ROLE OF CA++ GRADIENT IN FUNCTIONAL DILATATION IN THE MICROVESSELS OF RAT SKELETAL MUSCLE

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Abstract: During skeletal muscle contraction functional dilatation (FD) is a well established phenomenon, which is usually linked to the accumulation of various vasodilator metabolites. The present study aimed to establish the role of calcium ions (Ca++) in the functional dilatation of skeletal muscle microvessels especially during single & few twitches. The FD was studied in urethane anaesthetized rats whose spinotrapezius muscle was prepared for intravital microscopy. After stimulating the muscle with few twitch (1 & 3 Hz) and tetanic frequency (40 Hz), muscle contracted and showed dilatation. However the dilator response was quickest (1 Hz : 10 ± 0.40, 3 Hz : 02 ± 0.47 sec and 40 Hz : 02 ± 0.10 sec) and maximum in magnitude (1 Hz : 25 ± 0.30%, 3 Hz : 35 ± 0.49% and 40 Hz : 55 ± 0.39%) in terminal arteriole and with tetanic frequency. Calcium channel blockade by Diltiazem abolished the FD response except for tetanic stimulation contraction. Findings suggested possible involvement of Ca++ movement in functional dilatation which was initiated by passive efflux of Ca++ from smooth muscle of vessel and then maintained during higher frequency stimulation by release of local metabolites.

Key words: functional dilatation functional hyperaemia skeletal muscle micro vessels calcium channel blocker

INTRODUCTION

Functional hyperaemia in skeletal muscle (increased blood flow which accompanies muscle contraction) has been a subject of extensive study (1). It is well known from many studies that there is a decrease in muscle vascular resistance in response to muscle contraction. This has been attributed to dilatation of arterioles and arteries (2, 3, 4, 5). In 1984, Marshall and Tandon made a direct observation of the changes in diameter occurring in each section of microcirculation in response to neural stimulation of rat spinotrapezius muscle and concluded that venules and veins also dilate actively during muscle contraction (6, 7). However, the degree of vasodilator response varied amongst different sections of microvessels, i.e. arterioles responded quicker in comparison to venules in response to same frequency of stimulus.

The cause of this functional dilatation (FD) of microvessels has been attributed to release of vasodilator metabolites,
i.e. inorganic phosphate, lactate, K⁺, hyperosmolarity, changes in PO₂ and pH (7, 8, 9, 10, 11, 12). It appears likely that these factors may play a role in causing FD when muscle is stimulated briskly or with tetanic frequency since this would allow sufficient accumulation of the metabolites. But the cause of FD in microvessels in response to single twitch or few twitches is still unclear. For the same, we hypothesized that Ca++ ion gradient may play a role in FD in skeletal muscle and the study proposed to examine whether Ca ++ channel blockade, by Diltiazem, would attenuate FD during graded contractions in response to microelectrode stimulation.

MATERIAL AND METHODS

The study was carried out in Department of Physiology, GSVM Medical College, Kanpur on 25 male Sprague Dawley rats. The institutional guidelines for the care and use of laboratory animals were followed. The rats were anaesthetized with subcutaneous Urethane (diethyl carbamate; 1.25 ml of a 20% solution/100 g body weight) followed by the cannulation of the trachea with a 'L' shaped polyvinyl cannula (6).

Preparation of micropipette

Micropipettes were prepared from Corning glass capillary tubes (id : 1 mm) attached to a vertical micropipette puller fitted in adjusted palmer stand. The capillary was made to pass through a heating coil. The tip of pipette was broken under microscope against reflecting surface of glass rod to obtain a patent tip of 3–6 µm diameter.

Preparation of microelectrode for motor unit stimulation

Microelectrodes were prepared from stainless steel wire (0.3 mm thick). Straightening was done by applying 1 kg/m tension for 24 hrs following which equal pieces of 5 cm were cut and subjected to electrode etching using dilute hydrochloric acid. The sharpened electrodes with uniform tips (2–4 µ) were selected under microscope and finally coated with epoxy-resin and kept for 24 hrs in an oven at 80°C to become ready to use.

The steps of each experiments were as follows:
1) In vivo preparation of spinotrapezius muscle
2) Muscle stimulation
3) Drug – microdroplet Application

In vivo preparation of spinotrapezius muscle

The left spinotrapezius muscle (predominantly a fast muscle as 90% capillaries are straight) was dissected out using techniques already validated. The whole preparation was transferred to a special Perspex platform that was then fitted to a Hertel and Reuss binocular microscope stage for transillumination. Measurement of internal diameter (i.d.) of the microvessels was done by a precalibrated micrometer eyepiece (1 unit = 14 µm with × 10 objective eye piece). The working distance of × 10 objective was 1 cm. During the dissection and throughout the experimental period the muscle was superfused with a modified Kreb's solution [composition (g/l) : Nacl – 4.8, KCl – 0.35, NaH₂PO₄ – 0.35, NaHCO₃ – 1.7, Glucose – 1.4 and MgSO₄. 7 H₂O – 0.15 at 37°C] (13, 14).

The blood vessels in the muscle were defined on the basis of their relative positions and their internal diameters (16). The entire muscle was observed to have 3
groups of arterioles branching from the main artery, namely primary (1° A, id: 22–50 µm), secondary (2° A, id: 13–18 µm) and terminal arterioles (TA, id: 07–13 µm) which finally fed the capillaries. The capillaries had internal diameter of 4–5 µm at their arterial end and 6–7 µm at their venous end. The capillaries from adjacent terminal arterioles converged to form collecting venules (CV, id: 9–18 µm), which drained into the secondary venules (2° V, id: 18–30 µm) and then into the primary venular network (1° V, id: 40–60 µm) the primary venules eventually formed main vein.

**Muscle stimulation**

Steel microelectrodes were mounted on microelectrode manipulator and microscopically guided into the desired area of the spinotrapezius muscle. The stimuli were delivered through a research stimulator (Techno Electronics, India) at a constant voltage of 2 volts and constant pulse width of 0.1 ms. Stimulation evoked contraction of a small bundle of skeletal muscle fibres. At this pulse width and voltage, it had been established that only motor nerve terminals get excited whereas the sympathetic terminals remained unaffected (5, 15, 16).

In all the experiments, after measuring the control internal diameter (id), the microelectrode tip was inserted into muscle fibres which traversed a region containing a terminal branching system of microvessels so that it lay 100–200 µm from the nearest arterioles & venules and electrical stimulation with the chosen parameters evoked contractions in a bundle of muscle fibres, 300–500 µm in width (Fig. 1).

The muscle was stimulated at the frequency of 1 Hz, 3 Hz and 40 Hz (tetanic frequency) for 1 second following which magnitude of dilatation i.e. % increase in internal diameter compared to control internal diameter and time to onset were measured. It was impossible to make any measurement during the period of contraction because of movement in the viewed field but so this was done as soon resting state returned (2–4 s interval). Measurements could be confirmed later, by means of photomicrographs taken with a Leica MdA camera via a beam splitter and showed the accuracy of the measurements to be between 2% and 5% of the internal diameter. Stimuli trains of different frequencies were always applied in a random order and id of different microvessels were also measured in random order. A period of 5–8 min rest was allowed between

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**Fig. 1:** A schematic drawing, made from actual preparation to illustrate the effect of contraction of a bundle of skeletal muscle fibres on the terminal vasculature of rat spinotrapezius muscle. The star shows a point of placement of electrode. Stimulus at this site evoked contraction of muscle fibres of unshaded area but not those of shaded area. There was no change in diameter of these vessel within the shaded area.
consecutive stimulations when the muscle was superfused with Kreb's solution.

If the vessels failed to return to the control diameter or signs of tissue damage was present, the electrode was moved to a new site near another system of terminal branching vessels.

Drug – microdroplet application

In 15 experiments, Diltiazem hydrochloride (Sigma) was dissolved in modified Kreb's solution and it was applied in a form of a microdroplet in the concentration of 1 n mol through the glass micropipette fitted to a micromanipulator on muscle surface where control diameters of microvessels were measured. The Microdroplet spread on the muscle surface within a 1–2 sec and thence immediate responses were noted down.

After 80–120 sec, when response of the drug apparently vanished, muscle fibres were stimulated with 1 Hz, 3 Hz and 40 Hz frequencies for 1 sec.

Statistical evaluation

A small change in internal diameter of vessel may be physiologically highly significant though it may appear insignificant statistically. Hence the data was not subjected to conventional statistical procedures.

RESULTS

Under resting conditions, the flow through all arteriolar and venular vessels was fairly steady but the flow through capillary was intermittent. There were some capillaries through which blood was flowing at different rates, some contained stationary cells and others appeared to be emptied.

The effect of both twitch (1 Hz and 3 Hz) and tetanic (40 Hz) contractions was that immediately after contractions there was vasodilatation, not only of arterioles but also of the venules which crossed or ran alongside the activated muscle fibres. Accompanying the vasodilatation, the velocity of blood flow was noticeably increased in the capillaries supplied by dilated arteries. The flow became continuous in those capillaries in which it had been intermittent and often commenced in those that had no flow at rest.

| TABLE 1: Functional dilatation responses: time to onset (rapidity) and magnitude of dilatation (% increase in internal diameter in different segment of microvessels after muscle stimulation at lower (1 Hz and 3 Hz) and higher (40 Hz) frequencies. |
|-----------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Functional                        | Primary arteriole (1°A) Mean±SD | Secondary arteriole (2°A) Mean±SD | Terminal arteriole (TA) Mean±SD | Collecting venule (CV) Mean±SD | Secondary venule (2°V) Mean±SD | Primary venule (1°V) Mean±SD |
| dilatation                         | Time to onset (sec) | | | | | |
| 1 Hz | 20±0.81 | 15±0.64 | 10±0.49 | 22±0.82 | No response | |
| 3 Hz | 15±0.90 | 13±0.71 | 02±0.47 | 17±0.70 | 20±0.90 | 22±0.60 |
| 40 Hz | 08±0.78 | 05±0.61 | 02±0.10 | 10±0.90 | 12±0.82 | 15±0.53 |
| Magnitude of dilatation (%)       | 1 Hz | 10±0.62 | 22±0.53 | 25±0.30 | 15±0.75 | No response |
| 3 Hz | 18±0.73 | 25±0.42 | 35±0.49 | 32±0.65 | 22±0.67 | 12±0.59 |
| 40 Hz | 25±0.86 | 28±0.59 | 55±0.39 | 38±0.92 | 28±0.83 | 12±0.72 |
The dilator responses were frequency dependent (Table I). FD was quicker in 'onset' and larger in 'magnitude' in case of 40 Hz (tetanic frequency) stimulation in comparison to 1 Hz and 3 Hz in all sections of microvessels. Terminal arterioles generally reached maximal dilatation to a given twitch frequency a few seconds earlier and with larger percentage increase in diameter than other arterioles, while collecting venules were often quicker to reach a peak with more magnitude than other venules (Table I). Having achieved a peak, all vessels showed a plateau phase and then began to return to their control diameters.

Application of Diltiazem microdroplet produced consistent responses. It resulted in constriction in TA within 3–4 sec to the extent that flow in these vessels stopped completely. The corresponding capillaries revealed the stagnation of flow and feeding venules were also constricted to nearly zero diameter (Fig 2: a, b, c, d). The response stayed for as long as 80–120 sec and then returned to control value. Now, on stimulating muscle fibres at the same site with 1 Hz and 3 Hz frequencies, the arterioles and venules did not show any functional dilatation. In contrast, the tetanic

![Fig. 2: a, b, c, d: The series of microphotographs, taken at 1 sec interval. Panel a – control diameters of terminal arterioles (TA) and collecting venules (CV). Panel b, c, d – progressive vasoconstriction of TA & CV after superfusion of Diltiazem (10⁻⁶).]
contraction at 40 Hz resulted in functional dilatation that was of lesser magnitude and delayed in comparison to control experiments (Figs. 3, 4).

**DISCUSSION**

Over experiments confirmed earlier observations that electrical stimulus of localized groups of skeletal muscle fibres of rat spinotrapezius muscle was followed by dilatation of both arterioles and venules (6, 7).

Functional Dilatation (FD) has been linked to the release of vasodilator metabolites like inorganic phosphate, lactate, K⁺, hyperosmolarity, changes in PO₂ and PCO₂ and pH (7–12). Recently also it was reported that during systemic hypoxia, adenosine released from endothelium, acted upon endothelium at receptors to open KATP channels and resulted in nitric oxide (NO) release which caused vascular smooth muscle relaxation (17).

In the present study low frequency (1 Hz and 3 Hz) stimulation of skeletal muscle led to FD in almost all sections of microvessels. This early dilatation could not be due to 'substantial' accumulation of vasodilator metabolites.

Suzuki et al in a comprehensive study revealed that the local application of Diltiazem on mesentric arteries resulted into depolarization of arterial smooth muscle membrane (18). This can also explain our finding of vasoconstriction after Diltiazem application in skeletal muscle vessels. Diltiazem also prevented the FD response to low frequency stimulation of skeletal muscle. At 40 Hz, although FD was present, it was of late onset and lesser magnitude. This clearly suggests that release of vasodilator metabolites was not the only mechanism involved in FD. It seemed that the absence of FD was closely linked to the blockade of the movement of Ca⁺⁺ ions across the arteriolar and venular walls in response to application of Diltiazem.

On the basis of control and Ca⁺⁺ channel blockade experiments we are able to hypothesize the cause of FD at low twitch and early phase of tetanic contractions as follows:

"Production of single or few twitches in muscle fibres triggers the release of Ca⁺⁺ from terminal cisternae of sarcoplasmic reticulum which initiates muscle contraction. This may be associated with transient decline in Ca⁺⁺ in T-tubules connected to extracellular fluid (ECF) and therefore, eventually produces decline in ECF Ca⁺⁺ as well. The microvessels being surrounded by ECF are very prone to ECF Ca⁺⁺ decline and dilate, as very minute amount of Ca⁺⁺ from their smooth muscle pass out in ECF (19). That is the probable reason how the entire chain of events can simply be stopped by application of Ca⁺⁺ channel blockade, which caused prevention of Ca⁺⁺ entry from ECF to skeletal muscle as Bohle T reported in his study about paralysis of force and Ca⁺⁺ currents in skeletal muscles in the presence of 30 mmol Diltiazem application (20). Thus the ECF Ca⁺⁺ will not decline and Ca⁺⁺ movement from smooth muscle cells to ECF will not take place showing no dilatation. Nevertheless, the tetanic contractions evoked FD despite of the application of diltiazem as although Ca⁺⁺ efflux component is not present but substantial accumulation of vasodilator
metabolites can occur so in total, magnitude of dilatation is less.

This hypothesis can also explain the earliest and maximum FD in terminal arterioles in comparison to secondary and primary arterioles, as the movement of the Ca ++ efflux can be of greater magnitude in TA because muscle layer in them is comparatively very thin.

However, some more sophisticated experiments like estimation of smooth muscle Ca ++ , ECF Ca ++ and sarcotubular Ca ++ levels during muscle contraction are needed to confirm our hypothesis.

REFERENCES