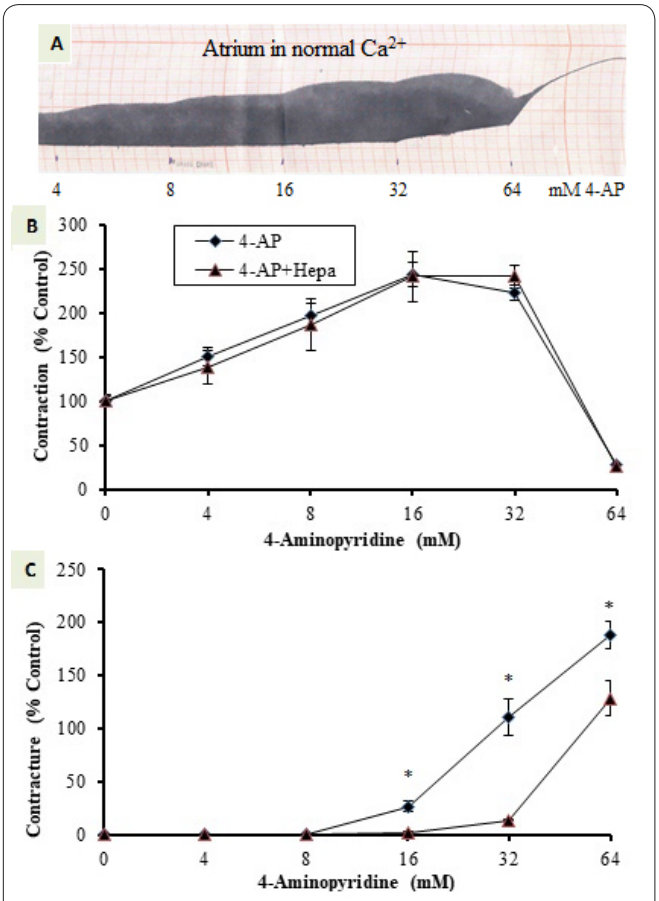

Figure 1. The effects of heparin (400 µg/ml) on twitch contractions (B) and contractures (C) induced by 4-AP in the electrically-driven atrial strips of rats. On the top, the dose-dependent effects of 4-AP on contractility were represented by an original recording (A). 4-AP was applied cumulatively in concentrations in the range of 4-64 mM with 2 min interval (n=16) and the preparations were exposed to heparin for 3 min before application of 4-AP (n=5). Contractions and contractures were plotted on the ordinate as % control in mean ± S.E.M and 4-AP concentrations on the abscissa in mM. Bold values of *: $p< 0.05$ represents statistical significance, 4-AP, 4-aminopyridine; Hepa: Heparin.

Interaction of 4-AP with heparin and neomycin on atrial twitch contractions and contractures in Ca2+-free media

Ca^{2+}-free media was obtained by removal of Ca^{2+} from Tyrode solution and addition of 0.5mM EGTA,

Table 1. The effects of heparin and neomycin on 4-AP-contracture in the electrically-driven rat atrial strips.

In medium with normal Ca^{2+}	4-Aminopyridine (mM)				
	4	8	16	32	64
C/Heparin	0.13±0.12	0.62±0.31	26.85±4.72	110.70±17.24	188.73±12.92
+Heparin	0.00	0.30±0.30	1.52±1.19*	13.90±1.05*	128.60±16.85*
C/Neomycin	0.12±0.12	0.59±0.30	25.14±4.78	104.56±18.07	177.33±14.59
+Neomycin	0.00	0.24±0.24	0.97±0.57**	15.29±2.42**	148.32±17.02**

Experiments were conducted in a Tyrode solution with normal Ca2+ and values were expressed as % control in mean±S.E.M. C, control; Bold values of * or ** represents statistical significance, *:p<0.05, heparin vs C/heparin; **: p<0.05, neomycin vs. C/neomycin.

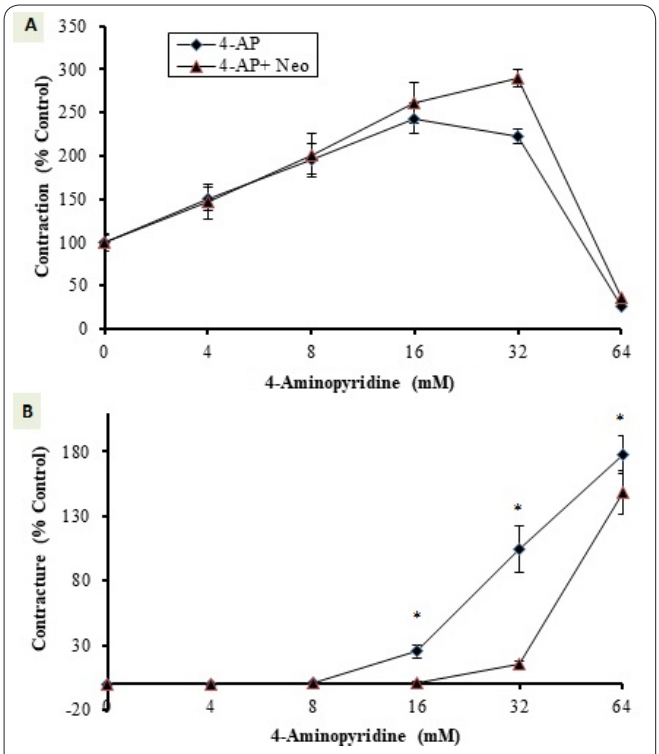

Figure 2. The effects of neomycin (400 µM) on twitch contractions (A) and contractures (B) induced by 4-AP in the electrically-driven atrial strips of rats. 4-AP was applied cumulatively in concentrations in the range of 4-64 mM with 2 min interval (n=16) and the preparations were exposed to neomycin for 3 min before application of 4-AP (n=15-38). Contractions and contractures were plotted on the ordinate as % control in mean ± S.E.M and 4-AP concentrations on the abscissa in mM. *: p< 0.05.

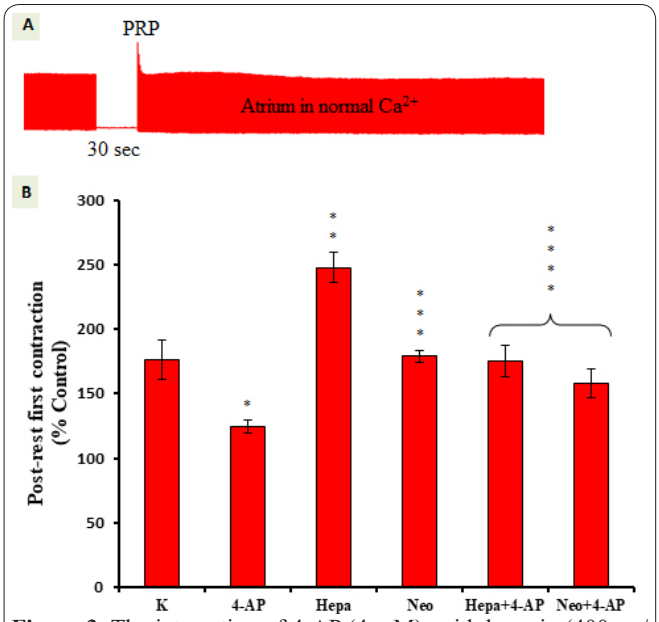

Figure 3. The interaction of 4-AP (4 mM) with heparin (400 µg/ml) and neomycin (400 µg/ml) on the post-rest first contractions induced by re-stimulation after interruption of electrical stimulation for 30 sec in a medium with normal Ca2+ (A). Histograms (B) represent post-rest first contractions as % control on the ordinate and types of manipulations on the abscissa as abbreviations. C: control, 4-AP: 4-aminopyridine, Hepa: heparin, Neo: neomycin, Hepa+4-AP: first contractions after 3 min exposure to heparin followed by 2 min exposure to 4-AP, Neo+4-AP: first contractions after 3 min exposure to neomycin followed by 2 min exposure to 4-AP, n= 7-16, *: p< 0.05 vs 4-AP, **: p< 0.05 vs C and 4-AP, ***: p< 0.05 vs 4-AP, ****: p< 0.05 vs 4-AP.

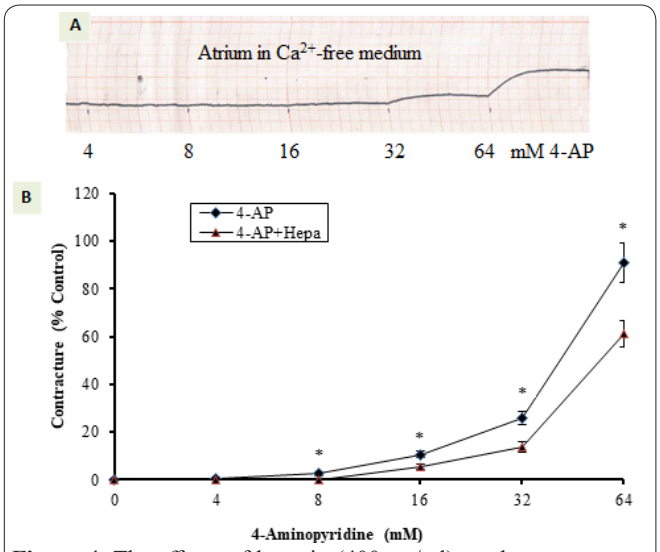

Figure 4. The effects of heparin (400 µg/ml) on the contractures induced by 4-AP under an absolute Ca2+-free condition in the electrically-driven atrial strips of rats (B). On the top, the dose-dependent contracture was represented by an original recording (A). 4-AP applied into the organ bath by 2 min interval cumulatively in concentrations in the range of 4-64 mM (n=18) and the preparations were exposed to heparin for 3 min before application of 4-AP (n=19). Contractures were plotted on the ordinate as % control in mean ± S.E.M and 4-AP concentrations on the abscissa in mM. *: p< 0.05.

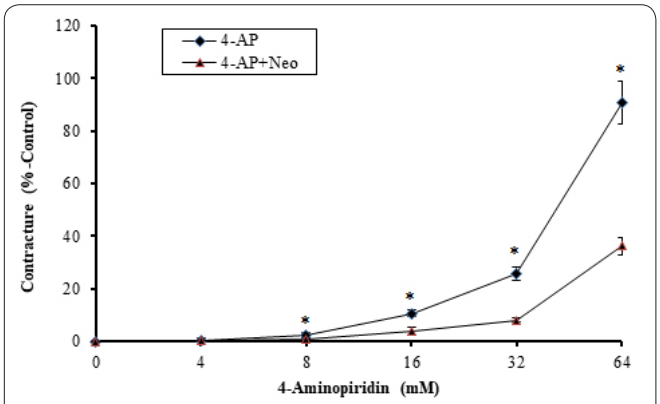

Figure 5. The effects of neomycin (400 µM) on the contractures induced by 4-AP under an absolute Ca2+-free condition in the electrically-driven atrial strips of rats. 4-AP applied into the organ bath cumulatively by 2 min interval in concentrations in the range of 4-64 mM (n=18) and the preparations were exposed to heparin for 3 min before application of 4-AP (n=15). Contractures were plotted on the ordinate as % control in mean ± S.E.M and 4-AP concentrations on the abscissa in mM. *: p< 0.05.

a Ca2+ ionophore, in it. After an equilibration period of 20 min in normal Tyrode solution, replacement of bathing medium with Ca^{2+}-free Tyrode solution caused twitch contractions of atrial strips to be diminished fully within 5 min, beyond which application of 4-AP in logarithmically increasing concentrations in the range of 4 to 64 mM induced dose-dependent contractures as shown by original tracing in Figure 4A. Contractures induced by 4-AP in Ca^{2+}-free media were of the order of 0.56±0.53, 2.42±0.83, 10.59±1.33, 25.67±2.65 and 90.89±8.04 respectively (Table 2). Incubation of atrial strips with 400 µg/ml heparin or 400 µM neomycin for three min in Ca^{2+}-free media antagonized these contractures by 4-AP at 8, 16, 32 and 64 mM to a significant extent as illustrated in Figure 4B, Figure 5 and Table 2 (p<

Table 2. The effects of heparine and neomycine on the 4-AP-contracture in the electrically-driven rat atrial strips.

In Ca^{2+}-free medium	4-Aminopyridine (mM)				
	4	8	16	32	64
Control	0.56±0.53	2.42±0.83	10.59±1.33	25.67±2.65	90.89±8.04
+Heparin	0.00	0.00*	5.34±1.30*	13.67±1.94*	61.19±5.63*
+Neomycin	0.30±0.17	0.82±0.37**	4.08±1.53**	8.18±0.58**	36.20±3.23**

Experiments were conducted in a Tyrode solution with normal Ca2+ and values were expressed as % control in mean±S.E.M. Bold values of * or ** represents statistical significance, *: p< 0.05, heparin vs control; **: p< 0.05, neomycin vs control.

0.05). Since 4-AP-induced contracture in the Ca^{2+}-free extracellular medium, it is obvious that the source of Ca^{2+} mediating it should be of SR in origin. Antagonism of such a contracture with a PLC inhibitor like neomycin and an IP3-R antagonist like heparin may be taken as an evidence for the activation of PLC-IP$_3$ cascade by 4-AP.

Interaction of 4-AP with heparin and neomycin on diaphragmatic contractures in normal Ca^{2+}-media

When rat diaphragmatic muscle is preserved in oxygenated normal Tyrode solution for 2-3 hours at ambient temperature, it gets denervated as evidenced by the loss of 4-AP-induced increase in twitch contractions due to stimulation of release of ACh from motor nerve terminals. 4-AP caused dose-dependent contracture in such denervated muscle strips with doses in the range of 2-16 mM applied cumulatively by two min interval as shown in Figure 6A by original tracing. Contractures by 8 and 16 mM 4-AP in the range of 43.43±11.62 % and 168.7±29.2 % reduced significantly to 8.62±0.01 % and 18.51±0.02 % by pre-treatment of strips with 400 µM neomycin for three min ($p<0.05$). The results were almost similar when muscle strips were pre-treated with 400 µg/ml heparin for three min (Figure 6B and C).

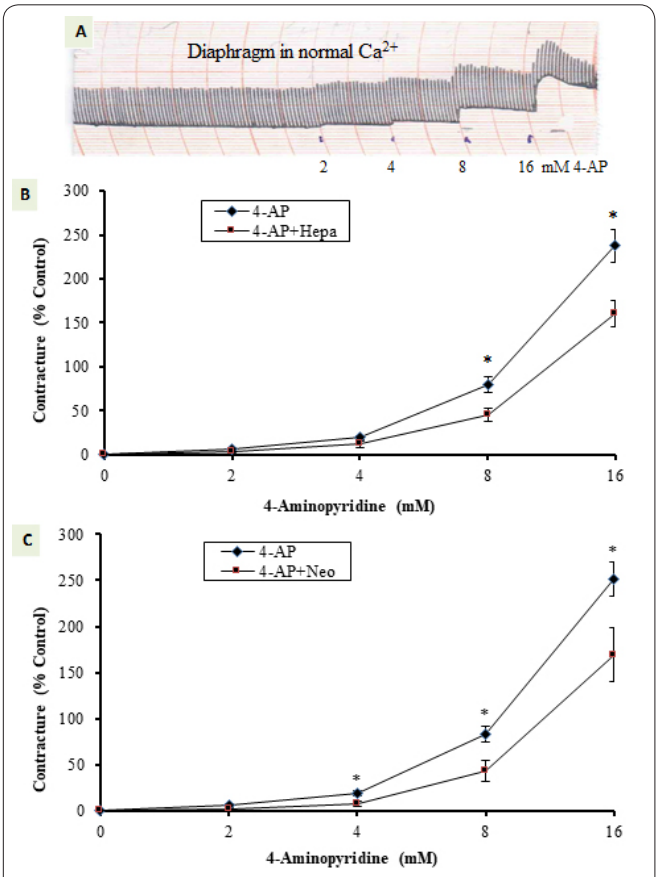

Figure 6. The effects of heparin (400 µg/ml) and neomycin (400 µM) on the contractures induced by 4-AP in the electrically-driven denervated rat diaphragmatic muscle strips (B and C). The dose-dependent effects of 4-AP on contractility were represented by an original recording as in A. 4-AP was applied cumulatively in concentrations in the range of 4-64 mM with 2 min interval (n=15-19). Then the strips were exposed to heparin (n=26) and neomycin (n=8) for 3 min before application of 4-AP, respectively. Contractures were plotted on the ordinate as % control in mean ± S.E.M and 4-AP concentrations on the abscissa in mM. *: p< 0.05.

Discussion

The mechanisms underlying the interaction of 4-AP with several excitable cell-lines appeared to be rather complex and have not been fully explored. Experimental results steadily suggested that the mechanisms subserving its effects at the cellular and molecular level may involve inhibition of K+-channels (1-4), promotion of transmembrane Ca^{2+}-influx (3,4), release of Ca^{2+} from SR (6,7), inhibition of Ca^{2+}-ATPase (11) or the interaction of 4-AP with intracellular Ca^{2+}-depots (3-7,9). Indeed, induction of contracture by 4-AP in Ca^{2+}-free media strongly suggested that it interacted with SR and that the source of Ca^{2+} responsible for such a contracture should be of sarcoplasmic Ca^{2+} stores (7,9). The level of intracellular Ca^{+2} in multiple types of mammalian cells controls an enormous number of functions including cell division, glandular secretion, and contractility of muscle cells. Initiation of muscle contraction is achieved by mutual participation of several cellular components including elevation of cytoplasmic free Ca^{+2} concentration mediated by depolarization of the cell membrane and spatial activation of dihydropteridine receptors (DHPRs), calcium-induced Ca^{+2} release through ryanodine-sensitive Ca^{+2} receptors (RyRs), IP$_3$/Ca^{+2} release receptors (IP$_3$-R) and the activation of membrane enzyme PLC generating IP$_3$ and DAG, respectively. Experimental results repeatedly suggested that 4-AP interacted with depolarization process (4), dihydropteridine-sensitive Ca^{+2} channels (4,9) and sarcoplasmic Ca2+/ATPase (11). It was rather unexpected to explore that 4-AP induced contracture in the spontaneously beating frog ventricular strips in the absence of Ca^{2+} (4), a finding indicating that the source of Ca^{2+} should be intracellular in origin, namely sarcoplasmic Ca^{+2} stores and/or Ca^{+2} loosely bound to phospholipids at the inner surface of the cell membrane. Although the contractile actions of 4-AP on the membranous K^+ and Ca^{2+} currents were adequately studied, its effects on intracellular Ca^{+2} movements as well as on Ca^{2+} release from the SR still needs further elucidation. It was thought that PLC, the key enzyme regulating intracellular calcium and modulating the phosphoinositide balance (18), IP$_3$/Ca^{2+}-release channels and Ryn/Ca^{2+}- release channels in the

SR might also be potential targets for 4-AP in addition to sarcoplasmic Ca^{+2}/ATPase. At least three isoforms of ryanodine receptors found in the mammalian tissues by cDNA cloning technique, namely R_yR_1 in skeletal muscle, R_yR_2 in heart muscle and R_yR_3 in the smooth muscles or epithelial cells (21). It was well documented that the effect of ryanodine on Ca^{2+} release from the SR presented a biphasic time course, e.g. stimulatory below and inhibitory over this concentration (22). If PLC-IP_3 cascade is involved in the mediation of 4-AP contracture, it can be prevented by a PLC inhibitor such as neomycin or by an IP_3-R antagonist like heparin (17).

In this recent work, we present new unexplored evidence for the induction of contracture by 4-AP which is found to be PLC sensitive and is likely mediated by massive release of Ca^{+2} from intracellular Ca^{+2} stores, namely the SR. To our knowledge, this is the first evidence revealing that PLC indeed has a share in the induction of 4-AP contracture in the cardiac and skeletal muscles. Ozgul et al. (2000), suggested that 4-AP induce contracture probably by two ways: l) the maximal capacity of Ca^{2+}-sequestering mechanisms necessary for relaxation may be exceeded by too much Ca^{2+}' entering into the sarcoplasm during an action potential and, 2) inhibition of cellular Ca^{+2}-sequestering mechanisms by 4-AP in the contractile tissues (6). As shown in Figure 1 and 2, although neomycin, a PLC inhibitor, and heparin, an IP_3-R antagonist were almost devoid of an effect on twitch contractions in the presence of 4AP under normal Ca^{+2} containing media, contractures developed gradually in a dose-dependent manner beyond 8 mM 4-AP were depressed significantly, a finding indicating that PLC play some role in the generation of normal twitch contractions and does contribute to 4-AP contracture. In more detail, neomycin significantly reduced the contracture by 4-AP at 64 mM from 188.73±12.92 to 128.6±16.85 and heparin from 177.33±14.59 to 148.32±17.02, respectively (Table l). Almost similar results were obtained in the diaphragmatic muscle of rats with these PLC inhibitors on the contractures induce by 4-AP in Ca^{2+}-containing media (Figure 6, Table 2). It is evident that 4-AP induces dose-dependent contractures beyond 8 mM, which is accompanied by the simultaneous reduction of twitch contractions as in Figure l. This reciprocal relationship between twitch contractions and contracture was not unexpected, because a part of contractile fibrils will be in a state of contraction and thus cannot relax during contracture. Consequently, twitch contractions will be reduced as the number of fibrils participating contraction is being reduced. When all actin-myosin fibrils take part in contracture, twitch contractions will disappear leaving only contracture to be observed (Figure 1A) (6). This seemed to be likely because contracture induced by 64 mM 4-AP was almost irreversible and could not be reverted by washout. Taking all these together, it is obvious that the source of Ca^{+2} in Ca2+-free medium inducing contracture (Figure 4 and 5) should be of intracellular Ca^{2+}-stores, namely SR. It has been shown that 4-AP can induce contracture in Ca^{2+}-free medium either by promoting Ca^{2+} release from SR or by inhibiting reverse Ca^{2+}-pumping with Ca^{2+}/ATPase into the SR (6,11). Furthermore, experimental results currently suggest that post-rest potentiation (Figure 3) mediated by the powerful triggering of Ca^{2+} release from the SR (3-7,9). In the present work, the first contraction induced by 4-AP alone was found to be reduced with respect to pre-drug contractions. This might be due to the reduction of the size of sarcoplasmic Ca^{2+}-stores with 4-AP by increasing Ca^{2+} influx triggering more Ca^{2+} to be released from SR, thus inducing a state of Ca^{2+} depletion in the SR. Indeed, this view was supported by the fact that the effect of 4-AP on post-rest potentiation was reverted and very strongly augmented in the presence of verapamil, a Ca^{2+} channel blocker (unpublished observation). If we consider that PLC generates IP_3 causing release of Ca^{2+} from SR by stimulation of RyR and DAG that activates Ca^{2+} channels in the superficial membrane, inhibition of 4-AP contracture in both Ca^{2+}-free and Ca^{+2} -containing media, and the antagonism of 4-AP effect on post-rest first contraction by neomycin and heparin can be expected.

It was concluded that the results obtained in this study correlated well with the results published previously (6,7). The effects of 4-AP on the cellular Ca^{2+} movements and its actions at the molecular level appeared to be complex and still need to be further elucidated. According to the so far known information, the overall cellular actions of 4-AP may be a consequence of the action potential prolongation due to inhibition of K^+ channels (1,6,16,23,24), the activation of Ca^{2+}-channels (3,4), reduction of Ca^{2+}- binding capacity of membrane phospholipids (eg: phosphatidylserine) due to partial depolarization and protonation of the plasma membrane by 4-AP (3) and of Ca^{2+}-release from the SR (5,6,25). In addition, our results clearly demonstrated that PLC was also a potential cellular target for and participated in some actions of 4-AP. To our knowledge, this work is the first which provides an experimental evidence for the interaction of 4-AP with PLC in contractile tissues.

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Conflict of Interest

The authors report no conflict of interest.

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