THE ACTION OF DIAZOXIDE ON ISOLATED VASCULAR SMOOTH MUSCLE

by

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For Brenda

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ABSTRACT

Diazoxide, a non-diuretic benzothiadiazine antihypertensive agent, is thought to act directly upon the vascular smooth muscle of the resistance vessels to exert its therapeutic effects in hypertension. Diazoxide may exert its antihypertensive action by antagonizing calcium in vascular smooth muscle. Wohl et al.(1967 and 1968) have suggested such an interaction based on experiments conducted with isolated rabbit aortae. The present experiments were designed to investigate the possible cellular locus of the postulated interaction of diazoxide with calcium using the isolated anterior mesenteric vein of the rabbit as a model of vascular smooth muscle. This vein is spontaneously motile and possesses characteristics similar to those observed for vessels of the microcirculation.

Diazoxide at 10^{-4} M inhibited spontaneous motility and its associated membrane electrical activity, and caused hyperpolarization in rabbit anterior mesenteric veins examined with a sucrose gap apparatus. Diazoxide also inhibited spontaneous electrical and contractile activity in guinea-pig taenia coli and in estrogen dominated rabbit uterus. In all these tissues, calcium is believed to play an important role in spontaneous electrical membrane activity. Diazoxide failed to affect contractility, rate of spontaneous contractions, or action potential configurations in isolated rabbit heart, even though the action potential in heart tissues possesses a definite calcium current component.

Diazoxide reduced contractions induced in the mesenteric vein by electrical stimulation of the smooth muscle itself or by

excitation of the nerve endings within the vein.

Various drugs were chosen for their ability to contract the mesenteric vein in different ways. Noradrenaline contracts vascular smooth muscle even when the tissue is depolarized with ouabain. Diazoxide failed to inhibit noradrenaline contractions in the depolarized vein, but showed the characteristics of a competitive inhibitor of noradrenaline in normally polarized veins. Diazoxide was also capable of inhibiting contractions to serotonin and procaine, agents which require membrane polarization to initiate contraction. The inhibitory effect of diazoxide was not observed to be modified in solutions containing high concentrations of calcium.

Diazoxide was tested upon the contractile responses to calcium in veins depolarized in K^+ Ringer solution. Examination of the resultant dose response curves showed that diazoxide inhibited calcium contractions in a reversible, non surmountable manner. Hydrochlorothiazide had no effect upon calcium induced contractions.

Diazoxide antagonizes drug induced contractions only if a polarized membrane is present. Calcium induced contractions in depolarizing solutions were inhibited in an apparently insurmountable manner, while drug responses in polarizing solutions were inhibited by diazoxide in a surmountable manner. In addition, action potentials from rabbit heart were unchanged whereas, the apparently calcium spike mediated electrical activity of certain smooth muscles is inhibited.

It is concluded that diazoxide affects the membrane of vascular smooth muscle to reduce excitability of the tissue to drugs or electrical stimuli. It is possible that cell membrane bound

calcium could be the locus of action of diazoxide and that this agent modifies membrane calcium to cause increased membrane stability.

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INTRODUCTION

Benzothiadiazines in Hypertension

Within the last decade, the benzothiadiazine compounds have found a firm place in the therapy of mild to moderate hypertension. The mechanism of action of such agents, for example chlorothiazide or hydrochlorothiazide, remains to be completely elucidated.

It was once held that the diuretic effect of many of these compounds played a major role in their antihypertensive action. This was thought to be accomplished by a decrease of circulating plasma volume, hence a decreased cardiac output and reduced blood pressure. It is now thought that the diuretic action of benzothiadiazines appears to play only a transient role, if any, in the relief of hypertension. Conway and Lauwers (1960) demonstrated, using chlorothiazide in 23 hypertensive patients, that the plasma volume loss due to the drug induced diuresis was restored after several weeks treatment without loss of antihypertensive efficacy. Cardiac output in these patients, if anything, was found to be somewhat elevated. Most important was the observation that chlorothiazide affected the total peripheral resistance. This function was reduced by some 25 per cent, resulting in blood pressure reductions by a mean of 26 mm. Hg. systolic and 17 mm. Hg. diastolic in patients with essential hypertension. Conway and Palmero (1963), using venous occlusion plethysmography, showed that in 43 patients with mild hypertension, chlorothiazide caused a mean reduction of 18 per cent in forearm peripheral resistance and a small decrease in venous tone. They also re-

ported that the hypotensive effects of the drug were not related to the magnitude of the diuresis, as measured by loss of weight. These authors thought that the reduction in total peripheral resistance was a result of the thiazide acting directly upon the vascular smooth muscle of the resistance vessels.

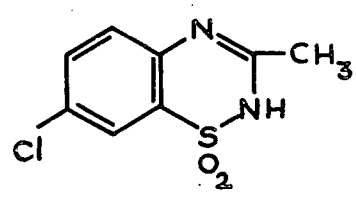
Because the diuretic benzothiadiazines may alter renal tubular transport of sodium (Preziozi et al., 1959), it was suggested by some workers that these agents may alter ionic gradients within vascular smooth muscle cells, perhaps altering excitation and diminishing contractility of the blood vessels. Daniel (1962) showed that hydrochlorothiazide caused no change in the sodium content of plasma, aortic tissue, stomach or psoas muscle from desoxycorticosterone hypertensive rats. Rubin (1963) on the other hand, found that chlorothiazide enhanced sodium uptake without affecting potassium loss in isolated rat aortic strips. Daniel and Nash (1965) suggested that if an effect upon ionic transport were the mechanism of action of benzothiadiazine antihypertensives, a reduction of vascular cell volume as well as decreased contractility might explain antihypertensive effects. They found, however, that hydrochlorothiazide and the non diuretic related compound, diazoxide, failed to show significant effects on reuptake of potassium or extrusion of sodium in cold treated aortic strips. Because of the inconsistencies of effects upon ion transport just discussed, it is unlikely that the antihypertensive mechanism may be ascribed to a direct effect on active transport of sodium or potassium.

Some diuretic benzothiadiazines have been shown to reduce

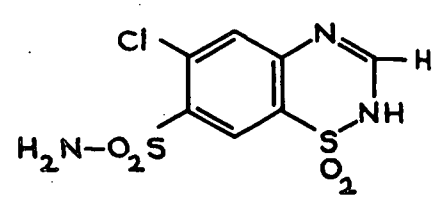
contractility in vascular smooth muscle. Preziozi et al. (1959) showed that chlorothiazide could antagonize pressor responses to noradrenaline, adrenaline, and angiotensin in intact dogs. Rubin et al. (1963) showed that both chlorothiazide and trichlormethiazide inhibited aortic contractions caused by noradrenaline. Daniel and Nash (1965) reported however that hydrochlorothiazide did not antagonize the contractile responses to noradrenaline of rabbit aorta or uterus. The same authors reported however, that hydrochlorothiazide inhibited spontaneous contractions in uterus.

Pharmacology of Diazoxide

Rubin (1961a,b,c.) investigating 7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide, or diazoxide (Figure 1), showed that this close analogue of the thiazide diuretics possessed marked antihypertensive activity. Interestingly, this compound failed to act as a diuretic, and in fact, was found to cause sodium and water retention. A benzothiadiazine compound had been found in which a clear cut separation of antihypertensive and diuretic properties could be demonstrated. It was felt that further investigation of this agent might lead to an explanation of the hypotensive properties of the diuretic benzothiadiazines.



Diazoxide



Chlorothiazide

Figure 1.

The hypotensive activity of diazoxide in anaesthetized cats was not blocked by previous administration of atropine, phentolamine, hexamethonium, chlorpheniramine, reserpine or by spinal transection (Rubin et al., 1961b). Intra-arterial injection of diazoxide into dog femoral, renal, and coronary vessels caused immediate and prolonged increase of blood flow through the affected vascular beds (Rubin et al., 1962). These observations indicated a site of action not primarily influenced by nervous pathways or humoral factors; in other words, a direct peripheral relaxation was implied. In addition, Rubin et al. (1961c) showed that diazoxide antagonized contractions to noradrenaline, angiotensin, and serotonin in aortic strips from rats and rabbits.

Unlike its diuretic congeners, diazoxide reduced blood pressure very rapidly (within one to two minutes) when injected rapidly into dogs or humans. This rapid action contrasts with the slow onset of antihypertensive activity of the diuretic benzothiadiazines, which require from several hours to several days to reduce high blood pressure. Diazoxide is also more effective intravenously than orally, in contrast to the optimal, oral route of administration of the diuretic benzothiadiazines.

Trials with desoxycorticosterone-hypertensive dogs (Rubin, Roth, Taylor, and Rosenkilde, 1962) showed that diazoxide injected intravenously at five milligrams per kilogram decreased the blood pressure and total peripheral resistance, and increased cardiac output and right atrial pressure. Dogs treated with diazoxide showed no evidence of orthostatic hypotension when tilted upright on their hind legs. These observations demonstrated that the autonomic vascular reflexes remain intact after

diazoxide treatment. The increase in right atrial pressure is interesting, as this implies that there is little effect on veins, as the requirements for an increased cardiac output are satisfied. These observations when taken together, provide rather good evidence for a highly selective site of action of diazoxide in reducing high blood pressure: the direct relaxation of the resistance vessels. This is particularly significant as the arterioles appear to be primary in the cause of essential hypertension (Freis, 1960). Rubin et al. (1963), using direct pressure recording demonstrated that intra-arterial and intravenous infusions of diazoxide caused a reduction of total forelimb resistance due almost exclusively to a reduction in small vessel resistance. Diazoxide had little effect on forelimb veins and larger arteries.

Under conditions of constant flow using an extracardiac circuit in anaesthetized normotensive dogs, Nayler et al. (1968), showed that diazoxide caused reductions of peripheral vascular resistance that resulted in increased flow in the coronary arteries, inferior vena cava and superior vena cava. Flow in the azygos, renal, and splanchnic circulations decreased. The same effects were reflected under conditions of constant perfusion pressure rather than flow. The same workers demonstrated that diazoxide displaced left ventricular function curves to the right, indicating a reduction of the capacity of the left ventricle for doing external work. The mechanism, the authors suggested, may be similar to that observed in smooth muscle, that is: relaxation or inhibition of contraction.

Trials with human patients were initiated and it was found

that diazoxide was effective in reducing blood pressure in most cases of hypertension. These included primary aldosteronism, essential hypertension, malignant hypertension, and toxemia of pregnancy (Saker et al., 1968; Finnerty, 1963). The rapid action and lack of tolerance to the drug gave indications of a very useful therapeutic tool.

Finnerty et al. (1963) reported the results of rapid intravenous injection of 300 milligram doses of diazoxide into 46 hypertensive patients. Blood pressure was lowered in these patients within one to two minutes by a factor of some twenty-five per cent. Gradually, the blood pressure rose to a level of fifteen per cent reduction from control values. There were no signs of postural hypotension, cerebral ischemia or collapse during the duration of activity of the drug: a mean of 4.7 ($\pm$ 1.7 S.E.) hours. At the peak of the hypotensive response there was a calculated reduction of 41 per cent in total peripheral resistance. These authors concluded "The standard dosage of three hundred milligrams ... the immediate onset of action, the maintainence of cardiac output, the lack of significant side effects, and the fact that it can be administered repeatedly without the development of drug resistance make diazoxide administered intravenously the ideal therapy for acute hypertension." Finnerty (1966) suggested that diazoxide might find its best use in hypertensive emergencies in which rapid relief of high blood pressure would be the critical feature. Such emergencies would include hypertensive encephalopathy and eclampsia. Finnerty, Davidov, and Kaviatos (1967) reported the long term effects of diazoxide therapy in sixteen

patients with severe intractable hypertension. These authors administered diazoxide in rapid injections of three hundred milligrams daily as required over a twenty day period to maintain arterial blood pressure twenty per cent below control values. Their results suggest that in some cases, rapid reduction of the blood pressure with diazoxide for a limited time may improve intractable hypertension and its complications such as retinopathy, cardiomegaly, congestive heart failure, and impaired renal function, to the point where more conventional therapy, for example chlorthalidone and reserpine or hydralazine or methyl dopa may be used successfully after discontinuing diazoxide.

Diazoxide, however, has adverse effects, which are serious enough to have kept this agent from general use. These side effects are manifest when the drug is used orally or chronically more than with intravenous injection. Sodium and water retention were the first side effects to be noted. Finnerty (1966) found that diazoxide induced edema could be eliminated or minimized by concomitant administration of chlorothiazide and acetazolamide. Hypertrichosis develops with long term oral use of diazoxide. The cause for the hirsutism is unknown but the effect appears specific for the vellus hair of the body (Koblenzer and Baker, 1968).

Perhaps the most serious side affect of repeated diazoxide administration is hyperglycemia, appearing often as overt diabetes mellitus. Dollery (1962) reported this effect developing after approximately one week of diazoxide therapy. The condition appeared to be reversible and his patients recovered after the drug had been discontinued. Dollery ascribed the development of

this diabetes to inhibition of insulin secretion by the pancreatic beta cells. Tabachnick and Gulbenkian (1968) reported that the hyperglycemic effect is due to an extrapancreatic effect as well as inhibition of insulin secretion. The additional activity was ascribed to inhibition of the cyclic 3',5' adenosine monophosphate phosphodiesterase enzyme. When phosphodiesterase is inhibited, the lipolytic and glycogenolytic activities of cyclic adenylate would be potentiated once the nucleotide had been produced by exogenous stimulation, as with adrenaline.

Diazoxide has been used in the therapy of hypoglycemia. For a review of this material, the reader is referred to the Annals of the New York Academy of Sciences, Art. 2, Volume 150, pages 191-467, "Diazoxide and the Treatment of Hypoglycemia".

Diazoxide: Its Effect on Smooth Muscle

The mechanism of action of diazoxide on vascular smooth muscle remains to be defined. Effects upon membrane ionic gradients by diazoxide have not been proven, and evidence for distinct modification in membrane ionic gradients is unconvincing. (Daniel and Nash, 1965; Freed et al., 1963; Kapitola, 1968).

It has been known since 1959 that some benzothiadiazines antagonize isolated smooth muscle contraction (Preziozi et al., 1959; Daniel and Nash, 1965). This was seen in at least three types of muscle: aortic, uterine and intestinal. The same authors also observed that hydrochlorothiazide, chlorothiazide, and diazoxide tended to inhibit spontaneous motility of smooth muscle.

In 1967, Wohl, Hausler, and Roth examined the effect of diazoxide on barium induced contractions in aortic strips from desoxycorticosteroid hypertensive rats. The results showed that

diazoxide possesses characteristics of a competitive surmountable antagonist of barium. Diazoxide was also shown to be a surmountable antagonist of noradrenaline induced contractions. The characteristics of this interaction suggested an indirect rather than directly competitive antagonism as demonstrated by Lineweaver-Burke plots of the inverse of noradrenaline and barium dose response curves. The authors suggested that barium was behaving as a calcium replacement which would effectively cause contractions. They speculated that diazoxide was a competitive antagonist of calcium itself. In 1968, the same group demonstrated that the efficacy of diazoxide in competitively antagonizing barium was enhanced in aortae from desoxycorticosterone hypertensive rats as compared with strips taken from normal rats (Wohl et al., 1968a). The results with diazoxide may reflect Hinke's (1961) suggestion that calcium is used more efficiently in experimentally hypertensive animals. It is tempting to speculate that if calcium is abnormally used in contraction in the hypertensive state, it may be more susceptible to diazoxide antagonism, although hypertensive aortas contain 13 per cent more calcium than do normals. (Tobian and Chesley, 1965).

Wohl, Hausler, and Roth (1968b) demonstrated antagonism between diazoxide and calcium in rabbit aortic strips. The strips were contracted maximally with noradrenaline in a series of solutions of varying calcium content. The maximum contraction achievable thus depended on the availability of calcium for contraction. When the strips were exposed to diazoxide, a competitive antagonism was observed between calcium and diazoxide.

Wohl's results help explain the effectiveness of diazoxide,

especially, if as suggested by the data of Hinke and of Tobian and Chesley, the functional lesion in essential hypertension is related to the role of calcium in vascular smooth muscle contraction. Another facet of diazoxide-calcium interaction, demonstrated in this laboratory, (Sutter, unpublished results) is that diazoxide is capable of reducing the ionic activity of a solution of calcium chloride as determined by an Orion calcium-sensitive electrode. A direct calcium interaction with diazoxide may occur which might explain the observed competition.

Smooth Muscle as an Excitable Tissue.

The problem remains, however, as to the locus of diazoxide action in smooth muscle. For this reason, a consideration of the phenomenon of contraction in vascular smooth muscle is pertinent at this juncture. Bohr (1964) has described four discrete components involved in contraction of smooth muscle. These are: excitation, excitation contraction coupling, contraction itself, and the intermediary metabolism supplying the energy for the contraction. Each of these components presents a possible locus for drug action but it is proposed to confine the scope of this thesis to the phenomena of excitation and of excitation contraction coupling.

Bozler (1948) has suggested two distinct types of smooth muscle, differing according to their degree of dependence on an extrinsic nerve supply and their ability to contract in an all or none manner. Multiunit muscles are those that normally respond only to excitation of their extrinsic nerve supply. These muscles tend to be divided into motor units, which may be individual muscle cells. In addition, multiunit smooth muscle does not normally support the propagation of action potentials. A good

example of this type of vascular smooth muscle is the pulmonary artery, which remains electrically quiescent even when contracted by exciting its nerve supply or by noradrenaline (Su, Bevan, and Ursillo, 1964).

Bozler's other classification of smooth muscle is that of visceral or single unit muscle. This type behaves as a syncitium and will support the propagation of action potentials. Such tissues are often spontaneously active, and this activity may be modified by but is not dependent upon an extrinsic nerve supply. Uterus, intestinal muscle, and ureter are good examples of single unit muscle (Burnstock, Holman and Prosser, 1963).

Most pharmacological work on vascular smooth muscle has probably been based on the aorta. This vessel however, is a "windkessel" vessel, a large elastic conduit whose function is to convert the largely pulsatile cardiac output into a fairly steady flow through the small vessels (Mellander, 1968). Its structure and functional features reflect this role and thus, the aorta may not be a good model for examining arteriolar smooth muscle. Aortic contractions are of a slow tonic nature while spontaneous vasomotion of the arterioles, as observed in the bulbar conjunctivum, is rather rapid (Lee, 1951; Jackson, 1958). Thus, aortic responses to drugs and to other modifications of environment may not reflect analogous responses of the vessels of the microcirculation.

Anterior Mesenteric Vein as a Model to Study Vascular Smooth Muscle

The smooth muscle tissue of primary concern in hypertension is that of the arterioles. Excitation would appear to be media-

ted in such small vessels (100-300 microns) by action potentials and slow wave membrane potentials. This was demonstrated by Steedman (1966), and by Speden (1964) both working with intracellular microelectrodes on the intact mesentery of the guinea pig. These workers showed that spike activity was mediated in large measure by the sympathetic nervous system. A mean maximum membrane potential of 39 millivolts was recorded from these vessels. In some cells, slow wave potentials appeared to generate spontaneous action potentials; in others, spontaneous action potentials did not occur, but when the nerves to the vessels were stimulated, junction potentials were observed which at a critical level led to action potential firing. Local application of adrenaline, noradrenaline, and vasopressin led to increased frequency of action potential firing and increased slow wave amplitude. Somlyo and Somlyo (1968a) suggest that "spontaneous vasomotion of the microcirculation indicative of conducted activity, may represent either myogenic single unit or neurally coordinated multiunit behaviour." Also, these authors cite Rodin's (1967) observation on the ultrastructure of blood vessels, that the nexus appears with increasing frequency in the terminal vascular bed. Somlyo and Somlyo claim a good positive correlation of nexuses with conducted action potentials, a property of single unit smooth muscle (see also Barr, Dewey, and Berger, 1965).

The anterior mesenteric-portal vein demonstrates behaviour rather like that of the arterioles observed by Speden (1964) and by Steedman (1966). This vein possesses spontaneous contractile activity which is associated with and preceded by action potentials and slow wave depolarizations. (Cuthbert and Sutter, 1964;

Funaki and Bohr, 1964; and Cuthbert, Matthews, and Sutter, 1964). This tissue apparently behaves as a single unit smooth muscle; action potentials are propagated from cell to cell (Johansson and Ljung, 1967b). Holman et al. (1968) and Johansson and Ljung (1967a) demonstrated that the spontaneous activity of the vein arises myogenically. Their evidence for this was that spontaneous activity could not be blocked with tetrodotoxin, adrenergic blocking agents, or nerve blocking concentrations of local anaesthetic. Sympathetic innervation, however, does play a considerable role in modifying the frequency and the tone of contraction in the isolated rabbit vein, intact cat vein, and in isolated sheep veins (Johansson and Ljung, 1967a; Hughes and Vane, 1967; and Holman and McLean, 1967).

Calcium: Its Role in Smooth Muscle Contraction

Calcium has been shown to be required in the normal contraction process in vascular smooth muscle (Waugh, 1962; Briggs, 1962; Hinke, 1964). Calcium may modulate membrane activity, mediate excitation contraction coupling, and activate the contractile proteins directly.

Holman (1958), Bulbring and Kuriyama (1963), and Brading et al. (1969) have examined the role of calcium in modulating membrane excitability in guinea-pig taenia coli. These authors demonstrated a competition between sodium and calcium at the membrane for the current carrying the action potential spike. It was found that in excess calcium and, or, depleted sodium media, the taenia coli exhibited slower spontaneous activity, membrane hyperpolarization, increased spike overshoot, and increased rate of

rise of the action potential spike. Slow wave activity tended to disappear under such conditions. In calcium depleted and, or, sodium rich solutions, the opposite effects were observed: membrane depolarization, increase in rate of spontaneity, and decrease in the rate of rise and of the overshoot of the action potential. In addition, in high sodium and calcium deficient solutions, electrical events tended to be uncoupled from contraction. The effect of changing either sodium or calcium concentrations was largely dependent on the concentration of the other ion (Brading et al., 1969).

Manganese was shown to block spontaneous spike discharge after a period of time and this could be overcome by increasing the concentration of extracellular calcium (Brading et al., 1969; Hotta and Tsuiki, 1968). Strontium or barium may replace calcium in maintaining action potential activity in taenia coli, but at the cost of decoupling of contraction from electrical events. "This decoupling is understandable if it is assumed that calcium ions which entered the cell during an action potential exert a direct influence on the contractile proteins and that other alkaline earth metal ions have a relatively weak effect on the smooth muscle actinomyosin system." (Hotta and Tsukui, 1968). These authors cite other work demonstrating that tetrodotoxin, an alkaloid that specifically blocks the sodium influx of the action potential in excitable tissue, had no effect on action potentials in the taenia coli (Kao, 1966; Moore and Narahashi, 1967). These results suggested strongly that the action potential spike in taenia coli is carried by the calcium ion.

Tetrodotoxin fails to block spontaneous activity in the

mesenteric vein (Hughes and Vane, 1967; Holman and McLean, 1967), indicating that as in the taenia coli, a calcium ion flux may be largely responsible for the current carrying the action potential spike instead of sodium. Strontium will substitute for calcium to some degree in maintaining spontaneous activity in the mesenteric vein (Severson and Sutter, 1969). Further support for the presence of calcium spikes in vascular smooth muscle is the observation made in this laboratory that spontaneous contractions and action potential activity are maintained in Krebs' solution modified with tris chloride buffer so that only 25 millimolar sodium remains (Sutter, unpublished results).

Many authors have demonstrated the importance of calcium in initiating and maintaining contractions in vascular smooth muscle (Waugh, 1962; Briggs, 1962; Northover, 1968; Hinke, 1964). An increase of calcium influx in aortic strips has been reported during potassium or noradrenaline contraction (Briggs, 1962). Such an influx of calcium may initiate contraction either by causing a translocation of intracellularly sequestered or bound calcium or by acting directly upon the contractile proteins (Somlyo and Somlyo, 1968b). It is also conceivable that the calcium influx component of the action potential in some tissues, for example the anterior mesenteric vein or taenia coli, may be sufficient to activate contraction.

The relative importance of calcium in the contractile process also depends upon the mode of contraction. Hudgins and Weiss (1968) demonstrated that in order to contract isolated aortic strips with either potassium or histamine, a higher minimum concentration of calcium was required, respectively in the bathing

solution than was necessary for noradrenaline contractions. Citing Briggs (1962), these authors proposed that potassium contractions were entirely dependent upon the availability of extracellular calcium, which presumably, caused contraction by a direct influx into the cell. Hudgins and Weiss showed that histamine was capable of causing a small additional contraction in potassium contracted vascular smooth muscle. This was interpreted as meaning that the amine was capable of releasing intracellularly bound or sequestered stores of calcium. Noradrenaline, on the other hand, has been shown to be capable of contracting several types of vascular smooth muscle for some time in zero calcium solution (Hudgins and Weiss, 1968; Johansson et al., 1967; Severson and Sutter, 1969). These authors interpreted these results as indicating that noradrenaline was capable of acting primarily upon intracellularly bound stores of calcium.

Excitation Contraction Coupling in Vascular Smooth Muscle

Somlyo and Somlyo (1968a,b) describe four important aspects of excitation contraction in vascular smooth muscle that differ from that of skeletal muscle:

> "1) their dose response curves, which indicate continuous gradation of excitation are difficult to reconcile with an underlying all or none process; 2) the lack of quantitative correlation between the contractile effects of drugs and either their action on the membrane potential or related monovalent ion fluxes, in polarized as well as in depolarized preparations; 3) the absence of action potentials in certain types of vascular smooth muscle; and 4) the inequality of the maximal contractile effect of different drugs on a given vascular smooth muscle."

These properties should be taken into account in consideration of the individual smooth muscle studied and in regard to

the specific contractile agents used. Different drugs have different mechanisms of action in contracting vascular smooth muscle. Noradrenaline will contract the anterior mesenteric vein of the rabbit. Associated with this contraction is increased frequency of action potential firing and depolarization of the cell membrane (Cuthbert and Sutter, 1965; Johansson et al., 1967). These, and other authors question however, the relative importance of the electrical events associated with contraction (see also Axelsson et al., 1968). Noradrenaline will contract the ouabain depolarized mesenteric vein (Matthews and Sutter, 1967), the potassium depolarized vein (Somlyo and Somlyo, 1968b), and will contract caffeine treated veins in which the membrane will not support action potential activity (Somlyo and Somlyo, 1968b). Thus, it appears that noradrenaline may exert an effect on excitation contraction coupling that is not membrane potential dependent. This, however, does not exclude noradrenaline from influencing or initiating contraction via alteration of resting potentials or via action potentials.

Similarly, Matthews and Sutter (1967) demonstrated that under conditions of ouabain depolarization, serotonin was capable of eliciting contractions which were of relatively less amplitude than those to noradrenaline. Like histamine and noradrenaline, serotonin exerts a depolarizing influence upon the vascular smooth muscle membrane but does not depend completely on the membrane potential for its contractile effects. Serotonin, however, would appear to have a greater membrane depolarization dependence than does noradrenaline in the initiation of contraction because it is less efficacious in the depolarized preparation (Matthews and

Sutter, 1967).

Procaine is another agent that is capable of contracting the anterior mesenteric vein (Sanders, 1969), and the guinea pig ureter (Washizu, 1968). Both these tissues appear to have calcium mediated action potential activity as does guinea pig taenia coli. Washizu demonstrated that procaine initiated membrane depolarization in the ureter, and converted action potentials to plateau type configurations, prolonging their duration. These lengthened action potentials were associated with prolonged contractions, probably due, Washizu stated, "to an increase in the mechanically effective period of the action potential." These prolonged contractions, however, were abolished after some twenty to thirty minutes exposure to 0.5 per cent procaine. It was found that fivefold elevation of external calcium concentrations restored the action potential to normal configurations in the presence of procaine 0.01 to 0.1 per cent. The effect of procaine upon guinea pig ureter was reported to be qualitatively similar to that of lowering external calcium concentrations. Depolarization and increased electrical activity have been observed in the rat portal vein when external calcium concentration is lowered (Axelsson et al., 1967). Procaine, then, may act by functionally removing calcium from a membrane site and thus "labilizing" the membrane to initiate a contraction. Sanders (1969) demonstrated that under conditions of ouabain depolarization, procaine could no longer contract the cat anterior mesenteric vein thus lending support to the concept that procaine contracts vascular smooth muscle solely by its depolarizing activity.

Another tissue in which calcium plays an important role in

the formation of the action potential, is vertebrate cardiac muscle. Orkand and Niedergerke (1964) demonstrated that calcium was important in determining the amount of overshoot in frog ventricular action potentials. Hagiwara and Nakajima (1966) showed that in frog ventricle, procaine and tetrodotoxin suppressed the initial rate of rise of the action potential, but that the plateau phase was unaffected. Manganese was shown to suppress the duration of the plateau phase. Reuter and Beeler (1969) showed with a voltage clamp technique on dog trabeculae that a slow inward current could be produced by voltage clamping above -30 mV. This current did not depend upon levels of external sodium concentrations but was affected by changes in calcium concentrations. In sodium free solution, this calcium current flow was directly related to activation of contraction but in sodium-containing solution served primarily to fill some intracellular depot for calcium. With regard to the present study, it was thought that diazoxide might exert some effect on the plateau phase of the action potentials of rabbit atria and papillary muscles.

If diazoxide antagonizes the calcium involved in the contractile process in vascular smooth muscle, it may act in one or more of three ways. It may interfere with membrane excitation and the events which connect depolarization to contraction. It may interfere with the way in which some drugs mobilize calcium for contraction. Finally, diazoxide may interfere with the ability of vascular smooth muscle to utilize calcium in the actual process of contraction.

STATEMENT OF THE PROBLEM

It was proposed to investigate the effects of diazoxide upon the anterior mesenteric vein of the rabbit, placing special emphasis on excitation contraction coupling. The spontaneous electrical activity of the vein preparation permitted examination of the bioelectric correlates of the relaxant effects of diazoxide upon contraction. This was achieved using a sucrose gap extracellular electrode apparatus. Estrogen-dominated rabbit uterus and guinea-pig taenia coli were similarly examined. In addition, intracellular potentials were recorded from rabbit cardiac tissue to determine the effects of diazoxide upon this type of excitable tissue.

Contractions of isolated veins to drugs acting on different components of excitation contraction coupling were examined using diazoxide as an antagonist. It was thought that the nature of diazoxide inhibition of contractions produced by noradrenaline, serotonin, or procaine would help specify the locus of action of diazoxide upon the contractile system in vascular smooth muscle. In addition, the action of diazoxide upon contractions induced by calcium in depolarizing solution was examined. Similarly, diazoxide was tested on veins contracted by electrical stimulation.

Thus, the effects of diazoxide were examined upon contractions of the mesenteric vein brought about by the following:

1. spontaneous electrical activity of the cell membrane;
2. electrical stimulation of the nerve plexus within the vein;
3. direct electrical stimulation of the veins themselves;
4. noradrenaline, a drug that can cause contraction independent-

ly of membrane electrical events.

5. serotonin, a drug that is dependent to a large extent upon membrane electrical events;
6. procaine, a drug completely dependent upon the membrane potential for its contractile activity; and
7. calcium (in depolarizing solutions) which probably acts directly upon the contractile proteins themselves or at least on a mechanism bypassing electrical membrane activity.

METHODS AND MATERIALS

Tissue Preparation

The anterior mesenteric vein was obtained from New Zealand white rabbits of either sex, weighing from 2.5 to 3.5 Kg. All animals used were killed by a blow on the neck. The veins were rapidly dissected and the portion between the liver and the bifurcation of the superior and inferior mesenteric veins was removed. This section was placed in oxygenated Krebs' solution at room temperature and any further dissection was performed as necessary. The veins destined for organ bath dose response studies were divided into two longitudinal halves; those for sucrose gap experiments were merely opened down one side. After no more than 20 minutes, the veins were mounted in the various apparati where they were maintained at 37°C. in oxygenated Krebs' or Ringer solutions.

Young female rabbits were used to obtain uteri for the sucrose gap experiments. These animals were pretreated with stilboestrol (125 micrograms daily injected subcutaneously) for six days prior to sacrifice. The uteri were then removed and placed in Krebs' solution. Strips some five millimeters wide and two centimeters long were cut and mounted in the sucrose gap apparatus or in organ baths where they were maintained at 37°C.

Taenia coli muscle was obtained from adult guinea pigs of either sex. The muscle strip was rapidly dissected from the colon and placed in oxygenated Krebs' solution. Lengths of approximately two centimeters were cut and mounted in the sucrose gap apparatus.

Heart tissue was obtained from rabbits of either sex. The

rabbits were killed and the hearts were immediately removed and flushed with Krebs' solution injected into the coronary arteries via the aorta. Both atria or the right papillary muscles were then dissected free from the rest of the heart. The atria were removed intact, including the sinus node area, so that pacemaker activity was assured in the preparation. The tissue was placed into organ baths containing gassed Krebs' solution at 37°C. A one gram tension was applied to the tissue once mounted in the organ bath. The right papillary muscles were removed intact with a piece of ventricular wall at one end and the chorda tendinae with valve tissue attached to the other end. These two ends served as points of attachment to hooks in the organ bath used to maintain the tissue for microelectrode recording.

Physiological Saline Solutions

Krebs' solution of the following composition was used for most of the experiments: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 2.56 mM; $MgCl_2$, 1.13 mM; $NaHCO_3$, 25 mM; NaH_2PO_4, 1.15 mM; and glucose, 5.55 mM. This solution was constantly gassed with 95 per cent oxygen and 5 per cent carbon dioxide to maintain the pH at 7.4. A stock solution was made up in large volumes (to 20 liters) without calcium chloride. As required, the stock Krebs' was drawn off and gassed, and calcium chloride and glucose were added to complete the solution. In the case of the depolarizing solution required in the sucrose gap, NaCl and $NaHCO_3$ were replaced with KCl and $KHCO_3$ in equivalent amounts.

When calcium concentrations were raised above five millimolar, Krebs' solution was found to be unsuitable because of precipitation of calcium salts, so it was replaced with a

mammalian Ringer solution of the following composition: NaCl, 154 mM; KCl, 5.4 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 6 mM; and glucose, 5.5 mM. This solution was gassed with 95 per cent oxygen and 5 per cent carbon dioxide to maintain pH at 7.0. When contractions were to be obtained from veins with calcium in depolarizing solutions, a K^+ Ringer was used. This resembled the normal Ringer but sodium salts were replaced with equivalent potassium salts.

Drug Solutions

All drugs used in the isolated tissue experiments were made up in normal saline (0.9 per cent NaCl). Where experimentally, this was not desirable, as when using K^+ depolarizing solutions, the drugs were dissolved in demineralized water. Drug solutions were added in various concentrations to the Krebs' or Ringer bathing the tissues, but always at a constant volume of one one hundredth of the volume of the organ bath. Drug concentrations are expressed as final bath concentrations in terms of base.

1. Diazoxide (Schering), pure powder, was dissolved in normal saline or in demineralized water by adding NaOH dropwise. The concentration of the stock solution of diazoxide was 10^{-2}M. The pH of this stock was in the range of pH 12. A dilution of one in one hundred into the organ bath of the stock did not affect the pH of either Krebs' or Ringer solution.

2. Noradrenaline (l-arterenol-D-bitartrate monohydrate, Mann Research Laboratories) was made up in stock concentrations of one milligram per milliliter. This stock was acidified by adding one drop of 0.1 N HCl to 10 milliliters of the solution.

3. Serotonin (serotonin creatinine sulphate monohydrate, Mann Research Laboratories) was used as a 10 milligrams per milliliter stock solution.

4. Procaine (procaine hydrochloride, K and K Laboratories) was used as a stock solution of 100 milligrams per milliliter.

5. Ouabain (Strophanthidin G, Mann Research Laboratories) was used as a stock solution at 10 millimolar concentration.

6. EGTA (ether glycol bis-amino tetraacetic acid, Geigy Industrial Chemicals) was made up in 300 mM tris solution to a stock concentration of 100 mM. Sodium hydroxide was added to solubilize the chelating agent. Additions of EGTA to organ bath Krebs' or Ringer solutions did not affect pH in the organ baths.

7. Chlorothiazide (Merck, Sharp, and Dohme) was used in stock solutions of 10 millimolar concentration.

8. Hydrochlorothiazide (Esidrex, Ciba) was made up to 10 millimolar concentration stock solutions.

9. Tetrodotoxin (Sankyo) was made up to 0.1 milligram per milliliter as a stock solution.

10. Stilboestrol (British Drug Houses) was obtained in ampoules and diluted in peanut oil to a concentration of one milligram per milliliter for subcutaneous injection.

APPARATUS

Sucrose Gap Electrical Recording

Electrical recordings were obtained from rabbit anterior mesenteric vein, guinea pig taenia coli, and from estrogen-dominated rabbit uterus using a sucrose gap apparatus (Stamfli, 1954; Burnstock and Straub, 1958). The principle of operation of this extracellular recording device is similar to that of obtaining an injury potential. One electrode, on the active side of the apparatus which contains normal physiological saline, was in contact with the surface of the tissue. The same tissue strip passes through an insulating layer of isotonic sucrose into the inactive side of the apparatus which contains a depolarizing solution (either isotonic K_2SO_4 or a potassium rich physiological saline). The other electrode contacts the tissue in the inactive side. Ideally, the only current pathway between electrodes is through the tissue lying within the insulating sucrose gap.

Potentials that are analogous to intracellular potentials may be obtained between the two electrodes. The active electrode is "outside" a normally polarized population of cells and the other electrode, contacting the depolarized tissue, by analogy is "inside" another population of cells. The algebraic sum of electrical events from the active side may be recorded monophasically from a sucrose gap apparatus.

The apparatus used was modified in design from the original tubular apparatus to resemble more a conventional organ bath (Figure 2). The apparatus was constructed of lucite plastic and consisted of three compartments. The upper chamber served

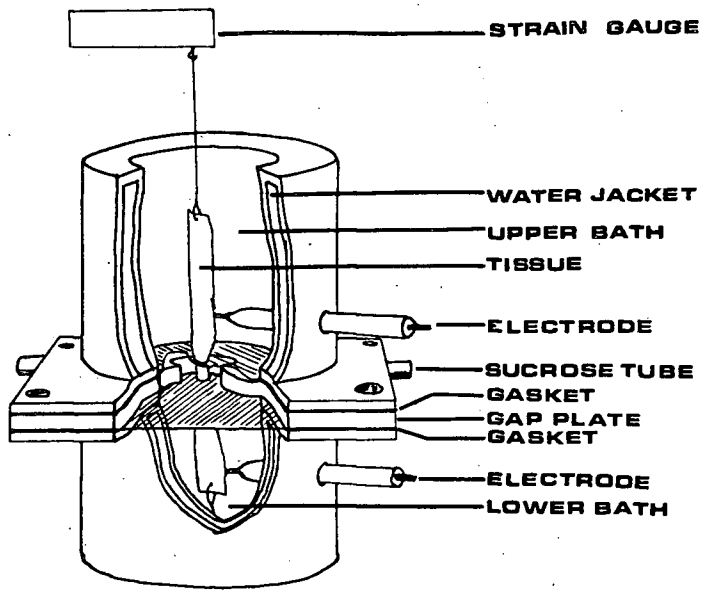


Figure 2. The sucrose gap apparatus. Part of the apparatus has been cut away to reveal the tissue passing through the rubber gasket membranes. Filling and drain tubes have not been illustrated. The apparatus is bolted together on four corners of the base plate.

as the active side of the apparatus and contained Krebs' solution into which drugs could be added directly. The lower compartment contained a depolarizing saline solution (Krebs' solution modified so that $NaHCO_3$ and Na Cl were completely replaced by potassium salts). These two saline compartments were separated one from the other by an eight millimeter gap through which demineralized 300 millimolar sucrose solution was perfused. Two dental dam rubber membranes separated the middle compartment or sucrose gap from the upper and lower compartments. (see Berger and Barr, 1969). The tissue to be examined, passed through a small hole in each membrane so that the upper portion was bathed in Krebs' solution, the middle in sucrose, and the lower portion of the tissue in high K^+ Krebs' solution.

Recording electrodes of silver wire (0.006 inch diameter) were used. These were insulated in glass except for the tips which were electrolytically coated with chloride. The electrode assemblies were passed through the walls of the upper and lower compartments and the tips of the electrodes contacted the tissue. Resistance measured between the two electrodes (an index of tissue resistivity) was of the order of 20 Kilohms. The electrodes were sealed with a rubber sleeve on the outside of each compartment to prevent leakage of saline.

The apparatus was enclosed in a water jacket, through which water at 37°C. was circulated to maintain the preparation at constant temperature. The Krebs' solution in the upper compartment was constantly aerated with 95 per cent oxygen, 5 per cent carbon dioxide. Because of the design of the apparatus, drugs could be added directly to the preparation and solutions could

be changed readily in either compartment by overflow drainage.

The electrodes were attached to an operational amplifier device which amplified the sucrose gap potentials tenfold. These augmented signals were displayed on one beam of a Tektronix 502A oscilloscope. Results were recorded on moving film using a Grass model C4 kymograph camera.

Contractile responses from the tissues in the sucrose gap were recorded using a Grass FT03C force displacement transducer. An operational amplifier circuit amplified the signals from the strain gauge and the output of the amplifier was displayed on the other beam of the oscilloscope so that contractions were monitored simultaneously with the electrical events.

Intracellular Recording from Heart Tissues

Microelectrodes for recording were pulled from 1 mm. O. D. pyrex capillary tubing. These electrodes had tip resistances of greater than 50 Megohms when filled with 1.5 M potassium citrate solution. The electrodes were filled in the following manner. First they were filled with methanol by boiling under a vacuum with the electrodes immersed in the alcohol. A return to normal pressures caused the evacuated electrode to fill with methanol. Once filled with methanol, the electrodes were immersed in distilled water for ten minutes at room temperature to fill them with water. Finally the electrodes were immersed overnight in 1.5 M potassium citrate and were ready for use the following day. Each electrode was carefully shortened by breaking off excess length from the shank so as to minimize electrode mass. They were then mounted on one mil. tungsten wire according to the method of Woodbury and Brady (1956). The indifferent electrode

consisted of a chloride coated silver wire which was directly immersed in Krebs' solution.

A Medistor type 34A microelectrode amplifier was used and was connected to a Tektronix 502A oscilloscope. Electrodes were lowered onto the tissue using a micromanipulator.

The whole atria was allowed to beat spontaneously in the organ bath. A Grass FT03C strain gauge and an operational amplifier circuit were used to monitor contractions as described for the sucrose gap recording. Action potentials and tension from the spontaneously beating tissue were recorded on continuously moving film. The papillary muscle and some atrial strips were electrically driven with platinum electrodes from the output of a modified Tektronix 114 pulse generator at a stimulus rate of one per second. Pulse duration and voltage output were varied so as to minimize stimulus artifact. The stimulated action potentials were recorded on single frames of film with the Grass kymograph camera.

Transmural Stimulation

Whole mesenteric veins from rabbits were mounted on a glass J-tube which was immersed in an organ bath containing Krebs' solution at 37°C. The apparatus resembled that described by Holman and McLean (1967). The isolated vein was cannulated by the end of the glass J-tube so that a platinum electrode from the centre of the tube protuded into the lumen of the vein. A loop of platinum wire approximately one centimeter in diameter surrounded the outside of the vein. The two wires served as stimulating electrodes. An AC stimulus was applied across the electrodes from an AEL stimulator. This apparatus was intended to

stimulate the nerve plexus within the vein (Holman et al., 1967; Holman and McLean, 1967). A silk thread attached the uncannulated end of the vein to a Grass FT03C strain gauge, from which contractions were monitored on a Grass model 7 polygraph.

Organ Bath Drug Response Experiments

Isolated rabbit anterior mesenteric veins were tested for drug responses in organ baths. The baths were filled with Krebs' or Ringer solution gassed with 95 per cent oxygen and 5 per cent carbon dioxide and were maintained at 37°C. by circulating water jackets. Solutions were prewarmed and entered the bath from the bottom of the chamber. Baths were drained either by an overflow method or from the bottom of the chamber when solutions were changed. Veins were cut into longitudinal halves and one end was mounted onto a stainless steel hook in the organ bath using a loop of suture thread. Another loop of thread connected the other end of the vein to a Grass FT03C strain gauge from which isometric contractions were recorded on a Grass Model 7 polygraph. Four half veins could be studied simultaneously in this manner. The veins were loaded with a baseline tension equivalent to a 500 milligram displacement of the strain gauge, and were adjusted periodically throughout the experiments to maintain resting baseline tension. Tissues were always equilibrated for one hour prior to testing drug responses. All drugs at all dilutions were added into the 18 milliliter organ baths in volumes of 0.18 ml. to minimize dilution of the physiological saline.

Protocol and Analysis of Drug Dose Response Experiments

Cumulative dose responses obtained from mesenteric veins in organ baths were performed in the following manner in most experiments. Tissues were divided into longitudinal halves and a control cumulative dose response curve was obtained from each half of the tissue. All subsequent responses were obtained by the cumulative dose method, and then were expressed as a percentage of the control maximum. One half of the veins were then treated with diazoxide at a concentration of 10^{-4} M. After 15 minutes exposure to diazoxide, a dose response series with the agonist drug was obtained from both the diazoxide treated and the untreated (time control) halves of each vein. A crossover manoeuver was then performed; the previously diazoxide treated half of each vein was left untreated, and the former time control half was pretreated with diazoxide. A second dose response series was then obtained from both halves of the vein. Use of this method serves two purposes. The first is that, as the experimental strip is always compared to a time control from the same vein errors in analysis due to changes in responses attributable to exhaustion or degeneration of the isolated preparation over a period of time may be minimized. The second is that the crossover design is a test for reversibility of the diazoxide inhibition of contraction. The data, as presented, represent the mean results from at least four different animals for each experiment and are presented with the standard error of the mean for each experimental point.

RESULTS

Sucrose Gap Experiments

Our observations on the rabbit anterior mesenteric vein in the sucrose gap apparatus were similar to those reported by Cuthbert and Sutter (1965) and by Holman *et al*. (1968). When the inactive side of the apparatus was filled with depolarizing K^+ Krebs' solution, resting membrane potentials of some 20 mV (range 15 to 25 mV) were recorded (Figure 3A). Superimposed on this resting potential were small spikes and depolarization "hillocks" of some 0.15 to 0.5 mV amplitude. Electrical activity was seen in most cases to just precede contractions (Figure 3B). Occasionally a lack of correlation was observed between the electrical spikes and contractions of the vein.

In the presence of diazoxide (10^{-4} M), the spontaneous electrical and contractile activity ceased and spontaneous activity did not return so long as diazoxide remained in the bath. This quiescence was associated with a slight hyperpolarization in nine of twelve veins tested. Normal spontaneous activity resumed some 10 to 20 minutes after diazoxide had been washed out of the bathing solution. A diazoxide concentration below 10^{-5} M had no effect and 10^{-4} M caused complete inhibition of spontaneity. Relaxation of tension from the resting baseline level of 500 mg. was not observed at any dose of diazoxide tested (10^{-6} to 2×10^{-4} M). Chlorothiazide, at a concentration of 10^{-4} M, had no effect upon spontaneous activity as observed in the sucrose gap apparatus (Figure 3D).

Noradrenaline, serotonin, and procaine all caused increased electrical activity and some degree of depolarization in mesenteric

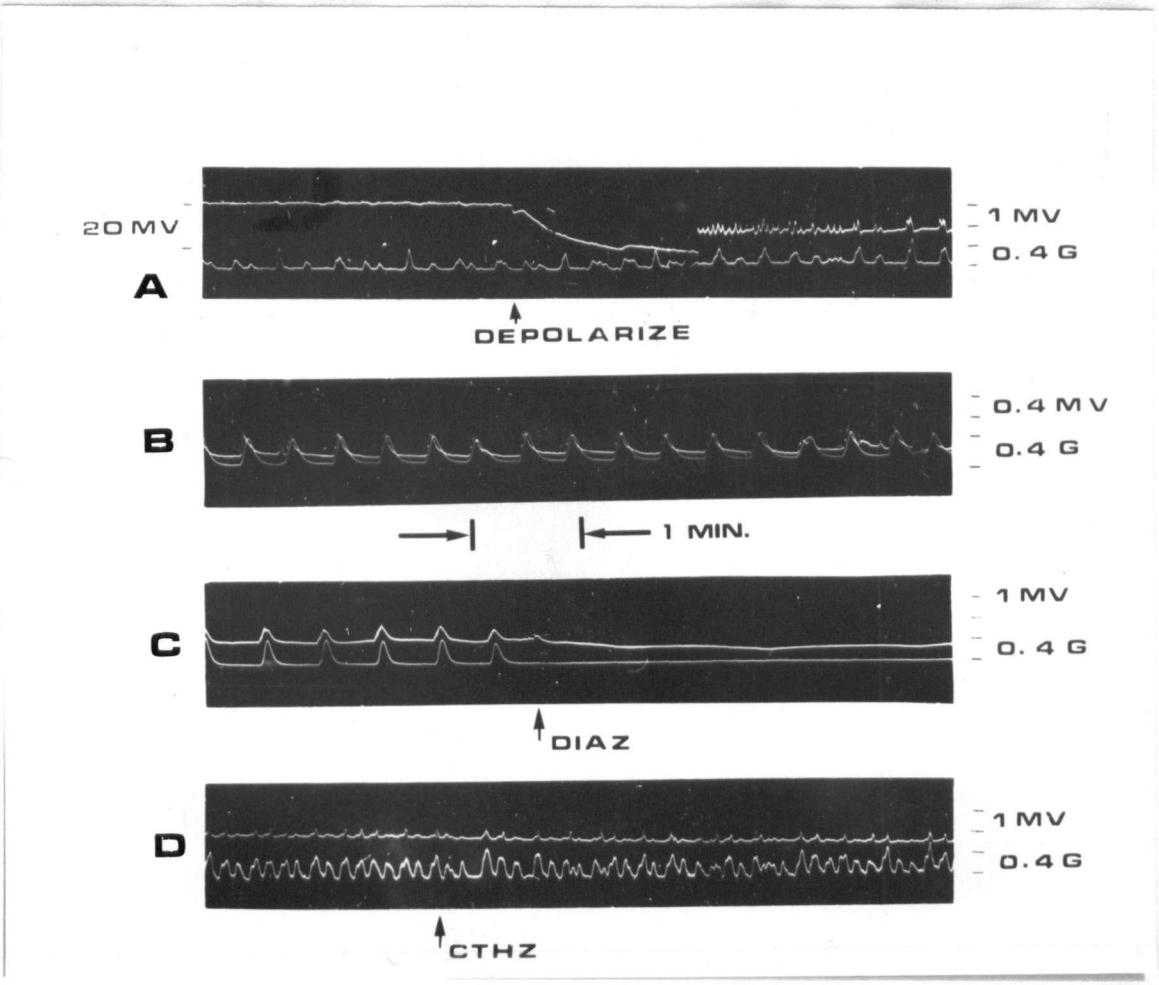


Figure 3. Sucrose gap (upper trace) and tension (lower trace) recordings from rabbit anterior mesenteric vein.

Panel A shows the measurement of membrane potential before, during, and after depolarization (arrow) of the lower half of the vein with K^+ solution in the "inactive" side of the apparatus. The electrical trace is amplified on the right side of the panel.

Panel B is a control trace showing the relationship between spontaneous electrical and contractile activity. Electrical activity immediately precedes each contraction.

Panel C shows the effects of diazoxide (DIAZ) at 10^{-4} M upon the vein. Hyperpolarization and inhibition of spontaneous mechanical and electrical activity can be seen.

Panel D shows that chlorothiazide (CTZ) at 10^{-4} M has no effect on mechanical or electrical events.

veins (Figure 4A,B,C). Contractions induced by these agents were associated with increased electrical activity in all cases. All three drugs were capable of contracting veins in which spontaneous activity had been abolished by diazoxide at 10^{-4} M. These contractions, like those in untreated veins, were associated with depolarization and increased spike activity.

In veins that had been treated with 10^{-4} M diazoxide, increasing external calcium concentration up to 5.0 mM in Krebs' solution failed to restore spontaneous activity. Addition of strontium, a cation which has been observed to substitute for calcium to some degree in contraction coupling but without the membrane stabilizing effects of calcium, also failed to restore spontaneous activity, even when added to Krebs' solution in concentrations up to 5.0 mM.

The addition of a 2.5 mM concentration of EGTA, a highly specific calcium chelating agent, to the Krebs' solution bathing the vein caused a marked depolarization without an associated contraction. This resembled very closely the response elicited from a vein by changing its bathing solution from one with normal calcium content (2.5 mM) to a zero calcium solution.

When calcium chloride was replaced completely in the bathing solution with strontium chloride, spontaneous contractions and electrical activity were maintained (Figure 4D). Electrical depolarization hillocks assumed an elongated configuration upon which many action potential spikes were superimposed. The electrical activity appeared more intense than in calcium containing Krebs' solution. In veins treated with EGTA at 2.5 mM, which was then washed out with zero calcium solution, strontium chloride at

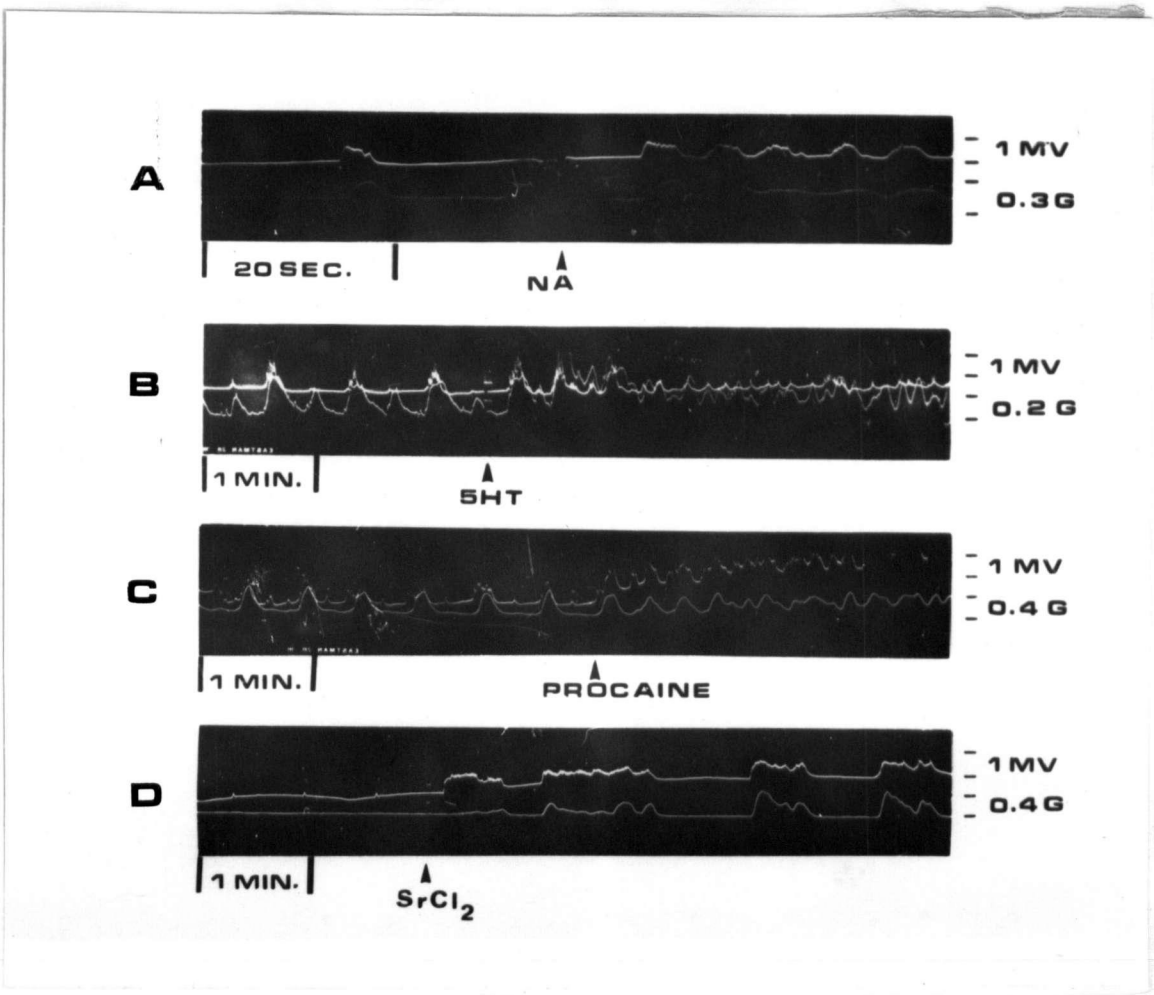


Figure 4. Sucrose gap (upper trace) and tension (lower trace) recordings from rabbit anterior mesenteric vein.

Panel A shows the effects of noradrenaline (NA) at 10^{-7} g/ml. Depolarization and increased rate of firing associated with contractions are evident.

Panel B shows the effects of serotonin (5HT) at 10^{-6} g/ml. Some depolarization and a marked increase in rate of firing may be seen in association with increased contractile activity.

Panel C shows the effects of procaine at 10^{-4} g/ml. in the vein. A marked depolarization and increased firing rate are seen in association with increased contractile activity.

Panel D shows the effects of adding strontium chloride (2.5 mM) to a zero calcium Krebs' solution bathing the vein. Spontaneous activity is restored and a prolonged multi-spike complex is evident.

2.5 mM was capable of initiating spontaneous mechanical and electrical activity. Diazoxide at 10^{-4} M inhibited all spontaneous activity induced by strontium.

The results of diazoxide treatment of guinea-pig taenia coli and of estrogen-dominated rabbit uterus are shown in Figure 5 top and centre panel respectively. Spontaneous activity in the taenia coli from three animals was inhibited in the presence of diazoxide at 2×10^{-4} M. At lower concentrations, inhibition of spontaneous activity took longer and was less complete. Even at a diazoxide concentration of 2×10^{-4} M some slow wave electrical activity remained although spikes were abolished completely.

Two uterine strips from two rabbits responded to diazoxide in a manner similar to the taenia coli and the veins. At a diazoxide concentration of 10^{-4} M, spontaneous activity was arrested in the uterine strips. Electrical multi-spike complexes were first converted to single spikes, and after approximately two minutes, the membrane became quiescent. Neither hyperpolarization nor relaxation were observed in the sucrose gap apparatus. In addition, the spontaneous contractions of uterine strips from four other rabbits were also inhibited with diazoxide at 10^{-4} M when examined in organ baths.

Intracellular Recording From Isolated Rabbit Heart Tissue

Diazoxide caused no change in the duration, rate of rise, or rate of fall of action potentials obtained by intracellular microelectrode recording from isolated rabbit atria and right papillary muscle driven by electrical stimulation. Diazoxide was tested on heart tissue in concentrations of up to 2×10^{-4} M (Figure 5, bottom panel). Diazoxide had no effect on the rate of

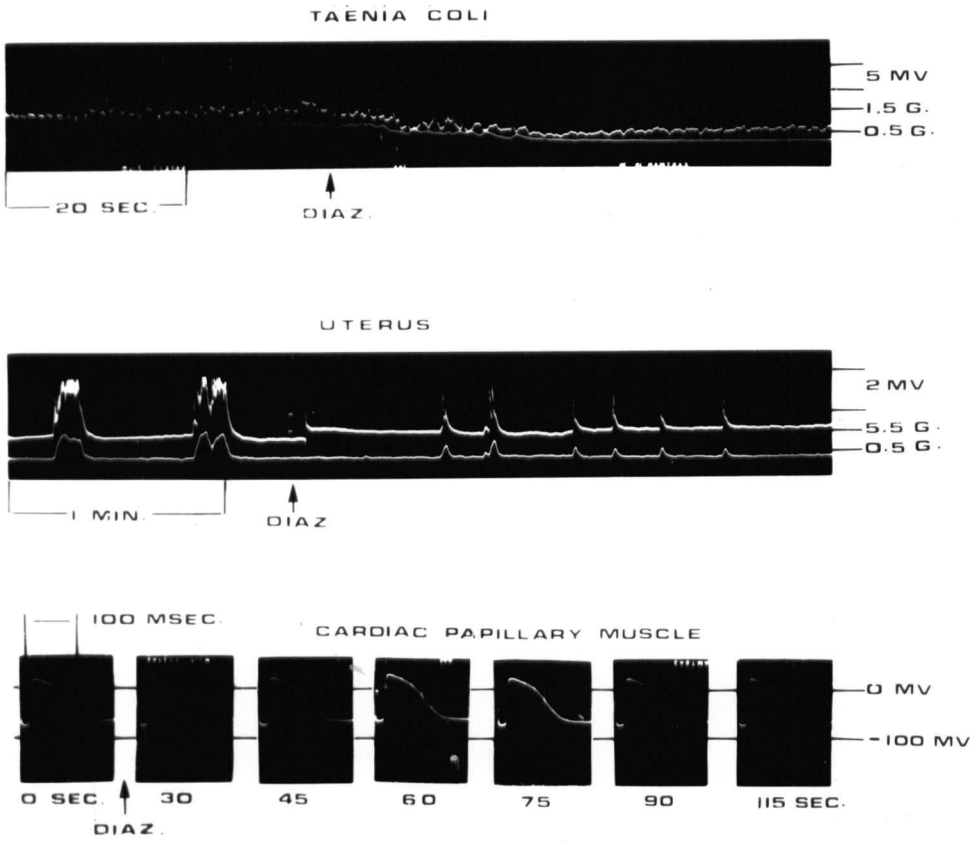


Figure 5. The effect of diazoxide upon electrical and mechanical recordings from isolated guinea-pig taenia coli, estrogen-dominated rabbit uterus, and rabbit heart papillary muscle.

The top panel is a sucrose gap record from taenia coli (upper trace, electrical; lower trace, tension). Diazoxide (DIAZ) at 10^{-4} M indicated by the arrow, inhibits spontaneous activity and hyperpolarizes the membrane.

The centre panel is a sucrose gap record from uterus. Electrical and tension traces are arranged as in the top panel. Diazoxide (DIAZ) at 10^{-4} M caused inhibition of spontaneous activity. No hyperpolariaztion is evident.

The bottom panel shows membrane action potentials from a single cell of cardiac papillary muscle obtained with intracellular microelectrode recording. Each frame was taken at 15 second intervals. Diazoxide (DIAZ) was added between the first and second frames at 2×10^{-4} M. No change in configuration of the action potential is evident after diazoxide treatment.

spontaneously beating atria mounted in the organ bath. As well, diazoxide caused no change in the resting potential of heart tissue measured intracellularly over time periods ranging from three to twenty minutes.

Transmural Stimulation of Veins

Rabbit anterior mesenteric veins contracted in response to an electrical stimulus applied with the apparatus described. Threshold for these responses was of the order of 4 volts at output, and at a frequency of 10 Hz. using the AEL stimulator. A maximum contractile effect was obtained at 4 volts and 60 Hz. with this device. Phentolamine at 10^{-6} g/ml. abolished responses to transmural stimulation in only one of the four preparations tested. In that same vein, 10^{-4} M diazoxide caused considerable inhibition of the maximum contraction achievable by electrical stimulation in this vein (only 25 per cent of control responses remained). The maximum contraction achievable by transmural stimulation in this vein was far below that obtained with noradrenaline at 10^{-6} g/ml. (a concentration that produced some 80 per cent of maximum contraction).

In three other anterior mesenteric veins, neither phentolamine at 10^{-6} g/ml. nor tetrodotoxin at 2×10^{-6} g/ml. completely blocked contraction induced by electrical stimulation although contractions were diminished by nearly half. Diazoxide at 10^{-4} M blocked one quarter of the remaining contractile response to each stimulus.

Effects of Diazoxide on Contractile Responses to Drugs

Noradrenaline

Cumulative dose responses to noradrenaline were obtained

from the anterior mesenteric veins of eight rabbits (Figure 6). The noradrenaline bath concentration at which a contractile response was just achieved was between 10^{-9} and 10^{-8} g/ml. Maximum vein contraction was achieved at a noradrenaline concentration of 10^{-5} g/ml. Time control and crossover control response curves very closely corresponded to the original control response curve. The experimental (10^{-4} M diazoxide treated) and crossover experimental responses were also very nearly identical with one another. Diazoxide, at 10^{-4} M or 4.28 X 10^{-4} M (10^{-4} g/ml.) caused a tenfold, parallel shift to the right of the noradrenaline dose response curve. At the maximum noradrenaline concentration tested (10^{-5} g/ml.) there was no overlap of the standard error of the means of the diazoxide treated veins and their time controls (p=less than 0.001).

Four experiments were conducted to test the effects of diazoxide (10^{-4} g/ml.) upon dose responses to noradrenaline of mesenteric veins bathed in Krebs' solution containing different concentrations of calcium chloride (0.25 to 5.0 mM). No consistent effect upon diazoxide inhibition of contraction was observed when the calcium content of the bathing solution was increased.

Noradrenaline dose response curves were also obtained from veins that had been depolarized by exposure to ouabain at 10^{-5} M for one hour. The contractions obtained with noradrenaline in the presence of ouabain were less than those from untreated veins. Reproducible dose response curves were obtainable, however (Figure 7). Between noradrenaline concentrations of from 10^{-8} g/ml. to 10^{-6} g/ml., the control responses, the time control responses, and responses from diazoxide (10^{-4} M) treated veins were nearly

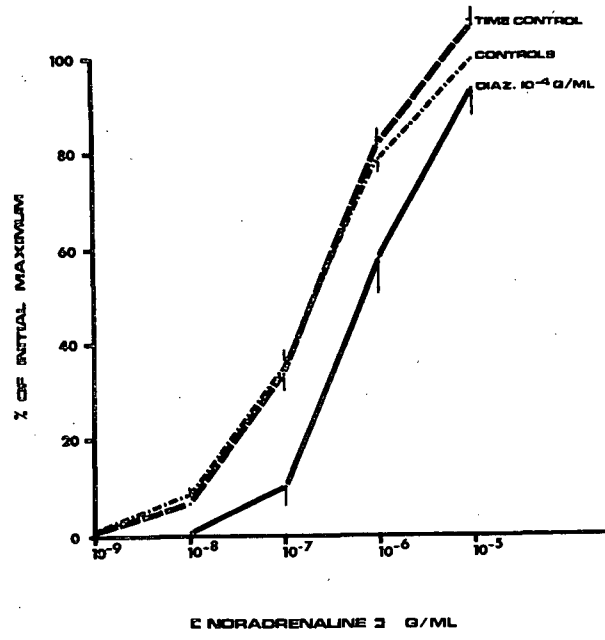


Figure 6. The effect of diazoxide upon noradrenaline dose responses of the rabbit anterior mesenteric vein.

Diazoxide (DIAZ) at a concentration of 10^{-4} g/ml (4.28×10^{-4} M) caused an increased threshold for contraction and a parallel shift to the right of the noradrenaline dose response curve. The crossover experiment is not illustrated, but results from the crossover indicate complete reversibility of the diazoxide inhibition of noradrenaline contraction. The effect of diazoxide at a concentration of 10^{-4} M was similar. The vertical bars represent the standard error of the mean for eight veins.

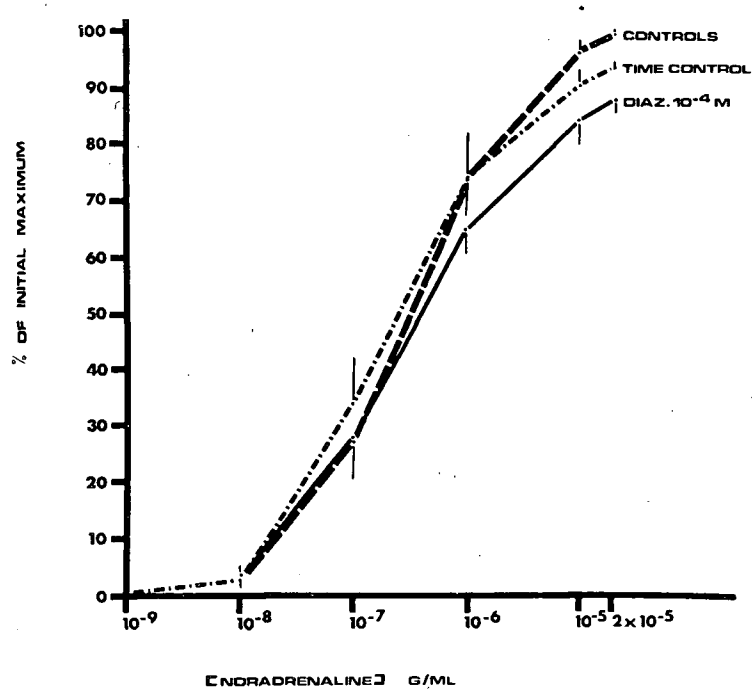


Figure 7. The effect of diazoxide upon noradrenaline dose response curves from rabbit anterior mesenteric veins treated with a depolarizing concentration of ouabain.

Diazoxide was observed to have no significant effect upon noradrenaline dose responses obtained from ouabain depolarized veins. No crossover experiment was performed. The vertical bars represent the standard error of the mean for five veins.

indistinguishable. At noradrenaline doses from 10^{-6} g/ml. to 2 X 10^{-5} g/ml., the time control and experimental responses are somewhat dissimilar, although the standard errors of the mean at maximum response (2 X 10^{-5} g/ml. noradrenaline) are virtually contiguous: experimental, 88.4 per cent $\pm$ 2.2 S.E.; time control, 94.7 per cent $\pm$ 3.5 S.E., (p=greater than 0.10). It would appear that the maximum response achievable under these conditions is little affected by diazoxide at a concentration of 10^{-4} M. No crossover was performed with the ouabain treated veins and no experiments were performed using increased calcium concentrations.

Serotonin

Cumulative dose response curves to serotonin were obtained from isolated anterior mesenteric veins of four rabbits. The threshold concentration for contraction to serotonin was 10^{-8} g/ml. and maximum responses were obtained at 3 X 10^{-4} g/ml. The results of the serotonin responses are illustrated in Figure 8. Diazoxide caused a marked increase in the threshold concentration of serotonin required for contraction and a marked decrease in the response to the maximum concentration of serotonin used (3 X 10^{-4} g/ml.). The crossover demonstrated that diazoxide inhibition of serotonin contractions was reversible.

When serotonin was tested upon four different rabbit anterior mesenteric veins treated with ouabain 10^{-5} M for at least one hour either no or very little contraction response was obtained. As a result, diazoxide was not tested further in this situation. When calcium concentrations were altered between 0.25 and 5.2 mM, in the bathing solution, no consistent changes

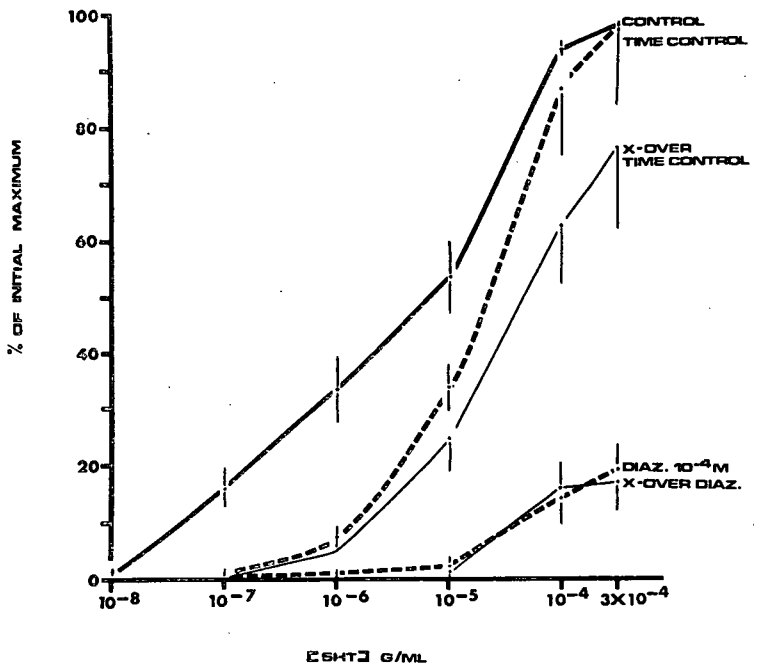


Figure 8. The effect of diazoxide upon serotonin dose response curves from rabbit anterior mesenteric vein.

Diazoxide at 10^{-4} M caused an increase in the threshold concentration of serotonin required for contraction. In addition diazoxide reduced the maximum achievable contraction to serotonin (5HT) within the concentration limits used. A crossover (X-OVER) was performed in this case and diazoxide inhibition was apparently reversible. The vertical bars represent the standard error of the mean for four veins.

could be observed upon the ability of diazoxide to inhibit contractions induced by serotonin.

Procaine

The effects of diazoxide at 10^{-4} M were examined upon procaine dose response curves obtained from nine anterior mesenteric veins (Figure 9). Threshold contraction response to procaine occurred at a concentration just below 10^{-5} g/ml. A maximum response to procaine was observed at 10^{-3} g/ml. concentration. When the veins were pretreated with 10^{-4} M diazoxide, the shape of the dose response curve was altered considerably. There was a shift of contraction threshold concentration of more than ten-fold to the right. Time control responses remained virtually identical to the original control. The inhibitory effects of diazoxide upon procaine induced contraction apparently were surmountable by increasing the concentration of procaine. At the maximum procaine concentration tested (10^{-3} g/ml.) there was very nearly an overlap of experimental (88.3 per cent $\pm$ 8.3 S.E.) and time control curves (95.7 per cent $\pm$ 2.3 S.E.) (p=less than 0.10 and greater than 0.05). When calcium concentrations were raised to 12 mM in a Ringer solution, there was no discernable effect upon the diazoxide antagonism of procaine. In four ouabain depolarized veins (treated with 10^{-5} M ouabain for one hour), procaine was unable to elicit a contractile response from the mesenteric vein.

Calcium Contractures in Depolarizing Solution

In initial experiments, six halved veins were equilibrated in normal Krebs' solution, after which the bathing solution was changed to a zero calcium K^+ Krebs' solution. A contraction then

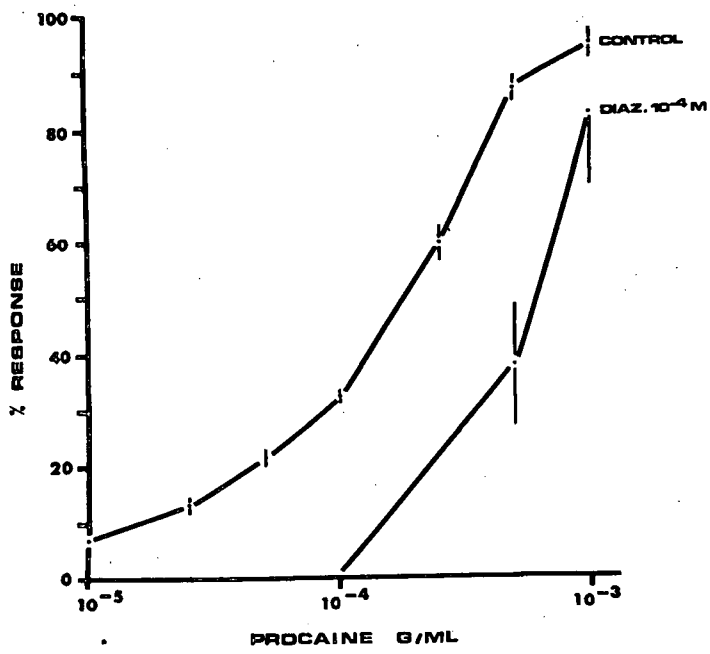


Figure 9. The effect of diazoxide upon procaine dose response curves from the rabbit anterior mesenteric vein.

Diazoxide at 10^{-4} M caused an increase in the concentration of procaine required to initiate contraction. The effect appeared similar to a parallel shift of the control dose response curve to the right. A crossover experiment was not performed with procaine. Maximum responses were statistically similar (p=less than 0.10, greater than 0.05). The vertical bars represent the standard error of the mean for nine veins.

occurred which relaxed gradually. After repeated washing, the tension on the vein returned to a baseline level (500 mg.). Calcium chloride was then added in cumulative stages to obtain a cumulative calcium dose response curve. The results obtained were not consistently reproducible, probably due to precipitation of calcium phosphate in the Krebs' solution at concentrations of calcium above 3 mM.

Because of the difficulties with Krebs' solution precipitation, eight veins from eight rabbits were subjected to the same experimental conditions using a phosphate free K^+ Ringer solution (Figure 10). Reproducible dose response curves were obtained when calcium was added back to the bath fluid. A maximum contraction was observed in the veins at a calcium concentration of 3×10^{-3} M and the minimum concentration that would produce contraction was close to 10^{-5} M. The experimental halves of the veins pretreated for 15 minutes with 10^{-4} M diazoxide, responded in a manner highly suggestive of a non surmountable antagonism when compared with the time controls. The threshold for contraction remained virtually the same in the time control and diazoxide treated veins. Diazoxide treated halves never achieved contraction amplitudes equivalent to those of the time control halves (p=less than 0.025, greater than 0.01). The crossover experiments showed that diazoxide antagonism of calcium chloride was reversible. The crossover control and crossover experimental curves were virtually identical with the time control and experimental curves respectively.

The effects of hydrochlorothiazide at a concentration of 10^{-4} M were also tested upon calcium contractures in zero cal-

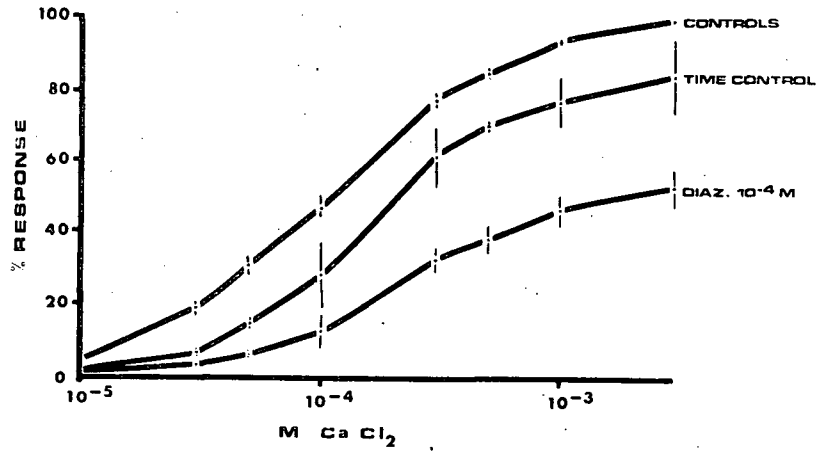


Figure 10. The effect of diazoxide upon calcium dose responses in K^+ Ringer solution.

Diazoxide caused a decrease in the maximum contractile effect of calcium chloride at concentrations of up to 3×10^{-3} M. A crossover experiment demonstrated that this inhibitory effect of diazoxide was reversible. The crossover is not illustrated. The vertical bars represent the standard error of the mean for eight veins.

cium K^+ Ringer solution. This benzothiadiazine, however, did not exert any demonstrable effect upon calcium induced contractions (Figure 11). The control dose response curves in this series resembled those of the diazoxide experiment. However, the hydrochlorothiazide series was carried to a calcium concentration of 3×10^{-2} M, a concentration ten times the maximum used previously. At this high concentration, an additional contraction was observed beyond the maximum at 3×10^{-3} M calcium. No further observations were made regarding this additional contracture.

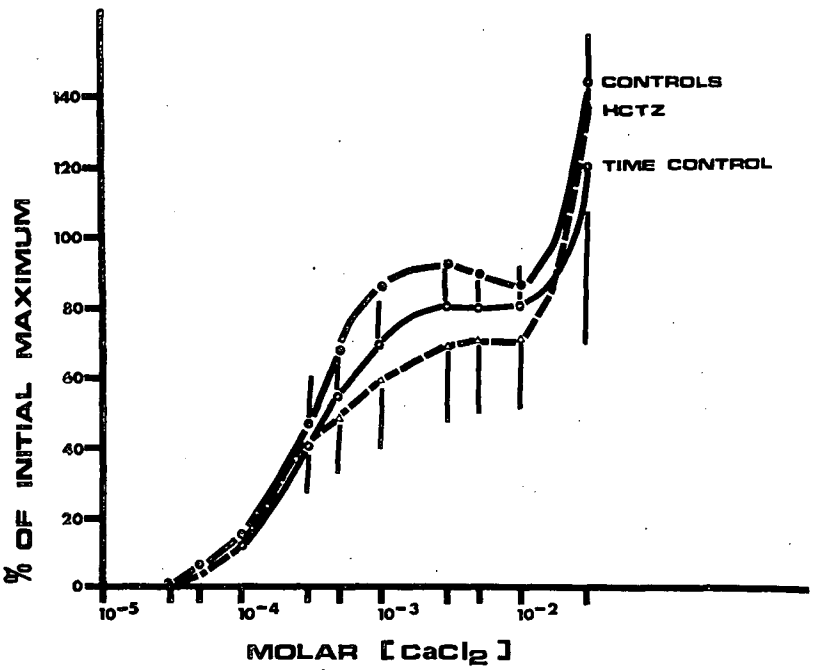


Figure 11. The effect of hydrochlorothiazide upon calcium dose responses in K^+ Ringer solution.

Shown are the control curve (CONTROLS) obtained from both halves of four veins. The hydrochlorothiazide (HCTZ) and time control curves obtained from four half veins are shown with the standard error of the mean (vertical bars) for each point. The time control and experimental curves are not significantly different: at 3.0×10^{-3} M Ca^{++}, p 0.5.

Note the unexplained contraction that developed at very high calcium concentrations (3×10^{-2} M).

DISCUSSION

Diazoxide and Electrical Membrane Phenomena in Smooth Muscle

The results from the sucrose gap experiments on isolated smooth muscle indicate that diazoxide is active at a membrane site as measured by electrical events in each of the three tissues examined: rabbit mesenteric vein, guinea-pig taenia coli, and estrogen dominated rabbit uterus. Spontaneous activity is inhibited in all three preparations within a very short time. In the vein and taenia coli, this inhibition of spontaneity was associated with some degree of membrane hyperpolarization, but this was not observed in the uterus. Spike generation in the uterine strips was converted from multi-spike complexes to single spikes before being abolished completely.

Similar results to these can be obtained if calcium concentrations are altered in the solution bathing the isolated smooth muscle. Increasing the calcium concentration has been shown to hyperpolarize the isolated rat portal vein (Axelsson *et al.*, 1967), the isolated uterus (Bulbring, Casteels, and Kuriyama, 1968), and the isolated guinea-pig taenia coli (Brading, Bulbring and Tomita, 1968). Conversely, these authors demonstrated that removal of calcium from solution bathing isolated smooth muscles has a depolarizing effect in each of these types of smooth muscle. An increased calcium concentration has been shown to increase the rate of spontaneous firing in uteri from estrus animals (Bulbring *et al.*, 1968), but to decrease the rate in rat portal vein (Axelsson *et al.*, 1967) and in guinea-pig taenia coli (Bulbring and Kuriyama, 1963; Brading *et al.*,1969).

If diazoxide inhibits spontaneous contractions by antagonizing calcium, as suggested by Wohl et al. (1967, 1968 a,b) in the case of drug induced contractions, it is unlikely that it acts in the manner of a chelating agent. Adding the chelating agent EGTA, or changing the bathing solution to one containing no calcium, results in depolarization and loss of electrical membrane stability in smooth muscle; whereas diazoxide would appear to stabilize the membrane potential. It is conceivable that calcium, either adsorbed on the cell membrane or at more specific sites, may act to stabilize cell membrane electrical activity in smooth muscle in a similar manner to that suggested by Frankenhauser and Hodgkin (1957) in the squid axon. It is tempting to speculate that an enhancement of calcium stabilization of the membrane of vascular smooth muscles might be the mechanism of action of diazoxide as an antihypertensive agent.

Diazoxide has been shown to reduce the ionic activity of calcium in aqueous solution, perhaps by a chelating or complexing action. If a calcium diazoxide complex were adsorbed onto the cell membrane, it is possible that this might serve as a stabilizer: a calcium channel or other membrane route for calcium ion flux could be blocked, perhaps in a similar manner to the sodium blockade effected by tetrodotoxin in nerves (Kao, 1966; Moore and Narahashi, 1967). Such a mechanism would explain the apparent specificity of diazoxide in antagonizing spontaneous activity in certain smooth muscles apparently possessing calcium mediated action potentials, and not in cardiac tissue, which has a definite sodium mediated action potential spike (albeit with a definite but delayed calcium component) (Reuter and

Beeler, 1969a, b.). The influx of calcium expected in tissues with a calcium spike mediated action potential (Goodford, 1968) should be limited by the calcium carrying capacity of the cell membrane. If this capacity were reduced in smooth muscle, one would expect diminished excitability and contractility. The observation that an increased calcium concentration in the solution bathing mesenteric veins had no effect on the ability of diazoxide to inhibit spontaneous motility, and failed to restore spontaneous activity in veins inhibited by diazoxide, may mean that a critical calcium component (perhaps membrane bound calcium) could become saturated with diazoxide or a diazoxide-calcium complex with which free calcium ions, necessary for spontaneous activity, do not effectively compete.

Strontium, which has been shown to possess membrane "labilizing" properties, is capable of supporting spontaneous action potential activity in mesenteric veins in the absence of calcium. The present results, which demonstrate that diazoxide at 10^{-4} M can inhibit spontaneous activity in mesenteric veins bathed in either strontium or calcium containing solutions, may mean that the drug acts in a manner that interferes with a function of calcium that may also be mediated by strontium.

Two membrane roles for calcium in vascular smooth muscle have been postulated. Calcium probably carries the action potential spike in rabbit anterior mesenteric vein, and has been demonstrated to act as a membrane stabilizer (Axelsson et al., 1967). It is possible that these two properties of calcium are distinct and separate. If this is so, membrane electrical activity in vascular smooth muscle could be blocked at either site

of action of calcium.

The inhibitory effects of diazoxide upon vascular smooth muscle motility may be explained in terms of an effect on calcium. First, if the action of diazoxide upon calcium is analogous to that of tetrodotoxin on sodium permeability in excitable tissues, a blockade of calcium mediated action potentials would be expected. If, on the other hand, diazoxide acts to increase membrane stability by an effect on calcium, this also would explain the inhibitory action of the agent on spontaneous motility in the mesenteric vein.

Strontium, as a calcium analogue, is apparently capable of carrying action potential spikes in the mesenteric vein, and this activity can be inhibited by diazoxide. Strontium, however, possesses little of the stabilizing properties possessed by calcium (Hotta and Tsuiki, 1968). Further, diazoxide is capable of suppressing strontium mediated spontaneous activity even in mesenteric veins that have been treated with EGTA and then washed in zero calcium solutions to remove all divalent cations. Under such conditions, when strontium is introduced into the solution, it is unlikely to exert a membrane stabilizing action. In fact, the inhibitory effect of diazoxide under these conditions appears similar to that observed in normal calcium solutions.

It is possible to infer from these data that the primary action of diazoxide in inhibiting spontaneous motility is likely the blockade of calcium action potential spikes. This tentative conclusion is predicated on a direct involvement of calcium with diazoxide. This need not, of course, be an exclusive action of the drug. However, in the context of the effect of diazoxide

upon excitation contraction coupling in vascular smooth muscle, a modification of the biological role of calcium is a likely mechanism of action of diazoxide.

The sucrose gap apparatus as used, does not lend itself to further analysis of the cell membrane effects of diazoxide. It must be remembered that the sucrose gap device records the algebraic sum of electrical events from a large population of cells and thus is not suitable for obtaining specific information concerning drug effects upon a given cell. This is especially true in tissues such as the rabbit anterior mesenteric vein which do not comprise an especially good syncitium (Cuthbert, Matthews, and Sutter, 1965). Because of these limitations with the sucrose gap, attempts were made to record intracellular potentials from isolated rabbit veins using intracellular glass microelectrodes. This proved unsuccessful, most probably because of the small size of the smooth muscle cells (2 to 3 microns wide and some 10 to 15 microns long) and because of the difficulties of intracellular recording from a tissue that is spontaneously motile. The advantages of intracellular recording would have permitted measurement of true membrane potentials rather than the relative measurement available with the sucrose gap technique. Further, it would have been possible to examine the effects of diazoxide upon evoked action potentials from the mesenteric vein with intracellular recording. This would have enabled determination of action potential configuration and excitation threshold changes not obtainable with the sucrose gap.

Transmural Stimulation of Rabbit Anterior Mesenteric Veins

Diazoxide inhibited electrically induced contractions in

rabbit anterior mesenteric veins. Those contractions due to direct electrical stimulation of the vascular smooth muscle were insensitive to tetrodotoxin or phentolamine, as were myogenic spontaneous contractions. Presumably these contractions were initiated by depolarization of vascular smooth muscle cell membranes. Diazoxide, in inhibiting these contractions, apparently blocked excitation of the membranes or disrupted the excitation contraction coupling process between membrane depolarization and muscle contraction.

Those contractions due to electrical stimulation of nerve endings within the vein were sensitive to tetrodotoxin or phentolamine, as would be expected (Holman and McLean, 1967; Hughes and Vane, 1967). Tetrodotoxin diminishes neuronal excitability and phentolamine blocks the action of the presumptive neurohumor released: noradrenaline. In other words, diazoxide would appear to block contractions due to release of endogenous noradrenaline.

With the apparatus used, the maximum response obtainable from veins was inhibited by diazoxide. Presumably this was due to a reduced membrane excitability attributable to diazoxide. This need not mean however, that the tissue was rendered incapable of membrane electrical activity: sucrose gap recordings showed that the quiescent membrane (inhibited by diazoxide) could still be excited by noradrenaline, serotonin, or procaine. This indicated that although excitation might be diminished by diazoxide, the membrane was still capable of supporting action potential activity under the appropriate stimulus.

Cardiac Muscle and Diazoxide

Diazoxide was observed to have no effect upon the configur-

ation of the rabbit heart action potentials examined. This observation may mean that diazoxide is unable to affect the ionic calcium current component of the cardiac action potential. In addition, spontaneous atrial rate and contractility were completely unaffected by diazoxide. Several points, however, might be raised concerning the nature of these experiments. Orkand and Niedergerke (1956) remarked that the full effect of calcium ion concentration changes on frog ventricular action potentials could not be observed when the heart muscle was stimulated more than once per minute. In the present experiments, rabbit atria and papillary muscles were driven at rates very close to one per second. It is possible that any influence of diazoxide might be more readily observed under stimulus rates similar to those used by Orkand and Niedergerke (1956). Another criticism of the heart experiments is that if calcium ion flux was being affected by diazoxide, any such effect might be examined better by measuring currents rather than potentials. The method of Reuter and Beeler (1969a,b.) for voltage clamping of cardiac muscle would be applicable to such an experiment.

Effects of Diazoxide Upon Drug Induced Contractions

The effects of diazoxide upon dose response curves of noradrenaline, serotonin, and procaine demonstrated the involvement of the excitable cell membrane of vascular smooth muscle as integral to the inhibitory effect of diazoxide upon contractions to these drugs. Noradrenaline dose response curves showed this clearly by the differences observed between the effects of diazoxide on ouabain depolarized rabbit anterior mesenteric veins and on normally polarized veins. Diazoxide was not capable of

eliciting a significant change in noradrenaline dose response curves obtained from depolarized veins. In normally polarized veins, on the other hand, diazoxide caused an apparently competitive and reversible type of inhibition of the dose response curves to noradrenaline. Although excitation contraction coupling in aortae and in mesenteric veins may differ, (as multiunit type differs from single unit type of smooth muscle) and the two tissues may respond differently in some regards to various drugs, it is likely that the antagonism observed between noradrenaline and diazoxide with the veins, is similar to that observed by Wohl et al. (1967) with aortic strips, i.e., diazoxide does not compete directly with noradrenaline.

Noradrenaline may cause contractions either with the membrane potentials of vascular smooth muscle intact or in depolarized muscle. The same pathways of excitation contraction coupling need not be followed in the two cases. In depolarized veins, one would expect that the sequence of events initiated by membrane depolarization would be eliminated or altered. Thus, a drug that causes contraction may do so either by releasing sequestered stores of calcium or by permitting an influx of calcium from the external bathing solution. It is not known what effect altering calcium concentrations would have upon noradrenaline contractions in the presence of ouabain or the effect of diazoxide upon such contractions.

Serotonin contractions of the anterior mesenteric vein of rabbits were markedly inhibited by diazoxide. Inspection of the dose response curves obtained suggests a reversible type of interaction. It is not possible to state the definite nature of the

competition observed, because serotonin is an agent which is not as efficacious as noradrenaline in causing contractions in the vein, and it may be that the apparently insurmountable nature of the interaction may be due only to concentrations of serotonin insufficiently high to effect maximum contracture. In any case, serotonin is capable of contracting mesenteric veins only poorly in tissues exposed to a depolarizing concentration of ouabain. Thus it would appear that serotonin depends to a large extent upon its ability to depolarize and excite the membrane in order to induce contractions. Therefore, one might conclude that in the case of serotonin as with noradrenaline contractions, diazoxide exerts its inhibitory action on serotonin induced membrane excitation.

The nature of diazoxide antagonism of procaine contractions leads one to similar conclusions. The efficacy of procaine as a contractile agent is due apparently only to its ability to excite the normally polarized membrane because procaine fails completely to contract ouabain depolarized rabbit mesenteric veins (Sanders, 1969). Diazoxide inhibits procaine induced contractions in the mesenteric vein in an apparently surmountable and reversible manner.

Contractions initiated by calcium chloride upon mesenteric veins immersed in depolarizing Ringer solution were also inhibited by diazoxide. The purpose of performing this experimental series was to attempt to determine whether calcium could be antagonized directly by diazoxide, as suggested by Wohl (1967, 1968a,b.). The results indicate a definite, apparently insurmountable, but reversible inhibition of calcium contractures by diazoxide at 10^{-4}

M. Hydrochlorothiazide, tested under the same conditions upon another series of mesenteric veins, demonstrated no ability to diminish calcium contractures.

The locus of action of the calcium used in this series of experiments with K^+ depolarized veins is not certain. Nor for that matter is the locus of the diazoxide interaction known with certainty. It is reasonable to speculate, however, that calcium exerts its contractile effect by a direct influx across the cell membrane to activate contractile proteins. If this is the case, diazoxide could be acting in several ways. First, diazoxide could be entering the cell along with calcium and competing with calcium at the muscle protein site. This is unlikely, however, as diazoxide is a reasonably large molecule and its inhibitory activity is readily reversible in regard to calcium contractures. This reversibility would be unlikely if the agent were bound to internal muscle proteins. More likely, diazoxide acts at the surface of the tissue to reduce the influx of calcium postulated to cause contraction in depolarized veins.

Although a further possibility of testing diazoxide-calcium would be to use calcium to contract glycerinated preparations of smooth muscle, this was not explored. It was felt that the results obtainable would reflect only the effect of diazoxide upon reducing the ionic activity of a calcium solution.

Another possible mode of action for diazoxide is its ability to inhibit 3',5'-cyclic adenylic acid (cyclic AMP) phosphodiesterase. It might be that the cyclic nucleotide mediates relaxation in the mesenteric vein. Caffeine, a well known inhibitor of the phosphodiesterase (Drummond, 1967) has an effect upon mesen-

teric veins much like that of diazoxide: relaxation and inhibition of spontaneous electrical activity (Somlyo, 1968b). Similarly, isopropylnoradrenaline, like adrenaline an adenyl cyclase stimulant (Drummond, 1967), is capable of inhibiting spontaneous activity in rat mesenteric veins (Johansson et al., 1967) and of relaxing depolarized uterus (Schild, 1967).

The effect of depolarization with ouabain on the cyclic AMP content of rabbit mesenteric vein is unknown. Diazoxide, under these conditions, fails to inhibit noradrenaline contractions. An explanation of this observation may await correlative information on the cyclic nucleotide in depolarized veins.

It is not inconceivable that at a metabolic level, phosphodiesterase inhibition by diazoxide might be its mechanism of action. Neither this consideration nor the calcium competition postulated by Wohl (1967, 1968a,b) need be mutually exclusive. No attempt, however, was made in these experiments to investigate the metabolic basis of diazoxide action upon smooth muscle.

In summary, diazoxide would appear to be effective as an inhibitor of smooth muscle contraction chiefly because of its effect upon biological membranes. The specific site remains unknown, but may relate to the role of calcium in regulating the excitability of active cell membranes. The fact that diazoxide inhibits spontaneous activity of the anterior mesenteric vein may or may not have functional significance in hypertension. Conceivably, one could examine the microcirculation in mesentery or in the bulbar conjunctivum (Lee, 1951; Jackson, 1958) to see whether inhibition of spontaneous vasomotion would take place in the microcirculation of the intact organism in response to

injected diazoxide. If inhibition of spontaneous vasomotion were affected by diazoxide in hypertensive patients, this might explain the rapid antihypertensive action of this agent compared with chlorothiazide or hydrochlorothiazide, which did not inhibit spontaneous motility in the smooth muscles examined.

BIBLIOGRAPHY

Axelsson, D., and Wahlstrom, B.: Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein. Circ. Res. 21:609-618, 1967.

Axelsson, J., Gudmundsson, G., and Wahlstrom, B.: Quantitative analysis of the correlation between electrical and mechanical activity in smooth muscle. Acta Physiol. Scand. 73: 36A-37A, 1968.

Barr, L., Dewey, M.M., and Berger, W.: Propagation of action potentials and the structure of the nexus in cardiac muscle. J. Gen. Physiol. 48:797-823, 1965.

Berger, W., and Barr, L.: Use of rubber membranes to improve sucrose-gap and other electrical recording techniques. J. Applied Physiol. 26:378-382, 1969.

Bohr, D.F.: Contraction of vascular smooth muscle. Canad. Med. Ass. J. 90:174-179, 1964.

Bozler, E.: Conduction, automaticity and tonus of visceral muscles. Experientia (Basel) 4:213-218, 1948.

Brading, E., Bulbring, E., and Tomita, T.: The effect of sodium and calcium on the action potential of the smooth muscle of the guinea pig taenia coli. J. Physiol. 200:637-654, 1969.

Briggs, A.H.: Calcium movements during potassium contracture in isolated rabbit aortic strips. Amer. J. Physiol. 203: 849-852, 1962.

Bulbring, E., Casteels, R., and Kuriyama, H.: Membrane potential and ion content in cat and guinea-pig myometrium and the response to adrenaline and noradrenaline. Brit. J. Pharmac. 34:388-407, 1968.

Bulbring, E., and Kuriyama, H.: Effects of changes in the external sodium and calcium concentrations on spontaneous electrical activity in smooth muscle of guinea-pig taenia coli. J. Physiol. 166:29-58, 1963.

Burnstock, G., Holman, M.E., and Prosser, C.L.: Electrophysiology of smooth muscle. Physiol. Rev. 43:482-527, 1963.

Burnstock, G., and Straub, R.W.: A method for studying the effects of ions and drugs on the resting and action potentials in smooth muscle with external electrodes. J. Physiol. 140: 156-167, 1958.

Conway, J., and Lauwers, P.: Hemodynamic and hypotensive effects of long-term therapy with chlorothiazide. Circulation 21: 21-27, 1960.

Conway, J., and Palmero, H.: The vascular effect of the thiazide diuretics. Arch. Int. Med. 111:203-207, 1963.

Cuthbert, A.W., Matthews, E.K., and Sutter, M.C.: Spontaneous electrical activity in a mammalian vein. Proc. Physiol. Soc. J. Physiol. 176:1-2, 1964.

Cuthbert, A.W., and Sutter, M.C.: Electrical activity in a mammalian vein. Nature 202:95, 1964.

Cuthbert, A.W., and Sutter, M.C.: The effects of drugs on the relation between the action potential discharge and tension in a mammalian vein. Brit. J. Pharmacol. Chemother. 25:592-601, 1965.

Daniel, E.E.: On the mechanism of antihypertensive action of hydrochlorothiazide in rats. Circulation Res. 11:941-954, 1962.

Daniel, E.E., and Nash, C.W.: The effects of diuretic and non-diuretic benzothiadiazine and of structurally related diuretic drugs on active ion transport and contractility in smooth muscles. Arch. Int. Pharmacodyn. 158:139-154, 1965.

Dollery, C.T., Pentecost, B.L., and Saman, N.A.: Drug induced diabetes. Lancet 2:735-737, 1962.

Drummond, G.I.: Muscle metabolism. Fortschritte der Zoologie. 18:359-429, 1967.

Finnerty, F.A., Kakaviatos, N., Tuckman, J., and Magill, J.: Clinical evaluation of diazoxide: a new treatment for acute hypertension. Circulation 28:203-208, 1963.

Finnerty, F.A.: Hypertensive emergencies. Amer. J. Cardiol. 17: 652-655, 1966.

Finnerty, F.A., Davidov, M., and Kakaviatos, N.: Hypertensive vascular disease: long term effect of rapid repeated reductions of arterial pressure with diazoxide. Amer. J. Cardiol. 19:377-384, 1967.

Frankenhaeuser, B., and Hodgkin, A.L.: The action of calcium on the electrical properties of squid axons. J. Physiol. 137: 218-244, 1957.

Freed, S.C., St. George, S., and Beatty, D.: Mechanism of antihypertensive action of theazides. Proc. Exp. Biol. Med. 112:735-737, 1963.

Freis, E.F.: Hemodynamics of hypertension. Physiol. Rev. 40:27-54, 1960.

Funaki, S., and Bohr, D.F.: Electrical and mechanical activity of isolated vascular smooth muscle of the rat. Nature 203:192-194, 1964.

Goodford, P.J.: The calcium content of the smooth muscle of the guinea-pig taenia coli. J. Physiol. 192:145-157, 1967.

Hagiwara, S., and Nakajima, S.: Differences in sodium and calcium spikes as examined by application of tetrodotoxin, procaine and manganese ions. J. Gen. Physiol. 49:793-805, 1966.

Hinke, J.A.M., and Wilson, M.L.: Effects of electrolytes on contractility of artery segments in vitro. Amer. J. Physiol. 203:1161-1166, 1962.

Hinke, J.A.M., Wilson, M.L., and Burnham, S.C.: Calcium and the contractility of arterial smooth muscle. Amer. J. Physiol. 206:211-217, 1964.

Hinke, J.A.M.: Effect of calcium upon contractility of small arteries from D.C.A. and hypertensive rats. Circ. Res. 18-19 (suppl.1):23-33, 1966.

Holman, M.E.: Membrane potentials recorded with high-resistance micro-electrodes; and the effects of changes in ionic environment of the electrical and mechanical activity of the smooth muscle of the taenia coli of the guinea-pig. J. Physiol. 141: 464-488, 1958.

Holman, M.E., Kasby, C.B., Suthers, M.B., and Wilson, J.A.F.: Some properties of the smooth muscle of rabbit portal vein. J. Physiol. 196:111-132, 1968.

Holman, M.E., and McLean, A.: The innervation of sheep mesenteric veins. J. Physiol. 190:55-69, 1967.

Hotta, Y., and Tsukui, R.: Effect on the guinea-pig taenia coli of the substitution of strontium or barium ions for calcium ions. Nature 217:867-869, 1968.

Hudgins, P.M., and Weiss, G.B.: Differential effects of calcium removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium. J. Pharmacol. Exp. Ther. 159:91-97, 1968.

Hughes, J., and Vane, J.R.: An analysis of the responses of the isolated portal vein of the rabbit to electrical stimulation and to drugs. Brit. J. Pharmacol. Chemother. 30:46-66, 1967.

Jackson, W.B.: The functional activity of the human conjunctival capillary bed in hypertensive and normotensive subjects. Amer. Heart J. 56:222-235, 1958.

Johansson, B., Jonsson, O., Axelsson, J., and Wahlstrom, B.: Electrical and mechanical characteristics of vascular smooth muscle response to norepinephrine and isoproterenol. Circ. Res. 21:619-633, 1967.

Johansson, B., and Ljung, B.: Spread of excitation in the smooth muscle of the rat portal vein. Acta. Physiol. Scand. 70: 312-322, 1967a.

Johansson, B., and Ljung, B.: Sympathetic control of rhythmically active vascular smooth muscle as studied by a nerve-muscle preparation of portal vein. Acta. Physiol. Scand. 70:299-311, 1967b.

Kao, C.Y.: Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena. Pharmacol. Rev. 18:997-1047, 1966.

Kapitola, J., Kuchel, O., Schreiberova, O., and Jahoda, I.: Influence of diazoxide on regional blood flows. Experientia 24:242, 1968.

Koblenzer, P.J., and Baker, L.: Hypertrichosis lanaginosa associated with diazoxide therapy in prepubertal children: a clinicopathologic study. Ann. N. Y. Acad. Sci. 150:373-382, 1968.

Landesman, R., and Wilson, K.H.: The relaxant effect of diazoxide on isolated gravid and nongravid human myometrium. Amer. J. Obstetrics Gynecol. 101:120-125, 1968.

Landesman, R., Coutinho, E.M., Wilson, K.H., and Lopes, A.C.V.: The relaxant effect of diazoxide on nongravid human myometrium in vivo. Amer. J. Obstetrics Gynecol. 102:1080-1084, 1968.

Lee, R.E., and Holze, E.A.: Peripheral vascular hemodynamics in the bulbar conjunctiva of subjects with hypertensive vascular disease. J. Clin. Inves. 30:539-546, 1951.

Marshall, J.M.: Relation between the ionic environment and the action of drugs on the myometrium. Fed. Proc. 27:115-119, 1968.

Matthews, E.K., and Sutter, M.C.: Ouabain -induced changes in the contractile and electrical activity, potassium content, and response to drugs, of smooth muscle cells. Can. J. Physiol. Pharmacol. 45:509-520, 1967.

Mellander, S., and Johansson, B.: Control of resistance, exchange and capacitance functions in the peripheral circulation. Pharmacol. Rev. 20:117-196, 1968.

Moore, J.W., and Narahashi, T.: Tetrodotoxin's highly selective blockage of an ionic channel. Fed. Proc. 26:1655-1663, 1967.

Nayler, W.G., M^{c}Innes, I., Swann, J.B., Race, D., Carson, V., and Lowe, T.E.: Some effects of the hypotensive drug diazoxide on the cardiovascular system. Amer. Heart J. 75:223-232, 1968.

Northover, B.J.: The effect of drugs on the constriction of isolated depolarized blood vessels in response to calcium or barium. Brit. J. Pharmacol. 34:417-428, 1968.

Orkand, R.K., and Niedergerke, R.: Heart action potential: dependence on external calcium and sodium ions. Science 146: 1176, 1964.

Potter, J.M., and Sparrow, M.P.: The relationship between the calcium content of depolarized mammalian smooth muscle and its contractility in response to acetylcholine. Aust. J. Exp. Biol. Med. Sci. 46:435-446, 1968.

Preziosi, P., Bianchi, A., Loscalzo, B., and DeSchaepdryver, A.F.: On the pharmacology of chlorothiazide with special regard to its diuretic and antihypertensive effects. Arch. Int. Pharmacodyn. 118:467-495, 1959.

Reuter, H., and Beeler, G.W.: Sodium current in ventricular myocardial fibers. Science, 163:397-401, 1969.

Reuter, H., and Beeler, G.W.: Calcium current and activation of contraction in ventricular myocardial fibers. Science 163: 399-401, 1969.

Rhodin, J.A. G.: The ultrastructure of mammalian arterioles and precapillary sphincters. J. Ultrastruct. Res. 18:181-223, 1967.

Robb, G.H.: A fatal reaction to diazoxide. Postgrad. Med. J. 45: 43-45, 1969.

Rubin, A.A.: Hemodynamic aspects of certain antihypertensive agents. Angiology 14:74-78, 1963.

Rubin, A.A., Beauregard, S.C., Hausler, L.M., Zitowitz, L., and Winbury, M.M.: A non-diuretic benzothiadiazine with antihypertensive properties. The Pharmacologist 3:65, 1961a.

Rubin, A.A., Roth, F.E., Taylor, R.M., and Rosenkilde, H.: Pharmacology of diazoxide, an antihypertensive, nondiuretic benzothiadiazine. J. Pharmacol. Exp. Ther. 136:344-352, 1962.

Rubin, A.A., Roth, F.E., and Winbury, M.M.: A non-diuretic benzothiadiazine with anti-hypertensive properties. Nature 192: 176-177, 1961b.

Rubin, A.A., Roth, F.E., Winbury, M.M., Topliss, J.G., Sherlock, M.H., Sperber, N., and Black, J.: New class of antihypertensive agents. Science 133:2067, 1961c.

Rubin, A.A., Zitowitz, L., and Hausler, L.: Acute circulatory effects of diazoxide and sodium nitrite. J. Pharmacol. Exp. Ther. 140:46-51, 1963.

Saker, B.M., Matthew, T.H., Eremin, J., and Kincaid-Smith, P.: Diazoxide in the treatment of the acute hypertensive emergency. Med. J. Aust. :592, 1968.

Sanders, H.D.: Procaine-induced contraction in vein and its modification by drugs. Can. J. Physiol. Pharmacol. 47:218-221, 1969.

Severson, D.L., and Sutter, M.C.: Smooth muscle contraction: effect of zero calcium and cation substitution. Proc. Can. Fed. Biol. Sci. 12:36, 1969.

Schild, H.O.: The action of isoprenaline in the depolarized rat uterus. Brit. J. Pharmacol. Chemother. 31:578-592, 1967.

Somlyo, A.P., and Somlyo, A.V.: Vascular smooth muscle. Pharmacol. Rev. 20:197-272, 1968a.

Somlyo, A.V., and Somlyo, A.P.: Electromechanical and pharmacomechanical coupling in vascular smooth muscle. J. Pharmacol. Exp. Ther. 159:129-145, 1968b.

Speden, R.N.: Electrical activity of single smooth muscle cells of the mesenteric artery produced by splanchnic nerve stimulation in the guinea pig. Nature 202:193-194, 1964.

Stampfli, R.: A new method for measuring membrane potentials with external electrodes. Experientia 10:508-509, 1954.

Steedman, W.M.: Micro-electrode studies on mammalian vascular muscle. J. Physiol. 186:382-400, 1966.

Su, C., Bevan, J.A., and Ursillo, R.C.: Electrical quiescence of pulmonary artery smooth muscle during sympathomimetic stimulation. Circulation Res. 15:20-27, 1964.

Tabachnick, I.A., and Gulbenkian, A.: Mechanism of diazoxide hyperglycaemia in animals. Ann. N. Y. Acad. Sci. 150 (art 2): 191-467, 1968.

Taylor, R.M., and Rubin, A.A.: Studies on the renal pharmacology of diazoxide, an antidiuretic benzothiadiazine. J. Pharmacol. Exp. Ther. 144:284-292, 1963.

Tobian, L.J., and Chesley, G.: Calcium content of arterial walls in normotensive and hypertensive rats. Hypertension 13:187-190, 1965.

Trail, W.M.: Intracellular studies on vascular smooth muscle. J. Physiol. 167:17-18, 1963.

Washizu, Y.: Procaine on smooth muscle. Comp. Biochem. Physiol. 27:121-126, 1968.

Waud, D.R.: Pharmacological receptors, Pharmacol. Rev. 20:49-88, 1968.

Waugh, W.H.: Role of calcium in contractile excitation of vascular smooth muscle by epinephrine and potassium. Circ. Res. 11:927-940, 1962.

Wohl, A.J., Hausler, L.M., and Roth, F.E.: Studies on the mechanism of antihypertensive action of diazoxide: in vitro vascular pharmacodynamics. J. Pharmacol. Exp. Ther. 158:531-539, 1967.

Wohl, A.J., Hausler, L.M., and Roth, F.E.: Mechanism of the antihypertensive effect of diazoxide: in vitro vascular studies in the hypersensitive rat. J. Pharmacol. Exp. Ther. 162:109-114, 1968a.

Wohl, A.J., Hausler, L.M., and Roth, F.E.: The role of calcium in the mechanism of the antihypertensive action of diazoxide. Life Sciences. 7:381-387, 1968b.

Woodbury, J.W., and Brady, A.J.: Intracellular recordings from moving tissues with a flexibly mounted electrode. Science 123:100-101, 1956.