Calcium Content and Force of Drug-Induced Contractions of Arterial Muscle During Recovery *in vitro*[1] (35133)

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The ability of mammalian vascular smooth muscle to contract maximally to vasoactive drugs, hormones and electrical stimulation is known to be severely hampered when blood vessels are excised from intact animals and subsequently prepared as helical strips for *in vitro* studies (1). Full sensitivity to stimuli is, however, restored when these helical preparations are incubated for 2 to 3 hr in a physiological salt solution (PSS) at 37–38° (1). Once full sensitivity is attained these strips yield quantitatively reproducible contractile responses to most vasoactive stimuli for periods of from 8 to 14 hr (1, 2). Since basic information about the contractile process in vascular smooth muscle is rapidly accumulating through the use of isolated blood vessel preparations, fundamental information regarding the physiologic mechanisms of recovery are important.

Dawkins and Bohr (3), working with excised rat aorta, noted that mechanical injury initially upset the sodium (Na) and potassium (K) gradients across the cell membrane (*i.e.* Na content increased while K decreased) and incubation at 38° in a PSS gradually restored the characteristics of the membrane and transmembrane gradients of these monovalent cations towards normality. Bevan, working with rabbit aortic strips, noted that over an initial 2-hr incubation period the Na content increased while the K content decreased (4). Bevan's data, however, do not show, quantitatively, a correlation between strength of catecholamine-induced contractions and the content of these monovalent cations. A more recent report of Barr *et al.* (5) suggests, that the failure of canine carotid strips, exposed to *cold storage,* to respond to electrical stimulation may be related to reaccumulation of intracellular K. Several other *in vitro* experiments, however, do not support a role for either Na or K in drug-induced contractions of vascular muscle (6–8). Moreover, a recent critical review of the physiology and pharmacology of vascular smooth muscle also fails to link intracellular concentrations or transmembrane gradients of either Na or K to strength of drug-induced contractions (9).

Since considerable evidence has accrued to suggest that drug-induced contractions of macro- and microvascular smooth muscles are dependent upon calcium ions (Ca^{2+}) (10–17), it is not too unreasonable to believe that restoration of full drug sensitivity upon incubation at physiologic temperature is related quantitatively to the calcium content of these injured vascular tissues. The present data support this speculation, at least for the most widely used *in vitro* vascular preparation—namely rabbit aortic strips.

Methods. Female rabbits (New Zealand strain, 1.8–2.6 kg in wt) were sacrificed by a blow on the head. The descending thoracic aortae were excised rapidly and immediately placed in Krebs–Ringer bicarbonate, cleaned, freed of surrounding connective tissue and either cut into helical strips as described previously (2, 18) for drug studies and analysis of Ca^{2+} content or cut into segments (only for analysis of Ca^{2+} content). The composition of the Krebs–Ringer bicarbonate (containing 2.5 m*M* $CaCl_2$) has been reported previously (2, 18). (The experiments utilizing

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barium required a modified Krebs–Ringer solution in which 1.2 m*M* $MgCl_2$ was substituted for 1.2 m*M* $MgSO_4 \cdot 7H_2O$). All solutions including those for analysis of tissue Ca^{2+} content by atomic absorption spectrophotometry were made up in triply-distilled, deionized water (Ca^{2+} content $< 10^{-6}$ *M*). The helically cut strips were mounted vertically, under isometric conditions, in 20-ml muscle baths and incubated for various periods of time (*i.e.*, from 15 min to 5 hr), under a resting tension of 2 g. The mean time from sacrificing the rabbits, preparing the strips and placing of the aortic strips in warmed, oxygenated, and buffered Krebs–Ringer was 40 min. The loaded tensions were maintained and periodically adjusted throughout the experiments. The Krebs–Ringer solution was oxygenated continuously with a 95% O_2–5% CO_2 mixture and kept at 37.5° (pH 7.2–7.4). As a precaution against the production of interfering metabolites (2), the Krebs–Ringer bicarbonate solution was changed every 5 min. Conventional isometric techniques, employing Grass FT.03C force-displacement strain gauges with either a Model 5 Grass Instruments six channel recorder or a Beckman Dynograph four channel recorder, were utilized.

Drug-induced contractions. Maximal or supramaximal concentrations were utilized for all seven drug stimulants (epinephrine, histamine, serotonin, angiotensin, acetylcholine, potassium chloride, barium chloride [$BaCl_2$]). Each vascular strip was stimulated repetitively (*i.e.*, at different times of incubation at 37.5°) with only one drug. In most instances the drug-induced contractions would not relax within a 15–30-min wash time and successive contractile events could not therefore be obtained every 15 min over the initial 60 min, thus necessitating the use of many different additional preparations for these initial intervals of incubation times. All contracted strips however, except for $BaCl_2$, usually relaxed within 45–60 min and thus repetitive contractile responses could be obtained every hr, on each strip, beyond the initial 60 min of incubation. The $BaCl_2$-stimulated strips usually required 1–2 hr to relax and thus additional strips were utilized for these drug studies.

The chemicals used to make up the Krebs–Ringer bicarbonate solutions, as well as the potassium chloride (KCl) and barium chloride ($BaCl_2$), were all A.C.S. Certified reagent grade. The following drugs were utilized: epinephrine (Adrenalin Chloride, Parke, Davis and Co.), histamine (histamine dihydrochloride, Nutritional Bochemicals Corp.), serotonin (serotonin creatinine sulfate, Nutritional Biochemicals Corp.), angiotensin (angiotensin amide, Hypertensin, Ciba Pharmaceuticals) and acetylcholine (acetylcholine chloride, Nutritional Biochemicals Corp.). All of the vasoactive drug stimulants were made up fresh in triply-distilled, deionized water as concentrated stock solutions so that the total volumes added to the 20-ml muscle baths never exceeded 0.65 ml. All of the vasoactive drug concentrations expressed below are in terms of the salt, except for angiotensin which is free base.

Preparation of aorta for determination of Ca^{2+} content by atomic absorption spectrophotometry. Two different types of rabbit aorta preparations were utilized for quantitative analysis of total tissue calcium content: (i) rapidly excised, nonincubated intact segments of aorta stripped of connective tissue, and (ii) aortic strips incubated for different periods of time as in the drug studies above, at 37.5° in Krebs–Ringer bicarbonate mounted under 2 g of tension. All excised segments and incubated strips were rapidly dipped twice in Ca^{2+}-free Krebs–Ringer bicarbonate containing 0.08 m*M* sodium ethylenediaminetetraacetic acid (EDTA), then placed on ash-free filter paper (Whatman, No. 42) at which point, in the case of the aortic strips, the ends with the suture ties were carefully dissected free and the strips as well as the segments were then pressed delicately between the filter paper for 10 sec. At this point, the subsequent handling, weighing, treatment, and ashing of the strips in platinum crucibles for analysis of total calcium content on a Perkin-Elmer Model 303 atomic absorption spectrophotometer was essentially similar to the procedures and meth-

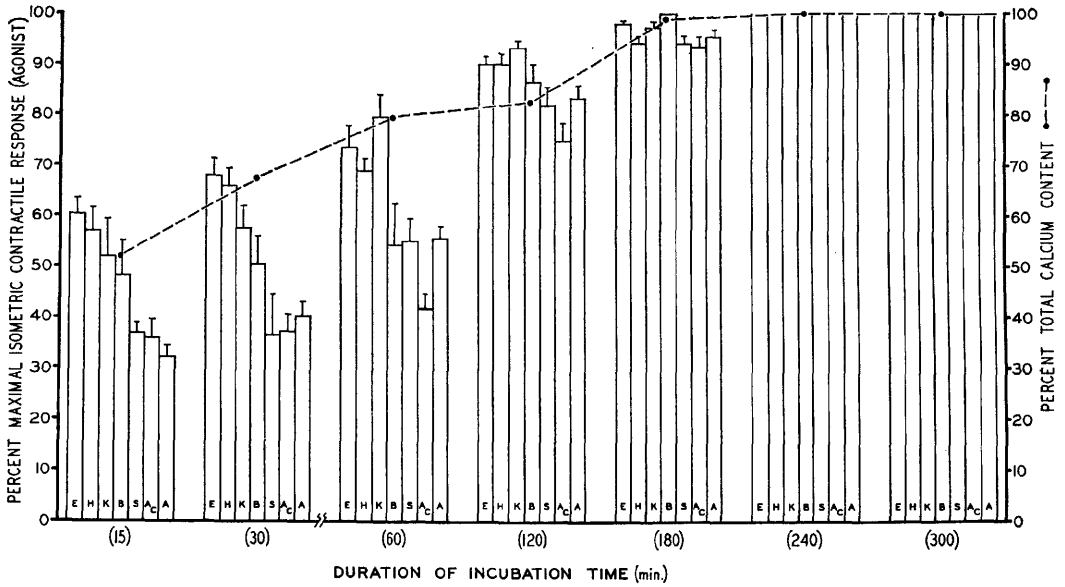

FIG. 1. Effect of time of incubation at 37.5° on the relationship between strength of drug-induced contractions and total calcium content in rabbit aortic strips during recovery. The vertical bars with the brackets represent the mean % contractile response ± SEM induced by maximal doses of various vasoactive substances (E = epinephrine, 10 μg/ml; H = histamine, 640 μg/ml; K = potassium, 80 m*M*; B = barium, 50 m*M*; S = serotonin, 40 μg/ml; AC = acetylcholine, 50 μg/ml; A = angiotensin, 0.5 μg/ml) at various times of incubation. 100% isometric contractile response to E = 3.03 g ± 0.26 SEM; 100% response to H = 2.87 g ± 0.19 SEM; 100% response to K = 2.38 g ± 0.23 SEM; 100% response to B = 1.60 g ± 0.11 SEM; 100% response to S = 2.20 g ± 0.18 SEM; 100% response to AC = 1.00 g ± 0.13 SEM; 100% response to A = 2.02 ± 0.17 SEM. A minimum of 12 different preparations were utilized for each of the drug-induced responses. (●——) the mean % total calcium content (see Table I for absolute values) at the various times of incubation.

odology previously documented in detail (11). All of the glassware, crucibles, pipettes, surgical instruments, etc., used for these analyses were specially washed in dilute HCl (1:4 dilution with triply-distilled deionized water, $Ca^{2+} < 10^{-6}$ *M*) as described previously (11). Means and standard errors of the means were calculated and compared to one another for statistical significance by means of Student's *t* test (19). A total of 135 rabbits and approximately 425 preparations were utilized for this study.

Results. Effect of time of incubation at 37.5° on strength of drug-induced contractions. Figure 1 depicts the maximal contractile forces (*i.e.*, tensions) that are developed for each of the seven different vasoactive substances after incubating the aortic strips for 15, 30, 60, 120, 180, 240, and 300 min. These data demonstrate that there is a progressive increase in developed tensions over the initial 3 hr for all of the vasoactive substances followed by a plateau between the third and fourth hours. In addition, these data show that the ability of epinephrine, potassium, and histamine to produce progressively greater contractile responses takes place in a much shorter period of incubation at 37.5° than do contractile responses induced by serotonin, acetylcholine, and angiotensin. For example, at 2 hr, epinephrine, potassium, and histamine have attained at least 90% of their maximal tensions while the other vasoactive substances have attained only 70–80% of their maximal contractile responses.

Effect of time of incubation at 37.5° on total calcium content of rabbit aortic strips. Table I shows that incubation of aortic strips at body temperature results in almost a

TABLE I. Effect of Time of Incubation at 37.5° on Total Calcium and Water Content in Rabbit Aortic Strips.

Incubation time (min)	Ca^{2+} (mmole/kg of wet wt ± SE)	H_2O (ml/kg of wet wt ± SE)
0[a]	2.35 ± 0.14[c](71)[b]	712.2 ± 7.9 (71)
15	4.45 ± 0.16[c](24)	721.4 ± 9.8 (24)
30	5.82 ± 0.28[d](24)	723.8 ± 6.7 (24)
60	6.84 ± 0.38[c](20)	707.1 ± 7.3 (20)
120	7.11 ± 0.19[f](20)	736.7 ± 9.1 (20)
180	8.55 ± 0.33 (21)	730.1 ± 8.9 (21)
240	8.62 ± 0.55 (16)	730.8 ±17.6 (16)
300	8.63 ± 0.70 (8)	737.1 ±18.2 (8)

[a] Determined immediately after excising the aorta (not incubated, see Methods).

[b] Number of different preparations.

[c] Significantly different from all other values ($p < 0.001$); [d] ($p < 0.05$); [e] ($p < 0.05$) except for 120 min; and [f] ($p < 0.05$) except for 60 min.

300% increase in total Ca^{2+} content over the normal value of 2.35 mmoles/kg of wet wt, upon attainment of equilibrium (*i.e.*, 3 hr). In addition, these data demonstrate a progressive, sequential rise in total Ca^{2+} content of incubated aortic strips over a 3-hr interval. Although there is a trend for the water content to increase during progressive incubation (Table I), these values are not significantly different from one another.

Figure 1 demonstrates that a close parallelism exists between the net total calcium content and the strength of epinephrine-, histamine-, and potassium-induced contractions over the entire incubation period to equilibration (*i.e.*, 3 hr). Such a close correlation between Ca^{2+} content and contractile response does not, however, exist for serotonin, acetylcholine, or angiotensin over the first 60 min of incubation (*i.e.*, the % Ca^{2+} content vs % maximal contractile responses are significant from one another [$p<0.05$] for these latter vasoactive substances during the first 60 min of incubation). Although there is a close correlation between % Ca^{2+} content and $BaCl_2$-induced contractile responses over most of the incubation periods, there is, however, a significant difference ($p<0.05$) between the two at 60 min.

Discussion. The Ca^{2+} content reported here for freshly dissected rabbit aorta (*i.e.*, 2.35 mmoles/kg of wet wt) is in close agreement with values reported for other types of mammalian smooth muscle; *e.g.*, Bauer *et al.* (20) reported values of 1.8–2.1 mmoles/kg of fresh wt for guinea pig taenia coli. Potter and Sparrow (21) reported an average value of 1.57 mmoles/kg wet wt. for fresh cat circular intestinal muscle, while Van Breeman *et al.* (22) have reported a mean value of 2.25 mmoles/kg of wet weight for rat uterus. The increased Ca^{2+} content observed, upon incubation, in our experiments on aortic strips may be a consequence of cellular injury and gains support from experiments on other types of smooth muscle (20–22). In addition, the increased Ca^{2+} may partly be a reflection of a greater amount of ionized Ca^{2+} in the Krebs–Ringer than is normally present in the total blood Ca^{2+} of the rabbit. Observations on blood and serum from various mammals, having at a total Ca^{2+} concentration approximately equal to 2.5 m*M*, indicate that usually only 40–60% is free, ionized Ca^{2+} (*i.e.* 1.0–1.5 m*M*) (23, 24).

It is interesting to note that the forces of the contractile responses induced by four different vasoactive substances, namely epinephrine, histamine, potassium, and barium, on isolated rabbit aorta, closely parallel the Ca^{2+} content even though the Ca^{2+} levels have been increased as much as 300% over the normal *in vivo* values (Table I). Although there is a progressive and consistent increase in the magnitudes of the contractile responses induced by epinephrine, histamine, potassium, and barium after all periods of incubation which is directly correlated to the total Ca^{2+} content at these various incubation times, this is not so for at least the first 60 min of incubation with respect to serotonin-, acetylcholine-, and angiotensin-induced contractile responses (but beyond 60 min of incubation there is close agreement between the magnitude of the contractile forces developed by even these latter drugs and the Ca^{2+} content). It is tempting to speculate that the failure to find a correlation for these latter three agents between strength of drug-induced contractions and Ca^{2+} content, during the first 60 min of incubation, is

a reflection of specific Ca^{2+} sites for these drugs not being completely filled with their respective Ca^{2+} complements. For example, considerable evidence is accumulating to suggest that different classes of vasoactive molecules may induce contraction of smooth muscles, including vascular muscles, by utilizing different cellular Ca^{2+} fractions (10, 11, 14, 17, 21, 25, 26). We have presented preliminary data (to be published in detail elsewhere) to suggest that angiotensin, serotonin, and acetylcholine-induced contractions of rabbit aorta are dependent on calcium stores different from those for either epinephrine, potassium, histamine, or barium (27, 28).[3] Furthermore, additional experiments (to be reported in detail elsewhere) have shown that Ca^{2+}-depleted aortic strips, which have been depleted of 75–85% of their Ca^{2+} stores, fail to respond to supramaximal doses of acetylcholine, angiotensin, or serotonin but will contract minimally to supra maximal doses of epinephrine, potassium, $BaCl_2$, and somewhat to histamine; maximal contractile responses to angiotensin, acetylcholine, and serotonin could, however, be completely restored if these Ca^{2+}-depleted preparations were reincubated in normal Krebs–Ringer (2.5 m*M* Ca^{2+}) for a period of 2–3 hr (a duration of time which corresponds very nicely with the time required in the present experiments).

Summary and Conclusions. Studies were designed, using rabbit aortic strips, to investigate the possible role calcium (Ca^{2+}) ions play in the recovery of depressed, drug-induced contractions of excised, isolated blood vessels. The present experiments demonstrated that: (i) excision of aorta from intact rabbits and the subsequent surgical preparation and incubation of helical strips, at physiologic temperature *in vitro*, severely impairs the development of full sensitivity, of these aortic smooth muscle cells, to a variety of vasoactive substances for a period of 3 hr. (ii) A close relationship was found to exist between the ability or inability of different vasoactive drugs (*e.g.*, epinephrine, histamine, potassium, and barium) to induce a maximal contractile response and the total Ca^{2+} content (as quantitatively determined by atomic absorption spectrophotometry). And (iii) mechanical (surgical) injury may alter the *in vivo* Ca^{2+} content of arterial smooth muscle.

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1. Furchgott, R. F., and Bhadrakom, S., J. Pharmacol. Exp. Ther. **108**, 129 (1953).

2. Altura, B. M., and Altura, B. T., Amer. J. Physiol. **219**, Dec. 1970.

3. Dawkins, O., and Bohr, D. F., Amer. J. Physiol. **199**, 28 (1960).

4. Bevan, J. A., J. Pharmacol. Exp. Ther. **129**, 417 (1960).

5. Barr, L., Headings, V. E., and Bohr, D. F., J. Gen. Physiol. **46**, 19 (1962).

6. Briggs, A. H., and Melvin, S., Amer. J. Physiol. **201**, 365 (1961).

7. Daniel, E. E., Arch. Int. Pharmacodyn. Ther. **158**, 131 (1965).

8. Dodd, W. A., and Daniel, E. E., Circ. Res. **8**, 451 (1960).

9. Somlyo, A. P., and Somlyo, A. V., Pharmacol. Rev. **20**, 197 (1968).

10. Altura, B. M., Amer. J. Physiol. **218**, 1082 (1970).

11. Altura, B. M., and Altura, B. T., Amer. J. Physiol., in press.

12. Bohr, D. F., Pharmacol. Rev. **16**, 85 (1964).

13. Briggs, A. H., Amer. J. Physiol. **203**, 849 (1962).

14. Hiraoka, M., Yamagishi, S., and Sano, T., Amer. J. Physiol. **214**, 1084 (1968).

15. Hinke, J. A. M., *in* "Muscle" (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, eds.), p. 269. Pergamon, London/New York (1965).

16. Hudgins, P. M., and Weiss, G. B., J. Pharmacol. Exp. Ther. **159**, 91 (1968).

17. Waugh, W. H., Circ. Res. **11**, 927 (1962).

18. Altura, B. M., and Altura, B. T., Eur. J. Pharmacol. **12**, 44 (1970).

19. Bancroft, H., "Introduction to Biostatistics," p. 172. Harper & Row (Haeber), New York (1962).

20. Bauer, H., Goodford, P. J., and Hüter, J., J. Physiol. (London) **176**, 163 (1965).

21. Potter, J. M., and Sparrow, M. P., Aust. J. Exp. Biol. Med. Sci. **46**, 435 (1968).

22. Van Breemen, C., Daniel, E. E., and Van Breemen, D., J. Gen. Physiol. **49**, 1265 (1966).

23. Bianchi, C. P., "Cell Calcium," Butterworth,

Washington (1968).

24. Moore, E. W., Nat. Bur. Stand. U.S., Spec. Publ. **314**, 215 (1969).

25. Daniel, E. E., *in* "Muscle," (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, eds.), p. 295. Pergamon, London/New York (1965).

26. Hurwitz, L., Von Hagen, S., and Joiner, P. D., J. Gen. Physiol. **50**, 1157 (1967).

27. Altura, B. M., Altura, B. T., and Zeman, R., Physiologist **12**, 157 (1969).

28. Altura, B. T., and Altura, B. M., Fed. Proc., Fed. Amer. Soc. Exp. Biol. Proc. **28**, 711 (1969).

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