Electrical depolarization of the muscle cell membrane has been generally considered to be an essential part of the physiological excitation of muscle cells which initiates contraction. However, there is direct evidence for electrical inexcitability of the membrane receptors in frog skeletal muscle, and complete depolarization by immersion in isosmotic potassium-rich solution does not abolish the contractile responses of visceral smooth muscle to acetylcholine. Further examination of the question of whether depolarization is an essential step in muscular excitation is therefore indicated.

In this investigation, contractile reactions of mammalian arteries of small caliber have been studied. The results indicate that epinephrine normally excites the contractile process of arterial smooth muscle by a mechanism which does not operate primarily through membrane depolarization or sodium ion influx. Adrenergic neurohormones appear to cause vasoconstriction by a primary reaction at receptor sites of the muscle cell membrane with or without concomitant depolarization. This adrenergic membrane reaction may primarily involve calcium which migrates and activates the contractile mechanism of vascular smooth muscle.

Methods

Segments of intestinal arteries, 1.4- to 2.2-cm. long with an external diameter of 0.5 to 1.0 mm. (intramesenteric branches of the superior mesenteric artery), were excised from anesthetized dogs. The segments were stripped of most of their adventitial sheaths and individually attached to the downstream end of a 23-gauge stainless steel T-cannula. Segments were not used if they contained side branches of microscopic flows. As illustrated in figure 1, constant-flow perfusion of the small arterial segment was then accomplished with a low-flow perfusion pump (model TM-11 Sigmamotor Pump) with stroke output substantially independent of variations in downstream pressure. Flow rates in various experiments were set at 0.75 to 0.90 ml/min. Without recirculation, the perfusion solution was conducted through Tygon plastic tubing of 1.3-mm. internal diameter to the downstream end of the pump, except for a 0.7-cm. long segment of 3-mm. bore rubber tubing interposed just upstream from the pump. This rubber segment was used as the site of injection of small volumes of test solutions. Polyethylene tubing of 0.1-mm. internal diameter (PE 50, Clay-Adams) was employed as the perfusate conduit downstream from the pump to the arterial T-cannula. The fluid volumes contained in the tubing system from reservoir to injection site and from the rubber injection site to the T-cannula were 0.45 ml. in each case. A strain-gauge pressure transducer, attached to the side arm of the T-cannula with 3-mm. bore rubber tubing interposed to dampen the pulsatile perfusate pressure, measured the arterial pressure immediately upstream from the arterial segment. The pressure signal was amplified and recorded on moving film by an Electronics for Medicine oscillographic recorder. Constrictor responses of the blood vessel segment were thus measured by upstream arterial pressure elevations which were proportional to the changes in arterial resistance. The arterial segment lying within a slightly inclined plastic trough was kept immersed in its own perfusion fluid which emerged from the distal end of the artery at atmospheric pressure. The temperature of the perfusate at the arterial segment was maintained at 35 to 36 C. by immersion of the 0.1-mm. bore tubing between the pump and arterial cannula in a stirred water bath at 38 C. Silver wire electrodes, 1.4-cm. long, were fixed in the trough alongside the mesenteric segment and were kept bathed in the small pool of effluent perfusate. These electrodes were used for electrical stimulation of the segment which was accomplished by use of a 60-cycle sine wave (A.C.) for 10 seconds; the root mean square voltage was measured under load.
Test doses of the following solutions were injected in 0.1-ml volumes as a bolus upstream: dl-epinephrine HCl (U.S.P.) in 5 per cent glucose containing $10^{-5}$ ascorbic acid; 5 per cent glucose containing $10^{-5}$ ascorbic acid (this was the control injection); piperoxan HCl (Norek) diluted with 5 per cent glucose; and proenine HCl (U.S.P.) in 5 per cent glucose. The dose of epinephrine chosen for study was one which caused a vasoconstrictor effect lasting two to five minutes, with a peak upstream arterial pressure rise between 30 and 120 mm. Hg when the artery was perfused with the isosmotic solution containing NaCl 140 mM/L. This response was usually produced by a 1- to 2-µg. injection of epinephrine, and this response was intermediate between threshold and maximal contractile responses to epinephrine stimulation.

The following perfusion solutions were employed: *Isosmotic solution, NaCl, 140 mM/L:* made by combining (mM/L.) NaCl, 140; KCl, 4.0; CaCl₂, 1.6; MgCl₂, 0.7; Na₂HPO₄-NaH₂PO₄ buffer of pH 7.4, 2.0; glucose, 5.5; creatine, 0.3; with final pH adjusted to 7.4 with NaOH. *Isosmotic solution, KCl, 144 mM/L:* made by combining (mM/L.) KCl, 144; CaCl₂, 1.6; MgCl₂, 0.7; K₂HPO₄-KH₂PO₄ buffer of pH 7.4, 2.0; glucose, 5.5; creatine, 0.3; with final pH adjusted to 7.4 with KOH. *Isosmotic solution, Na₂SO₄, 96 mM/L:* made by combining Na₂SO₄, 96 mM/L., with the same above concentrations of CaCl₂, MgCl₂, glucose, creatine, and K₂HPO₄-KH₂PO₄ buffer; with final pH adjusted to 7.4 with KOH. *Isosmotic solution, K₂SO₄, 96 mM/L:* made by combining K₂SO₄, 96 mM/L., with the same above concentrations of CaCl₂, MgCl₂, glucose, creatine, and K₂HPO₄-KH₂PO₄ buffer; with final pH adjusted to 7.4 with KOH. *Isosmotic solution, LiCl, 144 mM/L:* made similarly to the isosmotic solution containing 144 mM KCl/L., except for the substitution of an isosmotic amount of LiCl in place of the KCl. (The
NaCl-rich solution used as a control for this solution contained 144 mM NaCl/L, and a similar amount of K ion, 3.7 mM/L, by omission of the KCl and use of the K rather than Na phosphate buffer. The total osmolarity of each of the above solutions, calculated assuming complete salt dissociation, was 307 mosm/L. Isosmotic solution, sucrose, 280 mM/L: made similarly to the isosmotic solution containing 140 mM NaCl/L, except for the substitution of an isosmotic amount of sucrose for the NaCl and the substitution of tris buffer (trishydroxymethyl-amino methane), 0.75 mM/L, in place of the Na phosphate buffer; with adjustment of pH to 7.4 by HCl. (The NaCl-rich solution used as the control for the sucrose-rich perfusate also contained 0.75 mM tris buffer/L, in place of the Na phosphate buffer, and both solutions had an osmolarity of 302 mosm/L.)

Isosmotic solution, K_2SO_4: 119 mM/L: made similarly to the K_2 SO_4, 96 mM/L, except for the greater concentration of K_2 SO_4. This solution was hyperosmotic to the isosmotic solution containing NaCl, 140 mM/L, when based on the general assumption of complete ionization of the K_2 SO_4, NaCl, and KCl salts, but was isosmotic when calculated from electrical equivalent conductance measurements.

The following was the experimental procedure performed. After a number of minutes of initial perfusion with the isosmotic solution containing NaCl, 140 mM/L, control arterial responses to epinephrine injections and electrical stimulation were obtained in this solution. Then, a change to one or more different types of perfusion solution was made with determinations of the vascular responses to stimuli. Finally, the perfusate was changed back to the isosmotic solution containing NaCl, 140 mM/L, and the responses of the arterial segment were subsequently determined as a final control. The reactivity of the arterial segments, after the initial several minutes of perfusion, generally remained unchanged or increased slightly during perfusion for as long as four to five hours.

Results

RESPONSES OF ARTERIES IN SOLUTIONS OF HIGH POTASSIUM CHLORIDE CONTENT

Upon changing from the control perfusate containing NaCl, 140 mM/L, (in which the arterial segments were relaxed in the absence of applied stimuli) to the isosmotic solution containing KCl, 144 mM/L, an intense vasoconstriction developed immediately. This potassium-induced contraction gradually decreased to a relatively steady state of reduced contracture after 15 or more minutes of continued perfusion with potassium chloride-rich solution; the responsiveness of the arterial segment was then tested.

As illustrated in figure 2, in the isosmotic solution containing KCl, 144 mM/L, the amplitude of the arterial pressure elevation produced by epinephrine was similar to that produced by the same amount of epinephrine when the blood vessel was in the isosmotic sodium chloride-rich solution. Relaxation of the epinephrine-contracted smooth muscle was delayed, however, for the epinephrine-induced arterial pressure rise persisted much longer in the solution of high potassium chloride content.

Vascular responses to previously supra-threshold amounts of alternating current were severely reduced or abolished in the isosmotic solution containing KCl, 144 mM/L. However, in the latter, increasing the voltage of the stimulus two to four times elicited vasoconstrictor responses with delayed relaxation.

RESPONSES OF ARTERIES IN ISOSMOTIC SOLUTIONS OF HIGH POTASSIUM SULFATE CONTENT

As evidenced by direct intracellular recording and by extracellular recording using the sucrose-gap technique, isosmotic solutions of high potassium chloride content abolished most, but not all, of the resting potential of smooth muscle; and isosmotic solutions of high potassium sulfate content produced complete membrane depolarization of smooth muscle cells. Therefore, the behavior of arterial segments was also studied in solutions of high potassium sulfate content which were devoid of sodium ion. The solutions employed contained 96 and 119 mM K_2SO_4/L and only 4.6 mM/L of the chloride ion. The univalent cation concentrations of these solutions prevented gross calcium sulfate precipitation and seemingly permitted adequate ionization of the 1.6 mM of Ca/L, in spite of some association of calcium and sulfate as ion pairs.

In the solutions containing 96 and 119 mM K_2SO_4/L, the potassium-induced contractions
Serial records of the pressure responses to epinephrine produced by arterial muscle in isosmotic solutions of high sodium chloride and high potassium chloride content. Duration of perfusion in the respective solutions before these records were taken is indicated. Two µg. of epinephrine or an equal volume of the vehicle alone (5 percent glucose + 10⁻⁴ ascorbate) were injected at the marked instances.

with gradual partial relaxation and the vascular responses to epinephrine and electrical stimulation were similar to those reactions previously described in the isosmotic solution containing KCl, 144 mM/L. Figure 3 illustrates a typical experiment in which the solution containing K₂SO₄, 96 mM/L, was employed. The amplitude of the arterial pressure response to 1 µg. of injected epinephrine was quantitatively similar to the pressure response produced by the same amount of epinephrine in the isosmotic solution containing NaCl, 140 mM/L. However, in the solution of high potassium sulfate content, the relaxation of the epinephrine-induced contraction was delayed. Figure 3 also reveals that the vasoconstrictor responses to epinephrine were reproducible even in the isosmotic solution containing K₂SO₄, 96 mM/L. This figure also shows that the epinephrine response in the sodium sulfate-rich solution was not appreciably modified from the response obtained in the sodium chloride-rich solution; this is evidence that the free ionic calcium concentration was not altered sufficiently by the high sulfate concentration to affect significantly vascular reactivity to epinephrine.

As illustrated in figure 4, the vasoconstrictor reactions to electrical stimulation were not depressed by replacing sodium chloride in the perfusate by an isosmotic amount of sodium sulfate. However, isosmotic solutions of high potassium sulfate content depressed the contractile responses to electrical stimulation.
Considerably larger electrical currents evoked vasoconstriction in the potassium sulfate-rich solution, as shown in figure 4, with delayed relaxation times for the contracted muscle cells.

It was also found that storing arterial segments in isosmotic solution of high potassium sulfate content for 24 hours at 7 C. did not abolish their smooth muscle reactivity to epinephrine in this solution after rewarming to 36 C.

**EFFECT OF ADRENERGIC BLOCKING AGENT ON CONSTRICTOR RESPONSES OF DEPOLARIZED ARTERIES**

Piperoxan hydrochloride, a short-acting competitive antagonist of epinephrine at the...
Serial records of the pressure responses to electrical stimulation produced by arterial muscle in isosmotic solutions of high sodium chloride, high sodium sulfate, and high potassium sulfate content. Duration of perfusion in the respective solutions before these records were taken is as marked. Alternating current of 5.5 to 5.8 or 11 volts was applied for 10 seconds at the marked instances.

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excitatory or alpha adrenergic receptor sites of vascular smooth muscle was used to determine whether antagonists of epinephrine also specifically acted on depolarized vascular smooth muscle. As shown in figure 5, microgram quantities of piperoxan injected 45 seconds before the injection of epinephrine largely prevented (reversibly) the contractile responses to 1 μg. of epinephrine in the potassium-depolarized vessel as in the artery per-
fused with the nondepolarizing solution containing NaCl, 140 mM/L.

Figure 5 also reveals that piperoxan, in a dose which largely inhibited responses to the injected epinephrine, inhibited to a lesser degree the vasoconstrictor response to electrical stimulation in the sodium chloride–rich solution. This dose of piperoxan did not depress the vasoconstrictor response to injected barium ion. These findings suggest that the mode of action of electrical stimulation in these experiments, including its action on the potassium-depolarized artery, was (at least) partly mediated through release of adrenergic neurohormone from nerve endings or stores contained within the arterial wall. On the other hand, injected procaine impaired vasoconstrictor responses to electrical stimulation in proportion to its impairment of responses to injected epinephrine. This proportionate depression suggests that procaine was unable to inhibit any existing electrically excited vasoconstrictor neural elements without concomitant depression of the vascular smooth muscle itself, and that the electrical current exerted a significant and large excitatory effect directly on the muscle cells.

EPINEPHRINE RESPONSES OF ARTERIES IN ISOSMOTIC SOLUTIONS OF HIGH LITHIUM CHLORIDE AND SUCROSE CONTENT

Within the first several minutes of perfusion with the isosmotic solution in which the sodium ion had been replaced by lithium, epinephrine-induced muscular contractions were unimpaired or slightly augmented, and the relaxation phase was moderately prolonged. However, with perfusion in the non-sodium isosmotic solution containing LiCl, 144 mM/L, for many minutes, the contractile or vasoconstrictor response to injected epinephrine decreased moderately. Lithium impaired both the speed of muscular contraction and the total amount of amplitude of contraction, while the relaxation of the vascular contraction was slowed in simple proportion to the impaired contractile process (see fig. 6). A noteworthy observation is that epinephrine vasoconstrictor responses did occur, however, in the, apparently, virtual absence of sodium from the extracellular surface of the plasma membrane of the smooth muscle cells. This finding opposes the report of Horn, Krohn, and Zweifach.14

Upon changing from the isosmotic solution of high sodium chloride content to the electrolyte solution in which sucrose replaced osmotically all of the sodium salts previously used, extreme and nearly maximal vasoconstriction promptly developed. This vasoconstriction was long sustained in its intensity, in contrast to the potassium-induced contracture described above, which lessened in amount after several minutes. Injection of epinephrine produced only a slight contractile response, presumably slight because of nearly maximal vasoconstriction already coexisting, with little relaxation of the additional epinephrine-induced contraction. As illustrated in figure 7, a second injection of epinephrine produced a still smaller arterial pressure elevation with very little relaxation over many subsequent minutes of perfusion with the sucrose-rich fluid, despite the apparent washout downstream of the injected epinephrine. It is noteworthy that this isosmotic solution, which contained 1.6 mM Ca/L, 3.7 mM K/L, and 0.7 mM Mg/L, was of very low ionic strength because of the absence of sodium salt. Upon return to perfusion with the isosmotic solution containing NaCl, 140 mM/L, the extreme constriction of the arterial segment promptly disappeared, although the reactivity of the vessel to epinephrine was not completely restored.

Discussion

In this study employing constant-flow perfusion, the contractile responses of the arterial segments to the same dose of epinephrine produced absolute pressure elevations of similar magnitude in the potassium-depolarized state and in the control, previously polarized state. However, a closer index of the contractile responses of the muscle cells than the arterial pressure elevations would be determinations of the active muscular tensions developed in the responses. The mural tensions
Serial records of the arterial pressure responses to epinephrine and their antagonism by piperoxan in isosmotic solutions of high sodium chloride and high potassium sulfate content. Duration of perfusion in the respective solutions before these records were taken is as marked. One μg. of epinephrine, an equal volume of vehicle alone (5 per cent glucose + 10⁻³ ascorbate), or 2 to 4 μg. of piperoxan was injected at the marked instances; A.C. of 3.2 volts was applied for 10 seconds at the marked periods.

Are equal to the arterial transmural pressures times the arterial radii (law of Laplace). The developed muscular tension may be estimated by calculation of the increase in total mural tension (the difference in the tensions of the arterial wall at the height of the pressure response and at the initial pressure level), since the pressure elevations or resistance increases were mainly a result of active muscular reductions in arterial bore in accordance with Poiseuille's law, and since the arterial muscle cells have a practically circular arrangement. However, an increase in total tension cannot be used generally as an accurate index of the developed active muscular tension unless the details of the concomitant decrease in the elastic tension are known.

Whenever the baseline pressure level of the potassium-constricted artery did not exceed by much that of the control sodium-rich state, the active muscular tensions of the responses to the same dose of epinephrine approximated each other for both the pressure elevations and geometrical changes were similar. However, in the artery appreciably contracted by potassium, the calculated increase in total mural tension of the epinephrine response of similar pressure elevation was somewhat less than in the relaxed sodium-rich state of greater arterial radii. Therefore, the active tension developed in this epinephrine response of similar pressure amplitude at constant flow was also somewhat less in this state of appreciable potassium contracture, unless the lesser elastic tension which accompanied the similar pressure response to epinephrine in the potassium-contracted artery was sufficiently smaller to have made the developed active tension a greater part of the total tension increase. That the latter did, in fact, occur is suggested by the finding that similar pressure increases at constant flow resulted from the
same dose of epinephrine in the potassium-treated artery during quite different degrees of potassium contracture, provided the vessel was not extremely contracted. Since the pressure increases to the same dose of epinephrine were similar under different levels of residual vascular tone, including the relaxed sodium-rich state, the experiments substantiate, for segments of intestinal arteries, the findings and theory of Burton and Stinson.15 These are: when the method of constant-flow perfusion is employed, the active tension of the responses of vascular smooth muscle to concentrations of vasoactive drugs tends to be related linearly to the changes in driving pressures.15

The above considerations, the demonstrations that the arterial pressure responses in the potassium-depolarized state to microgram injections of epinephrine were similarly large, reproducible, and specifically blocked, and the fact that the arterial contractions were obtained in a geometrically natural manner (that is, neither isotonically nor isometrically) indicate the following conclusion, despite Csapo’s plea2 for the isometric study of smooth muscle behavior. The physiological contractile response of the resistance blood vessels to adrenergic neurohormones is produced by a mechanism which does not act largely through membrane depolarization because the essential operation is unimpaired in small-caliber arterial segments which have been completely depolarized by the external application of potassium.

Similarly, neither sodium nor chloride ion influx appears essential in the excitation process of arterial smooth muscle reactions to adrenergic mediator, for the contractile responses to epinephrine were essentially unimpaired, even after many minutes of perfusion with solution in which all the sodium ion had been replaced by potassium ion and the chloride content was reduced to less than 5 mM/L. Total replacement of sodium by lithium ion in the perfusate, however, depressed after many minutes the arterial responses to epinephrine both in the speed of contractile development and in the total amount of contraction. The length of time required for the manifestation of this vasodepressant effect of lithium strongly suggests that the lithium depressed at an intracellular location or acted through slow membrane displacement of a substance complexed in an undissociated manner, rather than that the lithium acted extracellularly or through an electrostatic cationic displacement at the plasma membrane of the smooth muscle cell. Lithium has been shown to abolish tension responses of intestinal smooth muscle while leaving the discharge of action potentials unimpaired for some time.16

The question could be raised that epinephrine excited the vascular smooth muscle cells in the potassium-depolarized state and in the sodium-rich experiments by an intracellular site of action rather than by a plasma membrane effect. However, the alpha adrenergic receptors, where epinephrine exerts its excitatory effect on smooth muscle and where piperoxan exerts its inhibitory effect, are generally considered to be situated in the plasma membrane of the cell. The above reported observations are perfectly consistent with this membrane theory, for epinephrine may physiologically excite vascular smooth muscle by a chemical reaction at the cell membrane which triggers the contractile process irrespective of membrane depolarization.

When the sodium of the solution bathing the arterial smooth muscle was replaced by an osmotically equivalent amount of sucrose while the extracellular calcium, magnesium, and potassium contents were kept at their normal low levels, there developed extreme arterial muscle contraction (sodium reversible) which was long sustained in its intensity in contrast to the vasooconstriction induced by potassium-rich solutions. This is evidence for a calcium-sodium antagonism for anionic sites at the cell membrane of arterial smooth muscle similar to the calcium-sodium antagonism at cell surfaces of heart muscle, indicated by the work of Liittigau and Niederggerke.17 In accordance with such a calcium-sodium membrane competition and the physical chemical mechanisms for concentrating
Serial records of the pressure responses to epinephrine produced by arterial muscle in isosmotic solutions of high sodium chloride and high lithium chloride content. Duration of perfusion in the respective solutions before these records were taken is as marked. One μg. of epinephrine was injected at the marked instances.

calcium at the surfaces of biological membranes,\textsuperscript{18} it is suggested that the membranes of the smooth muscle cells became more highly concentrated in calcium by substitution of sucrose for the sodium chloride in the perfusing solution. An increased influx of calcium perhaps activated and sustained the extreme vasoconstriction which developed when the extracellular sodium salt was replaced by sucrose. The markedly delayed relaxations of the contractions induced by epinephrine under this circumstance are noteworthy.

In membrane excitation by chemicals, the chemical reaction has been generally viewed as a process which increased the membrane...
permeability of the smooth muscle cell to one or more ionic species with electrical depolarization resulting as a consequence of ionic fluxes, the depolarization then somehow activating the contractile process. However, Eccles and Magladery demonstrated that epinephrine stimulation of mammalian smooth muscle caused detectable action potentials only early in the response, and they observed contractions maintained by epinephrine action far beyond the duration of detectable action potentials (epinephrine contracture).

The findings of this study indicate that epinephrine probably triggers the contractile event in arterial smooth muscle by a membrane reaction which essentially involves some other action rather than through depolarization or sodium ion influx. However, depolarizing sodium and/or potassium fluxes may normally take place concomitantly in a fortuitous manner, or else these fluxes may possibly augment epinephrine action by propagation of excitation to adjacent parts of the surface membrane of the same or other cells, analogous to what appears to happen in the membrane propagation of acetylcholine-induced impulses in skeletal muscle.

The results with potassium-depolarized
DEPOLARIZED ARTERIAL MUSCLE

arterial smooth muscle and with isosmotic solution of high sucrose content, discussed above, suggest that calcium may be the active agent involved in epinephrine membrane excitation of the contractile process of vascular smooth muscle. Indeed, recent evidence obtained in this laboratory strongly suggests that adrenergic neurohormone excites both depolarized and previously polarized vascular smooth muscle by the same membrane mechanism of calcium release, the calcium moving intracellularly to activate contraction.

The above hypothesis for the adrenergic excitation of vascular smooth muscle contraction agrees with the belief of Taylor21 that membrane excitation itself, rather than depolarization or current flow, forms the link between excitation and contraction of muscle. Current flow contributing to the coupling process2 or electrostatic forces set up by an electrical field of epinephrine depolarization3 would not appear to play an important role even in accelerating the inward migration of a coupling agent to the myofibrils of blood vessels. This follows from the demonstration that in the potassium-depolarized artery and in the previously polarized artery, the speed of development of the full contractile response (peak arterial pressure rise) from the initial activation of contraction (start of the arterial pressure response) was similar.

Summary

The excitation-contraction reactions of arterial smooth muscle to epinephrine and electrical current were studied in isolated segments of canine small-caliber arteries by means of a constant-flow perfusion technique. The arterial pressure elevations produced by the contractile responses of the smooth muscle to epinephrine were equally large, reproducible, and specifically antagonized by piperoxan whether or not the artery had been depolarized by isosmotic solutions rich in potassium and devoid of sodium. Responses to electrical current were impaired by potassium depolarization. In the potassium-depolarized vascular smooth muscle, relaxation of the induced contractions was moderately delayed. Complete replacement of sodium in the perfusion solution by lithium impaired, after many minutes, the excitation-contraction responses of the arterial segments both in the speed of contraction and in the total amount of developed muscular tension. Complete replacement of external sodium salts by an osmotically equivalent amount of sucrose resulted in extreme and long-sustained vasoconstriction; under this condition, epinephrine induced contractile responses with extremely delayed relaxation times. It is concluded that adrenergic neurohormones physiologically excite vascular smooth muscle by a mechanism which does not operate primarily through depolarization or sodium ion influx. It is furthermore suggested that adrenergic excitation of the contractile process of the smooth muscle of blood vessels consists of a basically non-electrical membrane reaction which is coupled, independently of current flow, to the contractile mechanism.

References


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