

**The pharmacology of buccal mass smooth muscle of *Arion ater***

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**ABSTRACT** A pharmacological investigation into the buccal mass of the common terrestrial slug, *Arion ater*, on several occasions the buccal mass smooth muscle, at 10°C, showed irregular spontaneous rhythmical activity. These natural activities were reduced at 5°C. This suggest that low temperature may have an adverse effect on the slug muscular activity. High K<sup>+</sup>-saline resulted in tension development in the smooth muscle and small twitches were seen at low K<sup>+</sup>-concentrations. In Ca<sup>2+</sup>-free saline, the responses were reduced indicating the dependency on [Ca<sup>2+</sup>]<sub>o</sub> for contraction. ACh, in this study, was found to be an up regulatory transmitter and at 5x10<sup>-6</sup> M ACh-elicited phasic twitch activity. Both [Ca<sup>2+</sup>]<sub>o</sub> and [Na<sup>+</sup>]<sub>o</sub> were essential for ACh to induce contracture. The mammalian Ca<sup>2+</sup>-channel blockers verapamil and diltiazem reduced ACh-responses. Suggesting that membrane depolarization was dependent on Na<sup>+</sup>-ions, whereas Ca<sup>2+</sup> was needed for activation of the contractile apparatus.

## **INTRODUCTION**

*Arion ater*, is a common temperate terrestrial slug. The reduction of this slug shell lessened the need for calcium salts and an increased tolerance of water loss means that slug can live in a wider range of habitats than most snails (South, 1992). The buccal mass of *Arion ater ater*, the large black / brown slug with characteristic foot fringe was used in this study. This slug is herbivorous, feeding on decaying vegetable matter and growing plants, often becoming a pest of agricultural and horticultural crops, or in the garden (Runham & Hunter, 1970).

The buccal mass is a complex globular organ lying in the head of the animal. It is

thought to house 28 known muscles, all of which are associated with feeding. Moreover, the buccal ganglia are connected, *via* a buccal connective and nerves running from the buccal ganglia to the buccal mass musculature, the radula, the salivary glands and the oesophagus (Smith, 1990).

Most smooth muscles show acute dependence upon  $[Ca^{2+}]_o$  for the maintenance of contractile activity (Huddart & Hill, 1988). Contraction is initiated by an inward flux of  $Ca^{2+}$ -ions, often associated with  $Ca^{2+}$ -dependent action potentials (Syson & Huddart, 1976; Brading *et. al.*, 1983). This signal may subsequently release stored  $Ca^{2+}$ , leading to muscle contraction from the activation of the actin / myosin contractile filaments of the muscle cell.

It is known that molluscan muscle contains more  $[Ca^{2+}]_i$  than vertebrate smooth muscle, but less than skeletal muscle (Huddart *et. al.*, 1977). Thus, it appears that  $[Ca^{2+}]_i$  storage sites are present but the heavy dependence on  $[Ca^{2+}]_o$  suggests that these stores need constant repriming from the external medium.

Several neurotransmitter substances have been identified from the pulmonate nervous system. Acetylcholine (ACh) has been shown to be both excitatory and inhibitory between neuron in the pulmonate central nervous system (Langton & Huddart, 1987 & 1988; South, 1992). ACh is the principle neurotransmitter in molluscs, there are two types of mammalian ACh receptor (AChR) can be found; the nicotinic (AChN) and muscarinic (AChM) types (Wright *et. al.*, 1996). The action of ACh was investigated in this study.

Verapamil and diltiazem are clinically used antiarrhythmic and antihypersensitive drugs whose primary action on mammalian cardiac and smooth muscle is the blocking of membrane ion channels which conduct a slow inward  $Ca^{2+}$  current (Devlin, 1993). These drugs have recently been shown to have interesting effects on molluscan cardiac (Devlin, 1993) and smooth muscle (Huddart *et. al.*, 1990a & b). Their function in this study was to demonstrate the dependence of molluscan smooth muscle on external effect  $Ca^{2+}$ -ions for contraction.

Wright *et al.* (1996) concluded that  $K^+$ -depolarization and ACh stimulated muscular contraction of buccal mass smooth muscle of *Deroceras reticulatum*. These responses were partly dependent upon  $[Ca^{2+}]_o$ . Moreover,  $Ca^{2+}$ -blocker agents, verapamil and diltiazem, gave a variety of responses, indicating differences between the mammalian and molluscan  $Ca^{2+}$ -channels which supply activating  $Ca^{2+}$  to the contractile apparatus.

The aim of this study is to test the possibility of interspecific similarities involving the pest *Arion ater ater*, which has significant agricultural implications. This study also hope to find a role for nervous feeding control in this animal, which is a step closer in tackling the problem of their control in the environment. In addition, is to identify the possible neurotransmitter involved in the control of buccal muscle, and attempts to elucidate their methods of action.

## MATERIALS AND METHODS

**Experimental animals:** A breeding population of *Arion ater ater* was established and they were housed in plastic containers lined with moist compost, and fed on lettuce, carrot and wheat seeds. Only healthy and active slugs were selected for dissection.

**Artificial saline preparation:** The formula of the artificial saline was complied by Gibson and Logan (1992), and was composed of as follows (g / l): NaCl 5.2, KCl 0.3,  $CaCl_2 \cdot 2H_2O$  1.03,  $Mg_2SO_4$  (Anh) 0.476, Tris-HCl 0.6, Glucose 1.8, adjusted to pH 7.5 at room temperature using NaCl and HCl. The saline could be stored for up to one week at 0 - 5°C. To prepare  $Ca^{2+}$ -free and  $Na^+$ -free salines,  $CaCl_2 \cdot 2H_2O$  and NaCl were replaced respectively with the equivalent amount of choline chloride, as follows (g / l): 1.96, 12.43.

**Test drugs:** The test drugs used in this study, were as follows: KCl,  $Ca^{2+}$ -free saline,  $Na^+$ -free saline, ACh, verapamil and diltiazem. These drugs were obtained from Sigma Chemical Company and were prepared freshly from stock solutions. They were added to the organ bath at known amount of stock solutions to the organ bath. After each test, the saline was drained and

replaced with fresh saline from an overhead reservoir.

**Dissection:** The dissection and ligature the buccal mass was after Bullough (1950) and Smith (1990) respectively. After selection the active slugs were anaesthetized with cold (in the fridge ~ 4°C) for 3 - 5 minutes, then cut along the dorsal mid line and pinned open. The buccal mass was extracted by severing the associated nerve ganglion and oesophagus, also by cutting around the mouth opening. During dissection and prior to mounting the preparation was kept moist with artificial saline.

**Preparation mounting:** Mounting the buccal mass in the organ bath was after Hill and Huddart (1995) and Wright *et. al.* (1996). Once removed, the buccal mass was ligature with cotton around the mouth opening from one end, and around the radula sac from the other end. Then was suspended in the 10 ml organ bath. This allowed the tissue to be held under tension in the organ bath but still gave scope for contraction. The ligature hook was attached to the plate of force displacement transducer, which detects changes in tension. Once in place the buccal mass was left to equilibrate in the bath for 30 minutes. This was essential for the tissue to become accustomed to the new environment prior to undertaking tests.

**The apparatus and experimental routines:** For muscle tension recording, the tissue is connected through an amplifier to a four channel Grass Instrument model 79D polygraph and Grass FT 0.03 force-displacement transducers, which records contractile force as a curvilinear ink trace. An overflow system is present to finely adjust the bath volume to 10 ml. The saline is drained by gravity with the aid of a water pump. The organ bath was maintained at 10°C with a Grant closed circuit cooler / circulatory system and the organ bath contents were gently constantly aerated. Each result was a replicate of 8 experiments, run for 20 to 30 minutes and followed by 20 minutes washing period with normal saline.

## RESULTS

On several occasions buccal mass smooth muscle, at 10°C, showed irregular spontaneous rhythmical activity. These natural activities were reduced at 5°C (Figure 1a & B). An increase in external  $K^+$  (Figure 2a) led to an increase in smooth muscle tonic force in a dose-dependent manner. Concentration as low as 10 mM  $K^+$  evoked small contractile force development, and minimal twitch activity was seen. Peak tension was achieved at 60 mM  $K^+$  and further increase in external  $K^+$  had no additive effect. Figure 2b, shows the fact that the  $K^+$ -induced contracture is dependent on  $[Ca^{2+}]_o$  with the  $K^+$ -response in  $Ca^{2+}$ -free saline being reduced. Similarly, this was seen with ACh-induced contracture (Figure 3a), while  $Ca^{2+}$ -free saline reduced the ACh-responses (Figure 3b). These contractures were also highly dependent on  $[Na^+]_o$ , shown by the response in  $Na^+$ -free saline being further reduced (Figure 3c).

The use of the mammalian  $Ca^{2+}$ -channel antagonists, verapamil and diltiazem supported these results. However, the ACh-induced response was strongly reduced by diltiazem than with verapamil, so these blockers are not as effective in molluscan smooth muscle as they are in mammalian. In both cases contractile force was decreased and diltiazem suppressed twitch activity (Figure 4a & b).

## DISCUSSION

The result of this study shows that the buccal mass of *Arion ater*, at 10°C pronounced irregular spontaneous rhythmical activity. These natural activities were reduced at 5°C, which may suggest that low temperature may reduce muscular activity. South (1992) reported that the development of terrestrial slug eggs takes longer in the winter because of the unfavorable temperature. He also suggested that low development and growth temperature are likely to have an adverse effect on the overall behavior of the slug and the activity of the buccal mass musculature.

The artificial increase in  $[K^+]_o$  caused a dose-dependent increase in tonic force. The

consequential  $K^+$ -influx and decrease of  $K^+$ -efflux from the muscle cells led to the disruption of the resting membrane potential (Langton & Huddart, 1987 & 1988). This resulted in depolarization of the membrane, leading to the opening of the voltage operated  $Ca^{2+}$ -ion channel. The resulting  $Ca^{2+}$ -influx along the concentration gradient caused activation of the cells contractile apparatus, demonstrating classic excitation-contraction (EC) coupling (Langton & Huddart, 1987; Huddart *et. al.*, 1990a & b; Wright *et. al.*, 1996).

The result of this study showed that  $K^+$ -responses were reduced in  $Ca^{2+}$ -free saline signifies the dependence of the buccal mass muscle on  $[Ca^{2+}]_o$ . This reduction could be due to depolarization prevention or because of the lack of available  $Ca^{2+}$  to induce contraction. Sucrose-gap studies by Huddart and Hill (1988 & 1996) showed that the level of depolarization in  $Ca^{2+}$ -free saline is not altered although the tension development nearly abolished. Therefore, the latter explanation seems more likely. Huddart *et. al.* (1977) reported that  $K^+$ -contractures and ACh-induced responses were reduced in  $Ca^{2+}$ -free saline, which indicates the presence of  $[Ca^{2+}]_i$ .

In this study, ACh elicited a dose-dependent increase in contractile force with phasic twitch activity superimposed at lower concentrations. ACh-induced responses were dependent on  $[Na^+]_o$  and  $[Ca^{2+}]_o$ , with a reduction in tension seen in  $Na^+$ -free and  $Ca^{2+}$ -free saline. This suggests a role for  $Na^+$  in membrane depolarization and subsequent  $Ca^{2+}$ -ion channel opening, supported by voltage clamping and sucrose-gap studies (Ram *et. al.*, 1991). In addition, this demonstrate that the depolarization event created by  $Na^+$ -influx in *Aplysia* buccal mass was shown to be great enough to cause the opening of voltage-dependent  $Ca^{2+}$ -channels (Orkand & Orkand, 1975; Ram *et. al.*, 1991).

Sucrose-gap studies, involving *Busycon* radular retractor and atrium muscle, also showed that in  $Na^+$ -free media ACh-induced depolarization decreased dramatically, as did contracture. In  $Ca^{2+}$ -free media, however, depolarization remained unaltered, or even increased,

but contractile force was greatly reduced. It is most likely, therefore, that  $\text{Na}^+$ -influx leads to membrane depolarization, whereas  $\text{Ca}^{2+}$  is required for electromechanical coupling (Hill & McDonald-Ordzie, 1979; Huddart & Hill, 1996).

The phasic twitch activity seen at lower ACh-concentrations indicates the presence of two voltage dependent  $\text{Ca}^{2+}$ -ion channels, one fast and one slow. Twitch activity appears to occur from  $\text{Ca}^{2+}$ -influx through the fast transmembrane channel, then as the cell becomes further depolarized the slow one takes over, the flow of  $\text{Ca}^{2+}$ -ions through which is possible for increased tension (Langton & Huddart, 1987 & 1988).

In this study, ACh-induced response was strongly reduced by diltiazem than with verapamil, so these blockers are not as effective in molluscan smooth muscle as they are in mammalian. In both cases contractile force was decreased and diltiazem suppressed twitch activity. Verapamil and diltiazem, the mammalian slow  $\text{Ca}^{2+}$ -channel antagonists, appeared to be active antagonists of ACh-induced  $\text{Ca}^{2+}$ -release pathway for tonic force development in *Arion ater*. They had no effect alone when added to the saline and so appear to have a similar role in *Arion ater* smooth muscle as in mammals. However, their excitatory effects on other molluscan smooth muscle, especially with nifedipine, Huddart and Hill (1988) show that  $\text{Ca}^{2+}$ -antagonists have no consistent actions on molluscan smooth muscle, indicating that the  $\text{Ca}^{2+}$ -channel activity of the buccal mass may differ from that seen in mammalian visceral muscle. A high degree of  $\text{Ca}^{2+}$ -channel variation is therefore thought to exist within the Animal Kingdom. Using  $\text{Ca}^{2+}$ -channel agonists and antagonists suggests that  $\text{K}^+$  and ACh act on the same intracellular  $\text{Ca}^{2+}$ -pool but by different membrane transduction routes (Huddart *et. al.*, 1990a & b).

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### FIGURE LEGENDS

**Figure 1.** Show natural activity of buccal mass smooth muscle. (a) normal spontaneous activity recorded at 10°C, while in (b) it natural activity recorded at 5°C. Note, the reduction in muscle activity. The force and time calibrations were standardized for each experiment reported in all experiments as indicated on the right hand side of the Figures.

**Figure 2.** (a) Show cumulative K<sup>+</sup>-addition, in artificial saline, on buccal mass (upper arrows) induced tonic-response, while (b) show reduction in this K<sup>+</sup>-response in presence of Ca<sup>2+</sup>-free saline. Lower arrow indicated washout with normal saline. The force and time calibrations were standardized for each experiment reported in all experiments as indicated on the right hand side of the Figures.

**Figure 3.** (a and c) Effect of control ACh-responses ( $5 \times 10^{-6}$  M) (upper arrow) of buccal mass muscle. (b) Same ACh-response (second arrow) was reduced in presence of Ca<sup>2+</sup>-free saline (first arrow). (d) Show reduction in ACh-response (second arrow) in presence of Na<sup>+</sup>-free saline (first arrow). Lower arrow indicated washout with normal saline. The force and time calibrations were standardized for each experiment reported in all experiments as indicated on the right hand side of the Figures.

**Figure 4.** (a and c) show the effect of control ACh-response ( $5 \times 10^{-6}$  M) (upper arrow) of buccal mass muscle. (b) Verapamil addition (first arrow) reduced ACh-response (second arrow), while in (d) diltiazem addition (first arrow) inhibited ACh-response (second arrow). Lower arrow indicated washout with normal saline. The force and time calibrations were standardized for each experiment reported in all experiments as indicated on the right hand side of the Figures.

## دراسة أثر بعض الأدوية على العضلات الملساء للتجفيف الفمي للبزاق أريون آثر

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الملخص:

أظهرت نتائج دراسة أثر بعض الأدوية على العضلات الملساء للتجفيف الفمي للبزاق أريون آثر أن الانقباضات الطبيعية قد ثبتت عندما انخفضت درجة الحرارة. هذه الدراسة تقترح بأن درجة الحرارة قد قللت من النشاط الأيضي لهذه العضلات. وعند إضافة محلول كلوريد البوتاسيوم بصورة متتالية، وجد أنها قد أحدثت انقباضة عضلية واضحة، مع ظهور انقباضات صغيرة متسارعة. ألا أن هذه الانقباضة العضلية ثبتت في غياب أيونات الكالسيوم؛ كما أن درجة هذا التثبيط زادت عند استخدام محلول خالي الصوديوم. هذه الدراسة تقترح اعتماد الانقباضات الطبيعية والانقباضات العضلية المستحثة بالبوتاسيوم على أيونات الكالسيوم الخارجية.

هذه الدراسة أشارت بأن إضافة محلول الأستيل كولين قد أحدث انقباضة كبيرة مع ظهور انقباضات صغيرة متسارعة. كما أنها أثبتت أن هذه الانقباضات المستحثة تعتمد في ظهورها واستمرارها على أيونات الكالسيوم والصوديوم الخارجية. كذلك وجد أن استخدام مثبطات قنوات الكالسيوم، الفيراباميل والديلتايزيم، قد ثبطوا الانقباضات المستحثة بمادة الأستيل كولين. هذه الدراسة أستنتجت أن قطبية الغشاء الخلوي تعتمد على وجود أيونات الصوديوم الخارجية، في حين أن أيونات الكالسيوم الخارجية مهمة في أحداث الانقباضة العضلية.