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MECHANISM OF MECHANICAL INHIBITION OF SMOOTH MUSCLE BY OUABAIN

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1 Ouabain (10 μg/ml) caused an initial increase followed by a decrease in tension in guinea-pig taenia coli bathed in normal sodium and potassium containing medium.

2 The excitatory effect of ouabain was prevented by the elevation of extracellular potassium while the inhibitory effect was abolished in the absence of sodium in the bathing medium.

3 In the presence of sodium, the inhibitory effect of ouabain disappeared when the drug was washed out, but in the presence of lithium (which is not extruded by the Na-K pump) the inhibition was not abolished.

4 The inhibitory effect of ouabain was accompanied by an increase in tissue Na and a decrease in tissue K concentration. The increase in intracellular [^{45}Ca] which occurred when extracellular [K] was varied, was also reduced by ouabain.

5 It is concluded that a high intracellular level of sodium inhibits contractions in visceral smooth muscle probably by a mechanism involving decreased Ca influx.

Introduction

The effect of cardiac glycosides on smooth muscle is inconsistent. In some multi-unit vascular smooth muscles ouabain produces an increase in tension (Cushny, 1925; Leonard, 1957; Brockaert & Godfraind, 1973) but in single unit smooth muscle various workers (Schatzmann & Ackermann, 1961; Godfraind & Godfraind De Becker, 1963; Daniel, 1964; Matthews & Sutter, 1967; Griffin, Szaro & Weltman, 1972) have found that initial excitation is followed by inhibition. The mechanism of the inhibitory action of ouabain is not clearly understood. In the present paper an attempt has been made to test whether inhibition of the sodium pump by ouabain is responsible for its inhibitory effect on the guinea-pig taenia coli. A preliminary account of this work has already been published (Bose, 1974).

Methods

Adult guinea-pigs of either sex were exsanguinated after stunning. Strips of taenia coli (resting length of 2.5 cm) were dissected out and suspended in a 'normal salt solution' (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Tris-HCl 10, glucose 11. The solution was bubbled with O_2 and the pH adjusted to 7.3 by addition of 10 N HCl. The strips were attached to Grass FT-03C force displacement transducers and subjected to a resting tension of 0.5 grams. The strips were made to contract by adding KCl (124.1 mM) to the bathing medium (hypertonic KCl medium). In some experiments all the NaCl was replaced by an equimolar amount of KCl (Na-free KCl medium). 'Hypertonic Na-free KCl medium' was made by replacing NaCl with 236 mM sucrose and adding 118 mM KCl. 'Li-medium' was made by replacing NaCl with 118 mM LiCl and adding 118 mM KCl.

Uptake of sodium by tissues was measured in two different ways:

(a) Strips were mounted on stainless steel holders and incubated in the normal salt solution for 1 h at a resting tension of 0.5 g; 0.6 μCi of ^{22}Na (New England Nuclear Corp.) was added to the medium and at 15 min intervals the tissues were taken out and rinsed for 2 s twice in non-radioactive medium. Radioactivity was immediately counted for 1 min in a γ spectrometer and the tissues were then returned to the radioactive medium. The experiment was arranged so that the tissues never stayed outside the bathing medium for more than 90 seconds. Some drying of the tissue was inevitable but parallel experiments showed that this did not appreciably affect the reactivity of the muscle to KCl or to ouabain. After the ^{22}Na content of the tissues reached a steady level the solution was replaced by hypotonic KCl medium. After 15 min ouabain was added to the bath and the ^{22}Na level in the tissue was measured at 15 min intervals for 30 minutes. After the tissues were returned to the normal ^{22}Na

medium recovery from the effect of ouabain was observed every 15 min for 45 minutes. In parallel experiments the effect on tension was observed. The radioactivity results were expressed as a ratio of ct $min^{-1} g^{-1}$ tissue wet weight: ct $min^{-1} ml^{-1}$ medium.

(b) Tissue content of Na and K was measured by flame photometry. Inulin space was measured after incubating the tissues for 30 min with inulin-[methoxy ^{14}C] (New England Nuclear Corp.). The tissues were blotted and weighed and the inulin was extracted by boiling in 1 ml distilled water for 20 min in a polypropylene test tube. A glass marble placed over the mouth of the test tube prevented loss of water vapour. An aliquot of this aqueous extract was mixed with scintillation fluid (Bray, 1960) for measuring radioactivity due to inulin and the rest was used for measuring Na and K. Aliquots were also taken from the incubation media for measuring radioactivity and Na and K concentrations. Total-issue water was determined by first measuring the wet weight and then measuring dry weight after drying *in vacuo* for 12 h at 80° C. The intracellular concentrations have been expressed as mmol/litre intracellular water and were calculated according to Boyle, Conway, Kane & O'Reilley (1941).

^{45}Ca uptake was measured by first incubating three sets of tissues in the normal salt solution (phosphate omitted). One group of tissues was then maintained in the normal medium. The other two groups of tissues were incubated in 'hypertonic KCl medium' (phosphate omitted) and one of these was also exposed to ouabain. ^{45}Ca (3 μCi/ml) was added to all the media and after 45 min 10 mM lanthanum chloride was added. After 5 min all muscles were then transferred to fresh medium of the same composition except that it lacked calcium and was non-radioactive. Phosphate was omitted in order to avoid interaction with lanthanum. The tissues were blotted, weighed and solubilized with NCS (ICN). Radioactivity in the tissues and in an aliquot of the medium was measured by liquid scintillation counting. Results are expressed as ratio of ct $min^{-1} g^{-1}$ tissue wet weight: ct $min^{-1} ml^{-1}$ of medium. This method gives an approximate estimate of intracellular Ca and is based on the assumption that lanthanum displaces all the extracellular Ca as well as trapping the intracellular Ca (van Breeman, Farinas, Gerba & McNaughton, 1972). Errors may result from intracellular penetration of lanthanum and a differential effect on influx and efflux of Ca, but these errors are probably similar in ouabain-treated and normal tissues and have been assumed not to invalidate comparisons between the two.

Ouabain (Nutr. Biochem. Corp.) was made up freshly as a 10 mg/ml solution in distilled water and the final concentration in the bath was always 10 μg/ml.

Statistical analysis of the data was done by either the paired of the unpaired *t*-tests (Steel & Torrie, 1960).

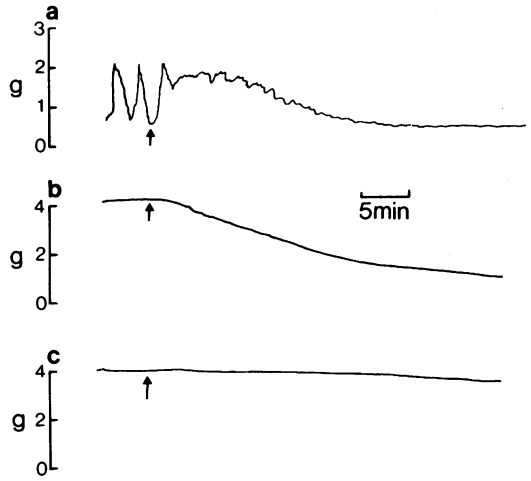

Figure 1 Isometric tension in guinea-pig taenia coli. Ouabain 10 μg/ml added at arrows. (a) Response in normal sodium and potassium containing medium. (b) Response after 15 min exposure to solution containing high potassium (KCl 124.1 mM) and normal sodium. (c) Response in presence of high potassium (KCl 124.1 mM) after exposure to sodium-free (replaced by sucrose) medium for 30 minutes.

Results

Effect of ouabain on resting tension

Ouabain (10 μg/ml) caused an initial increase in the tension of spontaneously active strips of guinea-pig taenia coli in the normal salt solution. The peak increase in tension due to ouabain (3.75 ± 0.3 g; $n = 10$) was similar to the peak amplitude of a spontaneous contraction. The increase in tension due to ouabain reached a maximum in 5 min and then gradually subsided in 15 min, and could be distinguished from the much briefer (< 2 min) duration of spontaneous contractions (Figure 1a).

Effect on depolarized taenia

Increasing the external potassium concentration to 124.1 mM without altering normal sodium concentration (hypertonic KCl medium) resulted

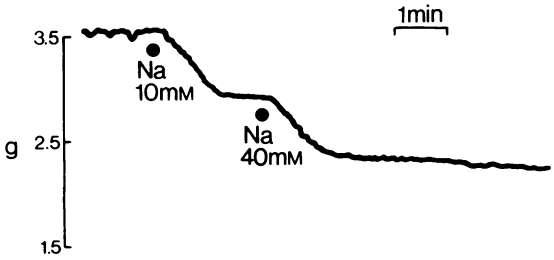

Figure 2 Effect of increasing sodium concentration to 10 and 40 mM (as indicated) on a muscle exposed to Na-free KCl medium and ouabain (10 μg/ml).

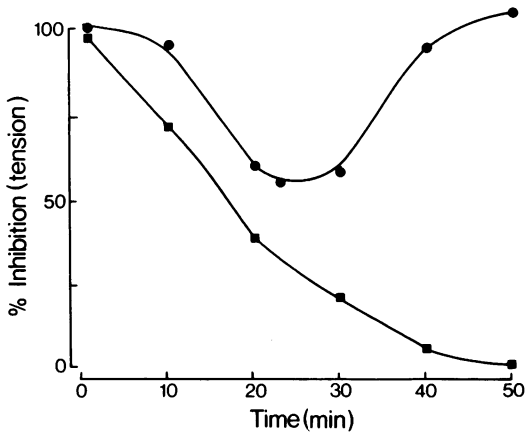

Figure 3 Effect of washing out ouabain (10 μg/ml) with normal sodium medium (•) or sodium-free lithium substituted medium (▪) (both containing 124.1 mM KCl) on tension in taenia coli exposed to a sodium-free (sucrose substituted) medium containing 124.1 mM KCl for 30 min followed by ouabain for 20 minutes.

in a sustained increase in tension (4.45 ± 0.25 g; $n = 8$). Addition of ouabain then caused a gradual decrease in tension to 12 ± 3.2% of the pre-ouabain level in 32 ± 3 minutes. The depolarized taenia did not exhibit any initial increase in tension when ouabain was added, in contrast to the normally polarized preparation (Figure 1b). When ouabain was washed out in the presence of the hypertonic KCl medium the tension returned to the previous level in 45-90 minutes.

Similar experiments were done with Na-free KCl medium. This solution also caused a sustained increase in tension of the taenia to a level similar to that in the hypertonic KCl medium. Addition of ouabain caused the tension to decrease only to 68 ± 6% ($n = 8$) of the pre-ouabain level, which was a significantly smaller effect than occurred in the presence of sodium in the medium ($P < 0.01$). Similar results were obtained in a hypertonic Na-free KCl medium (Figure 1c). Exposure to the Na-free media for less than 20 min failed to inhibit the action of ouabain. These results indicate that the stimulant effect of ouabain was not seen in the depolarized muscle whereas the inhibitory effect required the presence of extracellular sodium.

Effect of restoring sodium or substitution with lithium

Taenia coli strips were first exposed to Na-free KCl solution for 30 min as described in the preceeding section. The presence of ouabain in the bath for 30 min had a very small effect. If NaCl was then added to the bath cumulatively to attain concentrations of 10 and 40 mM, the tension decrease was 12.5 ± 0.5% and 20 ± 1.8% ($n = 6$) respectively (Figure 2). The relaxation seemed to occur more rapidly than that due to ouabain in the K-depolarized or normal preparation. This could be because the effect of ouabain on the sodium pump was already established before restoration of sodium.

Since the inhibitory effect of ouabain occurred only in the presence of extracellular sodium it seemed possible that the inhibition caused by ouabain was due to intracellular accumulation of sodium. To test this hypothesis the following experiments were done:

Eight taenia strips were first exposed for 30 min to hypertonic Na-free KCl medium and then ouabain was added. After 20 min, by which time the normal effect of ouabain is established the ouabain was washed out and the bathing media of 4 strips were replaced by hypertonic KCl medium (containing Na) and of the remaining strips by the lithium medium. The muscles exposed to hypertonic KCl medium relaxed at first but after 25 min the tension returned towards normal. The muscles kept in lithium medium also relaxed but their tension never recovered (Figure 3). Similar results were also obtained if the muscles were exposed to the lithium medium without prior exposure to ouabain ($n = 3$). This was expected because lithium is not pumped out of the cell after it gets in. On the other hand, sodium is expected to be pumped out of the cell as ouabain is washed out and the Na-K pump recovers.

Effect on ionic composition of taenia

^{22}Na influx Six strips of taenia coli were incubated for 30 min in the normal salt solution

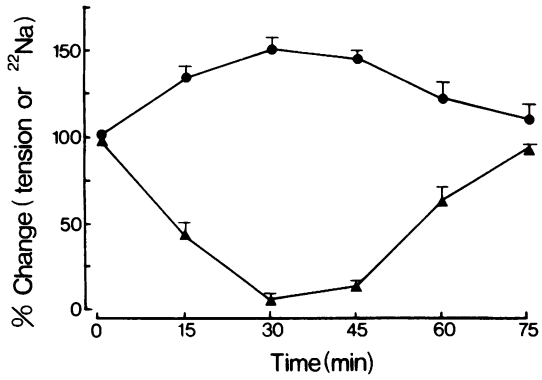

Figure 4 Effect of ouabain (10 μg/ml) on total tissue ^{22}Na (●) and tension (▲) in taenia coli exposed to hypertonic-KCl medium and ^{22}Na for 30 minutes.

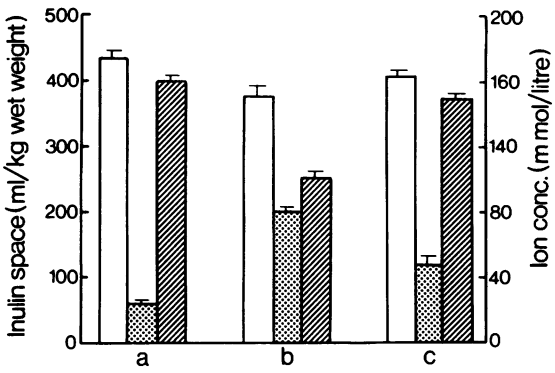

Figure 5 Effect of ouabain on inulin space (open columns), intracellular [Na] (stippled columns) and [K] (cross-hatched columns) concentrations of taenia coli exposed to the normal medium. (a) Control. (b) 30 min after ouabain (10 μg/ml). (c) 30 min after ouabain (10 μg/ml) followed by removal of ouabain for 45 minutes.

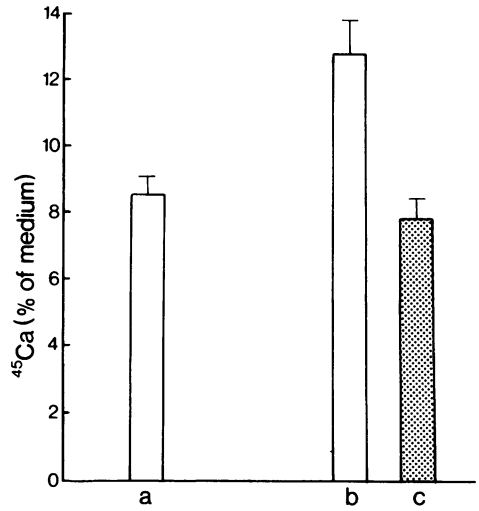

Figure 6 Intracellular accumulation of ^{45}Ca in taenia coli exposed to (a) normal, (b) hypertonic KCl medium, or (c) hypertonic KCl + ouabain, 10 μg/ml.

containing ^{22}Na. Na exchange was essentially complete by this time.

The KCl concentration was now increased to 124.1 mM. On adding ouabain to the bathing medium the ^{22}Na content increased to 130 ± 6.3% and 152 ± 6% of control after 15 and 30 min respectively. At the same time the tension declined to 43.8 ± 6.4% and 6.3 ± 2.5% of control respectively. After washing ouabain out the ^{22}Na level decreased to 146.9 ± 4.4%, 123.7 ± 8.8%, and 113.3 ± 10% after 15, 30 and 45 min respectively. The corresponding tensions were 15 ± 2.5%, 65 ± 7.5% and 96.52 ± 1.9% of control (Figure 4).

Intracellular Na and K concentrations Eighteen strips of taenia coli were incubated in the normal salt solution for 60 minutes. They were then divided into 3 groups. Group A stayed in the normal medium for 30 minutes. Groups B and C were exposed to ouabain for 30 minutes. After this period tissues in Groups A and B were removed for analysis while tissues in Group C were returned to the normal medium for 45 min to wash ouabain out. The results (Figure 5) show that ouabain caused significant ($P < 0.01$) increase in intracellular Na and decrease in intracellular K concentration. Inulin space also decreased slightly ($P < 0.05$). These effects were reversed partially on washing ouabain out. The ratio of dry to wet weight of the tissues did not differ significantly in the 3 groups and were 0.20 ± 0.01, 0.205 ± 0.02 and 0.19 ± 0.02 in groups A, B and C respectively.

Tissue ^{45}Ca content Ouabain significantly decreased the ^{45}Ca influx in taenia coli caused by increasing the potassium concentration (Figure 6).

Discussion

The initial increase in tension in the guinea-pig taenia coli caused by ouabain is associated with depolarization of the membrane along with an increase in spike frequency. Subsequently tension decreases even though membrane depolarization is maintained (Casteels, 1966). This suggests that the

secondary relaxation of the taenia due to ouabain occurs by a non-electrical mechanism. Casteels (1966) suggested that the mechanism of relaxation is a depolarization block. This seems unlikely because depolarization due to high-potassium leads to a sustained increase of tension in the taenia (Imai & Takeda, 1967). Griffin *et al.* (1972) suggested that depolarization prevented the inhibitory effect of ouabain. These authors did not consider the role of absence of sodium in their depolarizing solution. The results of the present experiments suggest strongly that it is not depolarization but the possible intracellular accumulation of sodium that is responsible for the inhibitory effect of ouabain. The lack of inhibitory effect of ouabain in the absence of external sodium both in the presence or absence of hypertonicity is not an artefact of an abnormal contractile mechanism or cell volume change. Tension produced under such conditions is associated with active state and can be reduced, (more slowly than usual) by removal of extracellular calcium (unpublished observation). The same explanation may be applicable for the inhibitory effects of K-free medium (Griffin *et al.*, 1972, unpublished observations) since it is well known that absence of external potassium inhibits the Na-K pump (Skou, 1965). The intracellular accumulation of sodium which is believed to be responsible for inhibition of tension in the taenia is due to inhibition of the Na-K pump. Griffin *et al.* (1972), observed that the maximum inhibitory effect of a 5 min exposure of ileum to ouabain was seen 10 min after washing ouabain out. The simplest interpretation of this phenomenon is that the maximum accumulation of sodium in the tissue lagged behind inhibition of the Na-K pump. Gradual washout of ouabain in the above experiments could also have slowed the rate of recovery of the Na-K pump from inhibition. The changes in intracellular sodium concentration in the taenia coli correlate reasonably well with the decrease in tension produced by ouabain. The strongest evidence for an intracellular inhibitory role of sodium comes from the experiments where the effect of sodium replacement was compared with the result of substituting lithium for sodium. Sodium ions can be pumped out when the Na-K pump recovers, thus explaining the gradual recovery from inhibition. On the other hand, lithium ions which can enter the cell cannot be pumped out by the Na-K pump (Skou, 1965). Lithium can substitute for sodium in many instances (Katase & Tomita, 1972), and it is possible that the inhibitory effect on taenia is no exception. Axelsson (1961) has shown that in the taenia coli, lithium substitution for sodium abolished mechanical responses without affecting electrical activity. Perhaps a similar effect of sodium is not seen normally because the Na-K pump prevents intracellular accumulation of sodium. The intracellular potassium concentration is also reduced by ouabain. This is unlikely to be the cause of the inhibition because elevation of extracellular potassium failed to prevent the inhibition (though the time course of inhibition was somewhat slower).

The mechanism whereby an increase in intracellular sodium inhibits contractility cannot be understood at present. It could either be at the level of the excitation-contraction process or the contractile protein. Inhibition of potassium-induced uptake of ^{45}Ca by ouabain in association with increase in intracellular Na suggest an effect on mechanisms that regulate intracellular [Ca] concentration. This effect of intracellular [Na] in the taenia coli contrasts with the increase in intracellular entry of [Ca] due to a Na-Ca exchange mechanism (Reuter, Blaustein & Haeusler, 1973) seen when intracellular [Na] is elevated in other tissues (e.g. squid axon, heart).

It is note-worthy that the inhibitory effect of cardiac glycosides is not seen in multi-unit vascular smooth muscle (Cushny, 1925; Leonard, 1957) or in cat spleen capsule (unpublished observations) although the excitatory effect is present. Thus visceral smooth muscle seems to be exceptional in this respect.

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