Inherent rhythmcity and interstitial cells of Cajal in a frog vein

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Interstitial cells of Cajal are responsible for rhythmic contractions of the musculature of the gastrointestinal tract and blood vessels. The existence of these cells and spontaneous rhythmicity were noticed in amphibian vein and the findings are reported in this paper. The postcaval vein was identified in the frog, *Rana tigrina* and was perfused with amphibian Ringer solution after isolation. Contractile activity was recorded through a tension transducer connected to a polygraph. The isolated postcaval vein showed spontaneous rhythmic activity. Addition of cold Ringer solution decreased, while warm Ringer increased, the rate of contraction. Adrenaline caused inhibition of rhythmic activity at a dosage that increased the rate of isolated sinus venosus. Sections of the postcaval vein, when stained supravitally with methylene blue, showed the presence of interstitial cells of Cajal. Photic stimulation of the vein in the presence of methylene blue led to a significant decrease in the rate of spontaneous beating of the vein. These findings indicate that the postcaval vein of frog is capable of inherent rhythmcity, which is dependent on the interstitial cells of Cajal but is independent of the sinus venosus.

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1. Introduction

Apart from the heart, several tissues exhibit the property of inherent rhythmicity. On supravital staining with methylene blue, specialized non-contracting cells can be demonstrated in these tissues. These cells are known as the interstitial cells of Cajal (ICC). Tissues that have ICC include the gastrointestinal tract from the oesophagus to the internal anal sphincter (Takaki 2003), urinary tract (Sergeant *et al* 2000; McCloskey and Gurney 2002) and the fallopian tube (Popescu *et al* 2005). ICC form networks that are widely distributed within the submucosal, intramuscular and intermuscular layers of the gastrointestinal tract, and generate spontaneously active pacemaker currents that may be recorded as plateau and slow potentials. These pacemaker currents drive the spontaneous electrical and mechanical activities of smooth muscle cells.

Among the blood vessels, the portal vein (Povstyan *et al* 2003) and mesenteric artery (Pucovsky 2003) show inherent rhythmicity and the presence of ICC. Harhun *et al* (2004) suggest that ICC, in addition to generating an electrical signal and thereby acting as a pacemaker in the rabbit portal vein, may also release some unknown diffusible substance(s), which depolarizes the smooth muscle cells of the blood vessel. While the role of the ICC is fairly well defined in rhythm generation of the gastrointestinal tract (Ward and Sanders 2006), their function in other tissues is being clarified (Huizinga and Faussone-Pellegrini 2005). We noticed that the postcaval vein of the frog, when isolated, exhibits spontaneous contractility and shows the presence of ICC when stained supravitally with methylene blue. Further, our experiments have shown that any physiological or pharmacological manipulation of the ICC affects the rhythm of the postcaval vein, reinforcing the emerging view that ICC may be responsible for the pacemaking activity of many tissues (Huizinga and Faussone-Pellegrini 2005). Demonstration of such activity in an amphibian species indicates that the functional role of ICC might have begun early in the process of evolution. It is hoped that the easy accessibility of frogs coupled with our finding of ICC in the postcaval vein will spur research on understanding the mechanism of impulse generation in these cells.

Keywords. Frog; interstitial cells of Cajal; postcaval vein; rhythmicity

2. Materials and methods

2.1 *Isolation of the postcaval vein*

Frogs (*Rana tigrina*) obtained from the local supplier were used for the study. They were double pithed as per the guidelines of the institutional animal ethics committee (Kasturba Medical College, Manipal, India). Species identification was done by an expert zoologist. The postcaval vein of the frog was identified as per the description provided by Parker and Haswell (1960) and isolated by applying ligatures below the sinus and above the hepatic veins.

2.2 *Recording of the beat*

The isolated tissue preparation was placed in an organ bath and perfused with Ringer solution at 30°C. Contractile activity was recorded through a tension transducer connected to a polygraph (Polyrite, Chandigarh, India). Basal tension was adjusted to obtain an optimal recording and never exceeded 1 g. The sinus venosus was also isolated, perfused and studied in a similar manner. After recording the basal rhythm, warm (33°C) or cold (25°C) Ringer or adrenaline was added and rhythmic activity recorded. In experiments requiring photic stimulation, the bath and transducer were covered by a dark cloth. Light was projected onto the tissue for 5 min through a laser torch (red laser) placed six inches from the tissue. Only the changes in rate were taken into consideration. The changes in amplitude were disregarded. The composition of the amphibian Ringer solution was: 117 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4 and 0.2 mM NaH_2PO_4. The organ bath concentrations of adrenaline and methylene blue were 10^{-8} M and 50×10^{-6} M, respectively.

2.3 *Histology*

For histological studies, the vein was stained supravitally by incubating it in frog Ringer solution containing methylene blue for 2 h with continuous bubbling of air. The tissue was fixed in 10% formalin for 48 h and processed for paraffin sectioning. Sections 5 μ in thickness were cut in a rotary microtome and mounted on gelatinized slides which were dried for 24 h. Sections were counterstained very lightly with 0.1% eosin.

2.4 *Statistical analysis*

All the results were expressed as mean ± SEM of 6 observations. Student *t*-test for paired observations was used to calculate the significance. $P<0.05$ was taken as significant.

3. Results

3.1 *Spontaneous rhythmicity of the postcaval vein*

The isolated postcaval vein was found to beat all by itself. The basal rate varied from 30 to 50 beats per minute (bpm). A typical recording is shown in figure1A. In about 20% of tissues, the rhythmicity was interrupted by periods of rest as shown in figure 1B. Further experimentation was carried out on continuously beating tissues only.

3.2 *Effect of adrenaline on the postcaval vein and sinus venosus*

It was found that application of adrenaline caused an initial reduction (before application 36 ± 4 bpm and after application 22 ± 7 bpm, $P<0.05$) and subsequent stoppage of the beat (figure 2A). In contrast, the same dose caused an increase in the sinus beat (before application 33 ± 4 bpm and after application 40 ± 5 bpm, $P<0.05$) (figure 2:B).

3.3 *Effect of temperature*

Application of warm and cold Ringer caused an increase (before 39 ± 6 bpm and after 66 ± 7 bpm, $P<0.05$) and a

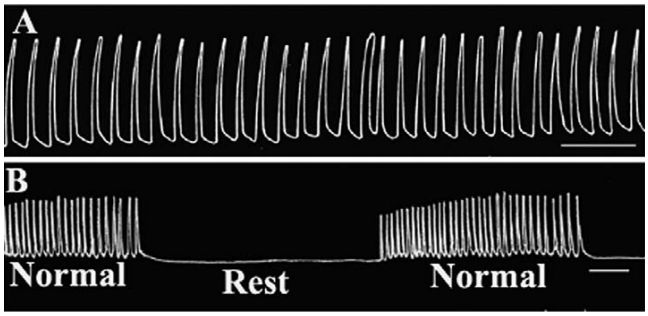

Figure 1. Spontaneous rhythmicity of the postcaval vein (**A**), and its interruption in 20% of tissues (**B**). Note that the postcaval vein beats at a rate of 30–50 bpm. Scale bar = 10 s

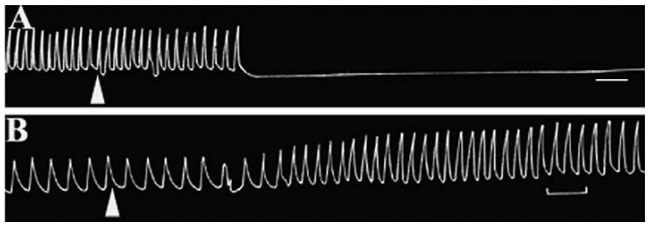

Figure 2. Effect of adrenaline on postcaval vein (**A**), and sinus venosus (**B**). Arrowheads indicate the time of application of the drug. Note that adrenaline stopped the contraction of the postcaval vein, and increased the rate of contraction of the sinus venosus. Scale bar = 10 s

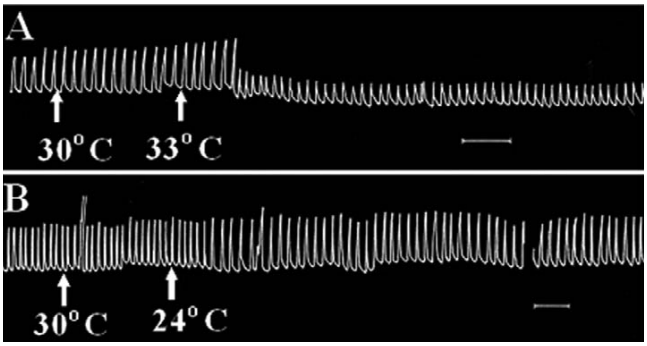

Figure 3. Effect of cold and warm Ringer solutions on postcaval vein and heart rate. Warm Ringer solution increased the rate of the postcaval vein rhythmicity (**A**) and cold Ringer decreased the rhythmicity of postcaval vein (**B**). Arrows indicate the time of application of the warm or cold Ringer solution. Scale bar = 10 sec

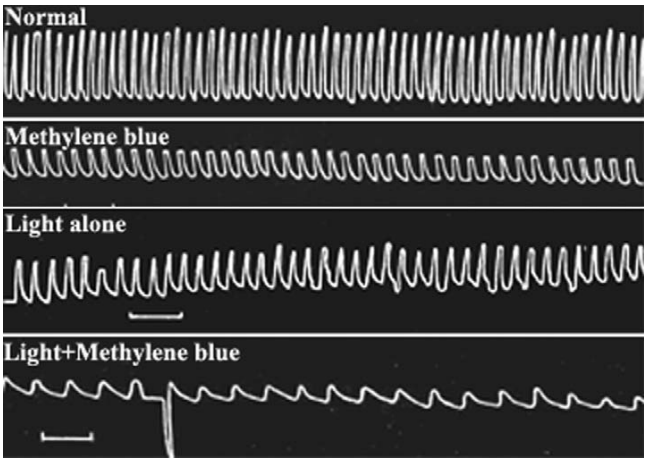

Figure 4. Effect of light and methylene blue on the spontaneous rhythmicity of the postcaval vein.

decrease (before 39 ± 6 bpm and after 25 ± 2 bpm, $P<0.05$), respectively, in the rate of contraction of the postcaval vein (figure 3).

3.4 *Effect of light and methylene blue*

When the spontaneously beating isolated postcaval vein was exposed to either laser light or methylene blue for a period of 5 min, no significant change was observed. But when both these stimuli were applied together for 5 min, a significant reduction in the rate of spontaneous beating was noticed (before 39 ± 6 bpm and after 29 ± 2 bpm, $P<0.05$, figure 4).

3.5 *Histological study*

Sections of the postcaval vein stained supravitally with methylene blue showed the presence of cells resembling

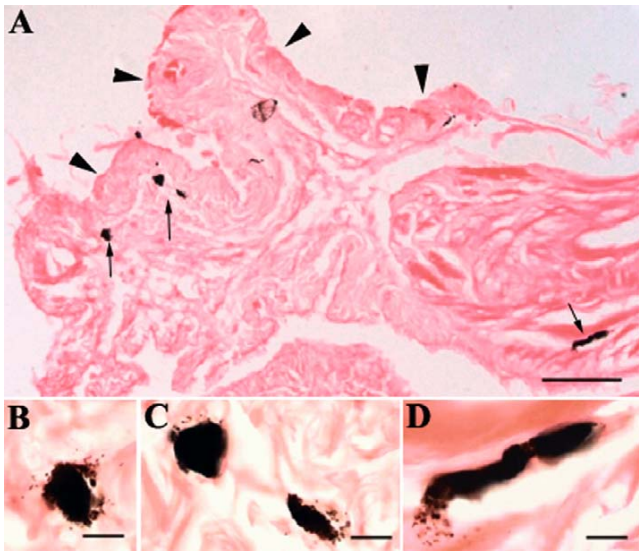

Figure 5. (**A**) Photomicrograph of a cross-section of the postcaval vein of frog stained supravitally with 0.1% methylene blue and counterstained with eosin. Note the presence of interstitial cells of Cajal-like cells (arrows) stained dark blue in the subendothelial region and within the tunica media (lower right arrow in A). Arrowheads indicate the endothelial lining. **B**, **C** and **D** are the magnified views of the cells indicated by arrows in A. Scale bar = 100 μm in A and 20 μm in B, C, D.

ICC in the subendothelial region and tunica media of the vein both in cross-section (figure 5) and longitudinal section (figure 6). In longitudinal sections of the vein, clusters of deeply stained cells could be seen (figure 6). These cells were deeply stained with methylene blue, and were spherical, elongated or fusiform. Their size ranged from 10 to 60 µ. Their processes varied in number and thickness, and ranged from a few microns to 200 µ in length. In longitudinal section there was a thick network of fibrers within the wall of the postcaval vein. They formed a thick bundle between the tunica adventitia and media (figure 7). Some cells did not possess any process. Cellular components were not visible as the cells were darkly stained.

4. Discussion

The ability of the postcaval vein to maintain rhythmic contractions after isolation and perfusion *in vitro* indicates that the rhythm is generated within the tissue. Such an idea is reinforced by the effects brought about by changes in the temperature of the bathing fluid. It is likely that pacemaker activity is induced by the ICC present in the postcaval vein. These cells are responsible for the rhythmic activity seen in the rabbit portal vein (Harhun *et al* 2004). The location and morphology of the cells in frog postcaval vein is similar to the description of the ICC in the blood vessels (Povstyan

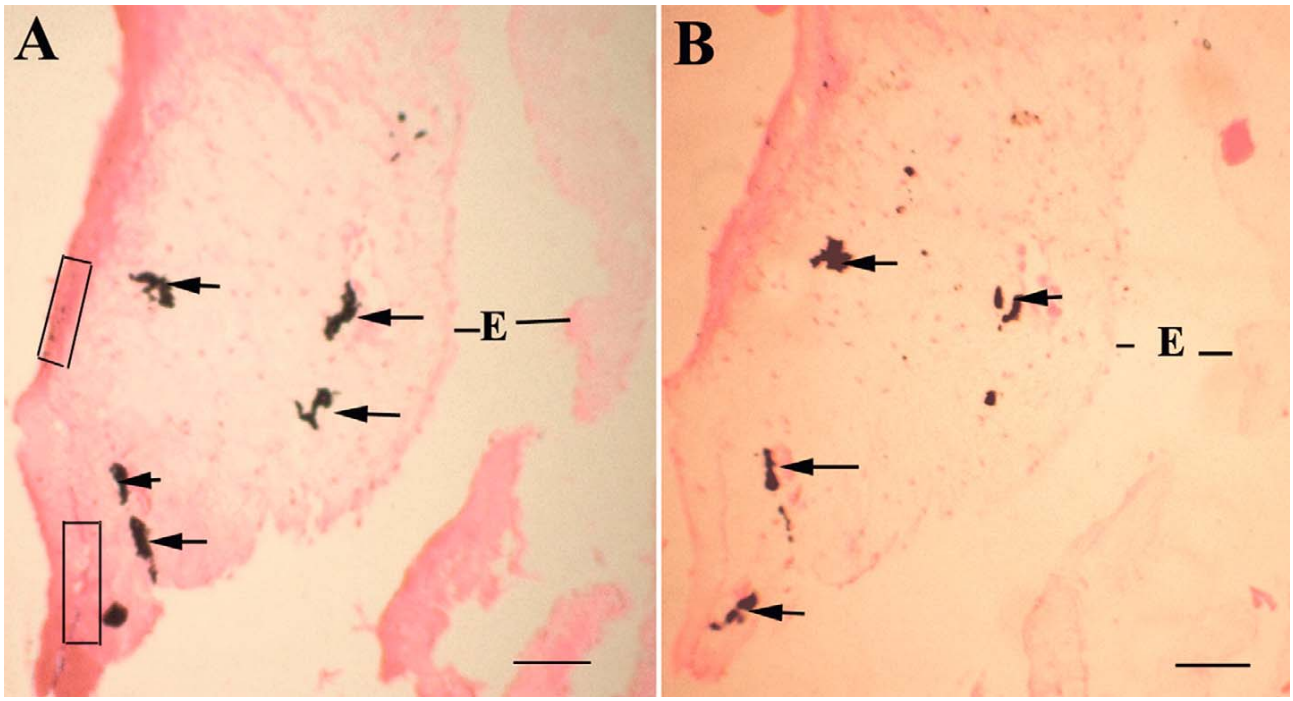

Figure 6. Interstitial cells of Cajal (ICC) (arrows) in longitudinal sections of the postcaval vein. (**A**) and (**B**) are adjacent sections which are 50 μm apart. ICC send their processes throughout the wall of the postcaval vein. They form bundles of such fibres (boxes, magnified in figure 7) between the tunica media and adventitia. Methylene blue stain, counterstained with haematoxylin. E, endothelium. Scale bar = 50 μm

et al 2003; Pucovsky *et al* 2003; Bolton *et al* 2004). Further, ICC have been shown to be susceptible to methylene blue and light (Liu *et al* 1993; Liu *et al* 1994). In our experiments, these two stimuli in combination significantly inhibited the spontaneous contractions of the frog postcaval vein, probably as a result of damage to the ICC.

The differing responses of the postcaval vein and sinus venosus to adrenaline indicate that the activity observed in the postcaval vein is not related to the rhythmic activity of the sinus venosus, the pacemaker of the frog heart. The finding that adrenaline inhibited the rhythmic contractions is similar to that~~ose~~ on rabbit portal vein (Holman *et al* 1968).

The results of studies on guinea pig mesenteric arteries by Pucovsky *et al* (2003) indicate that ICC-like cells can be found in the layer of the tunica media immediately under the basal lamina and are also scattered among the myocytes in the deeper layers of the tunica media. In our study, ICC was found in similar regions in the frog postcaval vein. The localization of ICC in the tunica media and especially in the layer of cells just under the basal lamina raises the possibility that they might act as intermediaries in neural transmission, a role suggested for ICC in the intestine (Ward *et al* 2000).

In one study, ICC in rabbit portal vein were c-kit positive and functioned as pacemakers responsible for electrical and contractile activity, and may be acting as intermediaries between nerve and muscle (Harhun *et al* 2004). ICC observed in the gastrointestinal tract express the antigen *c-kit*, have long processes and a high ability to accumulate methylene blue. They form gap junctions with smooth muscle cells and may serve to mediate signal transmission from the nerves to these cells (Daniel and Wang 1999; Daniel *et al* 2001). Even though c-kit staining was not employed in the present study, the presence of cells with long processes that selectively took up methylene blue strongly suggests that they were ICC. Such long processes have been considered as the platinum standard for ICC (Popescu *et al* 2005). Low-pressure vessels such as veins may require rhythmic mechanical activity to promote the flow of blood. ICC may play a role with their pacemaking capability.

ICC in the canine colon accumulate methylene blue and can be lesioned selectively by exposure to light (Liu *et al* 1993; Liu *et al* 1994). Similar procedures, when applied to the frog postcaval vein, resulted in a significant decrease in the rate of contraction. This observation strongly suggests the possibility that the ICC in the vein are responsible for the rhythm.

In conclusion, our study indicates that the postcaval vein of frog is capable of inherent rhythmicity and shows the presence of ICC, which may act as pacemakers responsible for the rhythm. However, we do not have any explanation for the periodic interruptions in rhythmicity observed in 20% of samples of the postcaval vein.

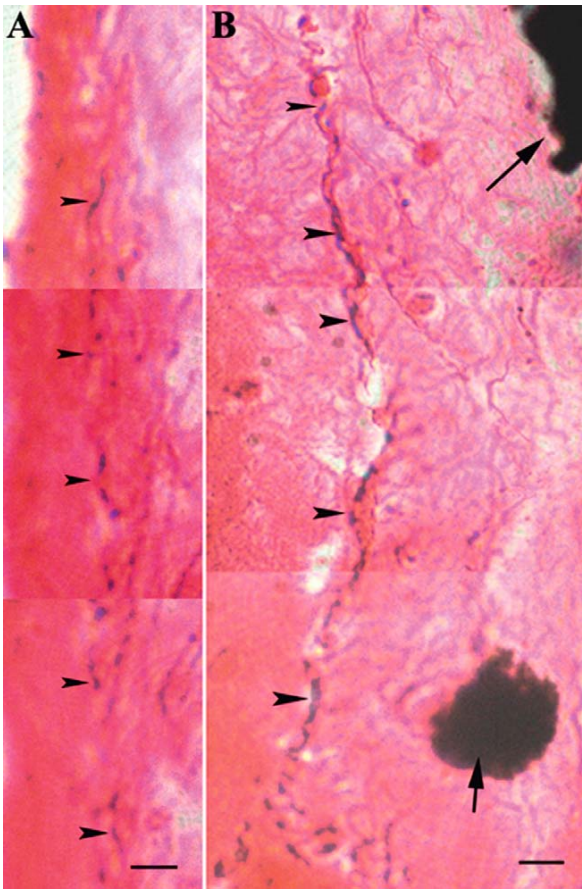

Figure 7. Magnified view of the two fields shown in figure 6. The interstitial cells of Cajal (arrows) send their processes throughout the wall of the postcaval vein. They form bundles of such fibres (arrowheads) between the tunica media and adventitia. Methylene blue stain, counterstained with haematoxylin. Scale bar = 20 μm

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