THE SEPARATION OF CELL MEMBRANE CALCIUM TRANSPORT FROM EXTRACELLULAR CALCIUM EXCHANGE IN VASCULAR SMOOTH MUSCLE.

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The soundest method of reducing blood pressure in hypertensive patients should be direct relaxation of arterial smooth muscle, rather than interfering with the function of neuro-transmitter agents. An appealing approach to this problem is to learn details of the mechanism which regulates the ionic calcium concentration in the myoplasm of the arterial smooth muscle cells with the hope of eventual pharmacological control at sites specific to arteries. This approach has been frustrated in the past by the absence of a reliable method for following cellular calcium movements in tissues with considerable extracellular spaces. We have designed a new method which isolates the cellular calcium exchange and consequently senses the relatively small calcium fluxes associated with contractions of the smooth muscle.

The principle of the method is that after the tissue has taken up ^{45}Ca, and before counting, all the extracellular ^{45}Ca is washed out and displaced from binding sites by lanthanum, while membrane bound lanthanum blocks the loss of ^{45}Ca from within the cells. The measured radioactivity will then give the unidirectional calcium flux into the cells during the experimental time period.

Lettvin et al. (1964) predicted that lanthanum ions due to their higher charge density have greater affinity than calcium ions for any anionic group which binds calcium. This was verified for sites on the lobster axon membrane (Takata et al., 1966; Hafemann, 1969) and the negative groups of a phospholipid artificial membrane (van Breemen and van Breemen, 1969). By studying transport across this latter membrane over a wide pH range it was shown that an

optimum affinity of the negative groups for calcium ions existed at which the transport rate reached a maximum. An ion with much greater affinity than calcium should thus be very poorly transported, and at the same time make the transport sites unavailable for calcium. As expected lanthanum completely blocked the ^{45}Ca flux across the artificial phospholipid membrane. Lanthanum also blocked calcium fluxes across mitochondrial membranes (Mela, 1968), and the squid axolemma (van Breemen and De Weer, 1970) and strong indirect evidence indicated a similar blockade in vascular smooth muscle membranes (van Breemen, 1969), intestinal smooth muscle (Weiss and Goodman, 1969) and heart (Palmer and van Breemen, 1970). In this report we show how the above properties of lanthanum can be used to remove the obscuring effect of extracellular calcium exchange.

METHODS

Rabbits were killed by a blow on the neck and their thoracic and anterior abdominal aortae rapidly removed and placed in a Tris-buffered physiological solution TS (in mM/l: NaCl 160, $MgCl_2$ 1, $CaCl_2$ 1.5, KCl 4.6, dextrose 10, Tris 5, pH 7.4, bubbled with O_2, temperature 37° C). The aortae were then cleaned of all fat and cut transversely into 4 mm wide rings for contraction studies or 3 mm wide strips for flux data.

^{45}Ca uptake: Aortic strips were placed in TS, Li-TS (Li substituted for Na), or K-TS (K substituted for NA). These solutions were labelled with ^{45}Ca. After varying time periods strips were removed, blotted, weighed and ashed. The ash was dissolved in 1 mM $LaCl_3$ in .1 N HCl. For some strips a portion of this was used for total Ca determinations with a Perkin Elmer 303 atomic adsorption spectrophotometer. 1 ml of the dissolved ash was dissolved in 10 ml Bray's solution and counted in a Packard liquid scintillation counter.

Sucrose-^{14}C uptake: The uptake was followed after 5, 7, 15, 30 and 60 minutes incubation in either TS or KTS solution at pH 7.4, and 37° C. After removing from the incubating fluid, the strips were blotted on filter paper until no residual moisture was present. The wet weight was recorded and the radioisotope extracted by elution in 2 ml of distilled water overnight in a

shaker. 10 ml of Bray's solution was then added for counting.

^{45}Ca cellular influx: After exposure to ^{45}Ca, aortic strips were placed in Ca free TS containing 2 mM $LaCl_3$(La-TS) for one hour. The strips were then blotted, weighed, ashed and counted.

All uptake studies were preceded by 10 minutes Ca free TS to minimize changes in specific activity due to mixing with unlabelled free extracellular calcium.

Ca efflux: Aortic strips were allowed to take up ^{45}Ca from labelled TS for 20 minutes before placing them in either Ca free TS or Ca free TS with 2 mM $LaCl_3$. They were then removed at varying times, blotted, weighed and prepared for counting as above.

Contractions: Aortic rings were mounted between stainless steel hooks. The lower hooks were connected to aerators and the top ones to Grass tension transducers. Transducer and aerator were rigidly connected and solutions were changed by moving the mounted ring between different beakers.

RESULTS

Figure 1 shows the total tissue calcium and ^{45}Ca labelled calcium of aortic strips exposed to labelled TS over a 100 minute time interval. The exchange

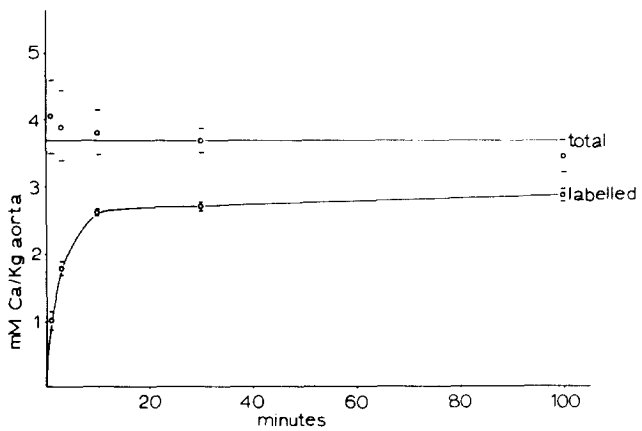

Figure 1. The uptake of ^{45}Ca and the total tissue Ca concentration of strips of rabbit aorta exposed to ^{45}Ca labelled TS for hundred minutes. The distance between horizontal bars equals twice the standard error of at least four observations.

is rapid and appears to plateau within the first 10-20 minutes, leaving about 20% of the total Ca inexchanged. A possible slow uptake is obscured by the initial large ^{45}Ca exchange. A comparison of the total tissue labelled Ca uptakes in TS, Li-TS and K-TS (figure 2) with the contractile states of the aortic rings

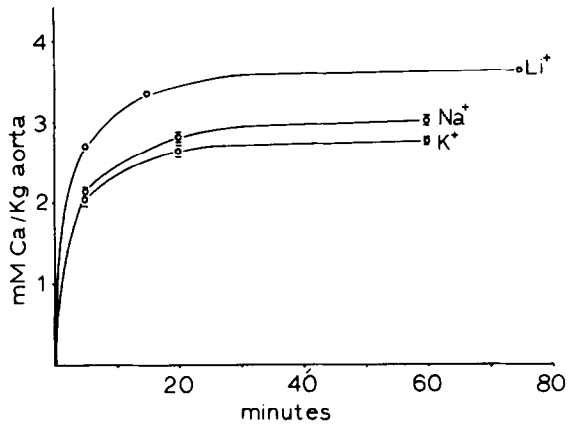

Figure 2. ^{45}Ca influx in strips of rabbit aorta from physiological solutions containing 160 mM Na^+, 160 mM K^+ or 160 mM Li^+. The distance between bars equals twice the standard error of at least four observations. Bars are absent if the S.E. is less than the radius of the circles.

in the same solutions (figure 3) demonstrates the total lack of correlation between these two parameters. This is only one of the reasons for believing that the initial rapid, large ^{45}Ca exchange occurs in the extracellular space. Other

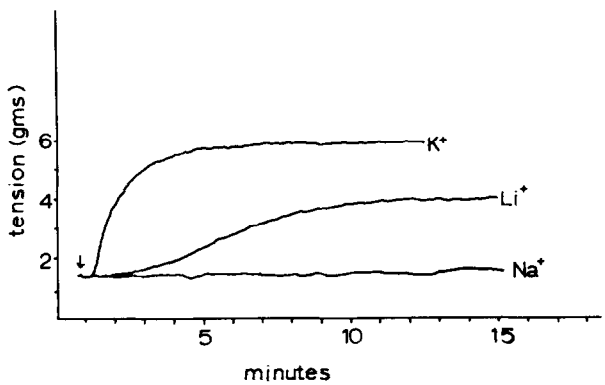

Figure 3. Tension developed by rabbit aortic rings when exposed to either 160 mM Li^+ or 160 mM K^+ containing physiological solutions.

reasons are: 1. There are many negative fixed sites in the extracellular space such as on mucopolysaccharides, proteins, mucoproteins (Borle, 1968) and the phospholipids of cell surfaces, which bind calcium ions. 2. The rapidity of the initial ^{45}Ca uptake is comparable to the extracellular uptake of sucrose-^{14}C (Hudgins and Weiss, 1969). 3. Acellular rabbit tendon shows similar ^{45}Ca exchange (unpublished data).

To determine if La ions would displace this bound extracellular Ca, the following experiment was done. Aortic strips were exposed to either a solution containing 290 mM sucrose, 10 mM glucose, 1 mM ^{45}Ca labelled $CaCl_2$ or the same solution with 1 mM $LaCl_3$ added for 20 minutes. The pH of both solutions was continually recorded and maintained at 7.3. Since Ca and La were the only cations in the extracellular space the total number of negative binding sites and the specificity coefficient $\frac{la^2}{ca^3}$ could be determined according to the method of Sparrow (1969). The concentration of binding sites was 8.1 $\pm$ 0.3 mM/kg

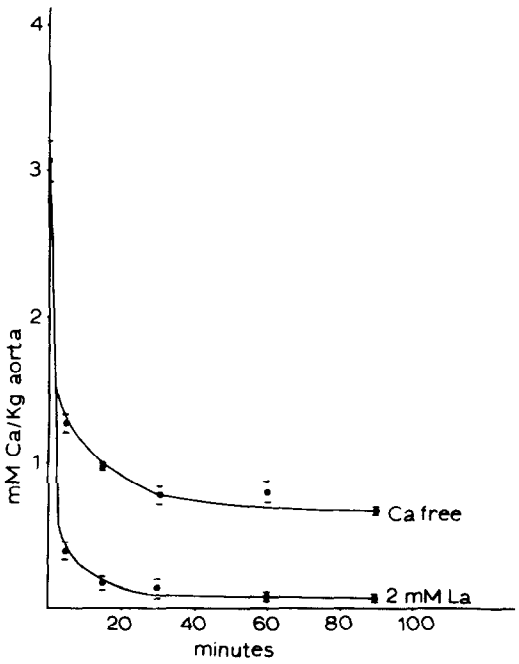

Figure 4. The loss of labelled Ca from aortic strips which had been exposed to ^{45}Ca for 20 minutes, to either Ca free TS or Ca free TS with 2 mM $LaCl_3$. The distance between the horizontal bars equals twice the standard error of the mean of at least four observations.

wet wt. and the selectivity coefficient of La over Ca was 3.1. The efflux curves of figure 4 also demonstrate the displacement of extracellular bound Ca by La. That this displacement takes place in the extracellular space is concluded from the observation by Smith (1970) that the electron dense La ions only stain the extracellular space and the outer surfaces of the cell membranes, and from the rapid course of the La induced ^{45}Ca loss, which is virtually the same as the La induced ^{45}Ca loss from the acellular rabbit tendon. After 30-40 minutes no more ^{45}Ca appears to be lost to the La-TS. To be sure of the completeness of the extracellular ^{45}Ca displacement we left the aortic strips for 60 minutes in La-TS before analyzing for the ^{45}Ca which had entered the cells. The labelled Ca cellular influxes thus determined under control conditions and Li and K substitutions are given in figure 5. These uptake curves consist of a rapid saturable phase of between 0.06 and 0.1 mM Ca/kg aorta and uptake which depends linearly on time. The nature of the initial rapid saturable phase is not entirely clear

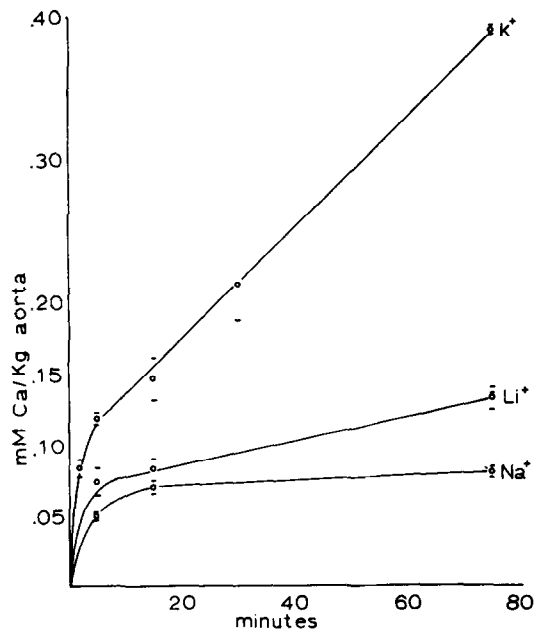

Figure 5. The cellular uptake of ^{45}Ca labelled Ca from physiological solutions containing 160 mM Na^+, 160 mM Li^+ or 160 mM K^+, measured after extracellular ^{45}Ca was removed with La $^{+++}$. The distance between the horizontal bars equals twice the standard error of the mean of at least five observations.

at the moment. It may represent calcium locked in the lumens of capillaries and small blood vessels by lanthanum or some calcium which penetrated the membranes after the initial 10 minute calcium free exposure. In any case it does not represent calcium which entered the cytoplasm, since no contraction was illicited in the control solution. The rates of the linear Ca uptake correspond very well with the rates of tension development in TS,Li-TS and K-TS shown in figure 3

DISCUSSION

The exigency of our new method for cellular calcium exchange may be illustrated by two simple calculations. Using a value of 5mg actomyosin/g artery (Murphy et al. 1969) and the calcium required for coupling values given by Bianchi (1969) we find that the aorta would require no more than 7uM Ca/kg for complete activation of the contractile mechanism. It is obvious that such small amount would be completely obscured by the variation in total tissue calcium measurments be they labelled or unlabelled. Secondly during the high K contraction the sucrose space of our preparation decreased from 440 ml/kg to 275 ml/kg. The labelled calcium concentration of the extracellular water was 1.5 mM/l and that of the remaining cells and extracellular solids 4.2 mM/Kg. Thus whereas the relaxed tissue labelled calcium concentration was 3.0 mM/Kg, that of the contracted aorta was $0.725 \cdot 4.2$ mM/Kg + $0.275 \cdot 1.5$ mM/Kg = 3.45 mM/Kg. Thus the mechanical effect of the contraction alone resulted in an arteffectual calcium uptake 64 times grater than that required for full activation of the contractile proteins.

Methods used in the past ignoring one or both of the above considerations have led to false correlations between calcium fluxes and contraction. The method described in this paper eliminates these difficulties and gives rates of Ca influx in quantitive agreement with the rates of tension development in high K and Li solutions. Potassium increased the slope of ^{45}Ca uptake over the control by 4 uM Ca/kg · minute and lithium by 0.8 uM Ca/Kg · minute. The rate of tension development during high K depolarization as calculated from the above figure would be 57% of maximum/minute. 65% of maximum/minute was observed in

figure 3 during the fast phase of the contraction. The respective calculated and observed rates of contraction in the Li substituted solutions are 11% and 10% of maximum/minute.

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