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SHORT COMMUNICATIONS

Effect of age upon the uptake and binding of calcium in rat aorta*

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Changes in vascular reactivity to sympathetic stimulation occur with age [1]. Some of these changes may be due to alterations in the availability of neurotransmitter or in the receptors for the neurotransmitter. Other changes may be beyond the receptor in the cascade of events culminating in contraction of vascular smooth muscle. Increasing vessel rigidity and an increasing amount of connective tissue with age may provide physical barriers to contraction or relaxation [2].

Adrenergic agonists, such as norepinephrine, and nonspecific agonists, such as potassium chloride, depend upon the availability of calcium to initiate a contraction. The response to these agonists is affected by the extracellular calcium concentration [3] and both affect the efflux of calcium from binding sites in the tissues [4]. In the present study, the effect of increasing age on uptake and binding of [$^{45}Ca^{2+}$] in the mature rat aorta was determined.

Materials and methods

Female Wistar rats aged 4, 12 and 21 months were studied. After sacrifice, the thoracic aorta from the diaphragm to the aortic arch was rapidly removed, placed in physiological salt solution (PSS; 154 mM NaCl, 5.4 mM KCl, 11 mM glucose, 6 mM Tris buffer, 1.5 mM $CaCl_2 \cdot 2H_2O$) at pH 7.4, at 37°, aerated with 100% O_2, gently cleaned of excess connective tissue, and cut into a helical strip by the method of Furchgott [5]. Tissues were mounted on stainless steel rods and maintained at 1 g resting tension.

After 1 hr, tissues were incubated for 15 min in PSS containing calcium concentrations of 0.01 to 15 mM before the addition of 0.45 μM $CaCl_2$ ([$^{45}Ca^{2+}$], 39.5 mCi/mg). After 60 min of incubation with [$^{45}Ca^{2+}$], tissues were rinsed, blotted, weighed and digested in 0.1 N NaOH at 60° for 1 hr. The amount of [$^{45}Ca^{2+}$] taken up into the tissue was determined by liquid scintillation spectroscopy. The amount of [$^{45}Ca^{2+}$] taken up by the tissues was expressed as tissue/medium ratio (ml/g tissue) obtained by dividing the amount of [$^{45}Ca^{2+}$] taken up per g tissue (wet weight) by the concentration of [$^{45}Ca^{2+}$] per ml of incubation solution (PSS). To correct for the amount of [$^{45}Ca^{2+}$] in the extracellular space, the sucrose space (in ml radioactive bathing solution taken up per g tissue) was subtracted from the total [$^{45}Ca^{2+}$] uptake (in ml/g).

Extracellular space was determined by the addition of [^{14}C]sucrose (480 mCi/mmole) or [^{14}C]inulin (62.5 mCi/mmole) to tissues incubated in PSS containing 1.5 mM Ca^{2+} under 1 g resting tension. After 60 min, the tissues were removed from the bathing solution, rinsed, blotted, weighed and digested as described above. The amount of [^{14}C] taken up into the tissues was determined in a manner similar to that for [$^{45}Ca^{2+}$].

Scatchard plot analysis [6] to determine the number of binding sites and the relative dissociation constants was done according to the method of Weiss [7]. Bound [$^{45}Ca^{2+}$] was calculated as the product of the amount taken up, adjusted for the amount in the extracellular space, and the calcium concentration of the incubation medium (concentration of nonradioactive calcium plus [$^{45}Ca^{2+}$]. Free calcium equaled the calcium concentration in the incubation medium. From a plot of bound versus the ratio of bound/free calcium, linear regression analysis was used to determine slope and the x and y intercepts for the high and low affinity binding sites. The number of binding sites was estimated from the x-axis intercept. The apparent dissociation constant, K_D, was calculated by dividing the x-axis intercept by the y-axis intercept. Analysis of variance was used to determine differences among the number of binding sites or among the dissociation constants with increasing age.

Results

Extracellular space. After 60 min no further uptake of [^{14}C]sucrose, which was used to determine extracellular space, occurred. The extracellular space at 4 months contained 0.82 ± 0.10 ml [^{14}C]sucrose/g tissue, at 12 months 0.57 ± 0.08, and at 21 months 0.44 ± 0.04. One-way analysis of variance indicated that significant differences existed between the extracellular spaces with age ($P < 0.05$). A least significant difference test indicated that the extracellular space decreased significantly with increasing age ($P < 0.05$). Extracellular space was also measured with [^{14}C]inulin in 4 and 21-month-old rats. At 4 months the extracellular space contained 0.74 ± 0.03 ml [^{14}C]inulin/g tissue and at 21 months 0.52 ± 0.03. The extracellular space measured with inulin was not significantly different than that measured with sucrose.

The weight of the thoracic aorta from the diaphragm to the aortic arch was significantly greater in the 12- and 21-month-old rats than in the 4-month-old rats. The aortae from the 4-month-old rats weighed 30.58 ± 2.06 mg, from the 12-month-old rats 42.10 ± 2.88 mg, and from the 21-month-old rats 46.98 ± 5.11 mg. Significant differences were determined as described above for the extracellular space ($P < 0.05$). To minimize the effects of increasing weight, [$^{45}Ca^{2+}$] uptake data has been expressed on a per g tissue basis.

Effect of age on [$^{45}Ca^{2+}$] *uptake.* Uptake of [$^{45}Ca^{2+}$] into the tissues at various extracellular calcium concentrations is illustrated in Fig. 1. Preliminary experiments indicated that no further uptake occurred after 60 min. Uptake was corrected for the variation in extracellular space to eliminate this variable. [$^{45}Ca^{2+}$] uptake varied inversely with calcium concentrations at all three ages, since, as calcium concentrations increased, total (radioactive and nonradioactive) calcium uptake also increased, but the proportion of radioactive calcium decreased. Below physiological concentrations, tissues concentrated more [$^{45}Ca^{2+}$]. Age did not significantly influence the uptake of [$^{45}Ca^{2+}$] into the tissues as determined by Duncan's Multiple Range test ($P > 0.05$).

Effect of age on high and low affinity binding of [$^{45}Ca^{2+}$]. Binding at high and low affinity binding sites was studied by Scatchard plot analysis [6] using the method of Weiss [7]. The number of high affinity binding sites was the same at 4 months and at 12 months but increased significantly by 21 months (Fig. 2). The number of low affinity binding sites increased significantly with age. The dissociation constant for the high affinity binding sites at 21 months was increased but not significantly. The dissociation constants for low affinity binding sites increased significantly with age.

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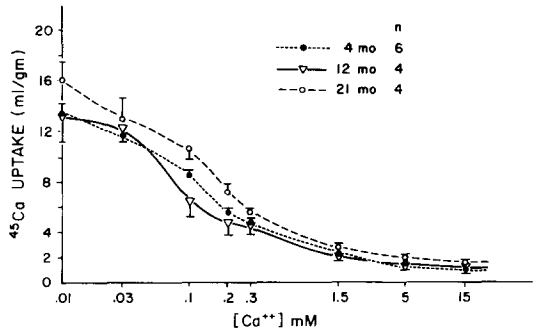
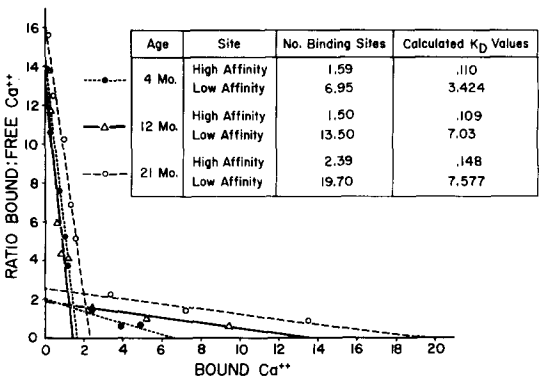


Fig. 1. [$^{45}Ca^{2+}$] uptake by rat aorta from rats aged 4, 12 and 21 months as a function of calcium concentration. Tissues were incubated under 1 g resting tension in PSS containing 0.01 to 15 mM $CaCl_2$ for 15 min before the addition of [$^{45}Ca^{2+}$] (39.5 mCi/mg) for 60 min. To correct for the amount of [$^{45}Ca^{2+}$] in the extracellular space, the extracellular space was subtracted from the total [$^{45}Ca^{2+}$] uptake. Vertical bars represent the S.E.M.



Age	Site	No. Binding Sites	Calculated K_D Values
4 Mo.	High Affinity	1.59	.110
	Low Affinity	6.95	3.424
12 Mo.	High Affinity	1.50	.109
	Low Affinity	13.50	7.03
21 Mo.	High Affinity	2.39	.148
	Low Affinity	19.70	7.577

Fig. 2. Scatchard plot analysis of the uptake and binding kinetics of [$^{45}Ca^{2+}$] by rat aorta from rats aged 4, 12 and 21 months. Aggregate data from six rats aged 4 months, from four rats aged 12 months, and from four rats aged 21 months were used. Slopes and intercepts were determined by linear regression analysis. Bound Ca^{2+} = μmoles/g, free Ca^{2+} = μmoles/ml, number of binding sites = μmoles Ca^{2+}/g tissue, K_D = apparent dissociation constant.

Discussion

[$^{45}Ca^{2+}$] uptake into rat aorta was dependent on the extracellular calcium concentration over a wide range of calcium concentrations at all of the ages studied. After correction for differences in extracellular space, the total amount of [$^{45}Ca^{2+}$] taken up into rat aorta was similar in young and aged rats. However, the disposition of the [$^{45}Ca^{2+}$] varied with age. Both high and low affinity binding of [$^{45}Ca^{2+}$] were altered.

The decrease in extracellular space with age correlated well with the microscopic findings of Gerrity and Cliff [2], who found that in older rats the watery matrix between cells was filled with debris resulting in a thickening of the intima. The change in extracellular space with age was inversely related to increased weight of the thoracic aortae. Even after corrections were made for the amount of free calcium in the extracellular space, alterations in calcium binding were evident. Decreases in the amount of extracellular fluid and accumulation of debris may hinder the rate of diffusion of calcium through the extracellular space to binding sites.

A gradual change in calcium binding occurred in low affinity binding. Both the relative number of binding sites and their dissociation constants increased with age. Vessels from older animals had more binding sites for calcium but, due to the increased dissociation constants, took up only slightly more calcium than did vessels from younger animals. With age high affinity sites also increased along with a trend toward an increase in K_D.

The alterations in high affinity binding may have more functional significance than changes in low affinity binding. The larger low affinity binding may be representative of surface bound calcium [8] associated with voltage sensitive channels [3]. Even though the high affinity binding is a small fraction of the total binding, it may be associated with both the resting calcium and receptor-linked calcium channels. Compared with K_D values for arteries from other species, the high affinity K_D values for aging rat aorta were quite high. This is not unexpected since contractile activity in rat aorta is highly dependent on extracellular calcium not only for potassium chloride induced contractions but also for norepinephrine [9]. However, small amounts of sustained contraction can be produced by intracellular stores [10]. In most other tissues norepinephrine initially mobilizes intracellular calcium stores [11]. Godfraind and Miller [12] have reported that in rat aorta contractions produced by α_2 selective agonists are entirely dependent on extracellular calcium.

Caution must be exercised in applying Scatchard plot analysis, intended for a homogenous system, to a heterogeneous system such as vascular tissue, but relative affinities and numbers of binding sites can be compared by this method. It is entirely possible that the nonsmooth muscle sites may extensively bind calcium serving as a reservoir [3]. To more specifically investigate the effect of potassium chloride and norepinephrine on calcium binding, Karaki and Weiss [13] used lanthanum to displace superficially bound calcium. Although [$^{45}Ca^{2+}$] binding was decreased 80%, high affinity and low affinity binding sites were still observed. Low nondepolarizing doses of norepinephrine decreased the binding of [$^{45}Ca^{2+}$ primarily at these high affinity sites. Both high depolarizing doses of norepinephrine and potassium chloride increased [$^{45}Ca^{2+}$] uptake at these low affinity sites. Different effects of alpha agonists and nonspecific agonists on calcium binding, together with alterations in calcium binding that occur with age, provide an explanation for the observation that the sensitivity to norepinephrine increased more than that to potassium chloride in aged animals [1].

Changes in calcium uptake and binding may parallel contractility both in different types of blood vessels and in pathological conditions. Hester and Weiss [14] demonstrated a correlation between increased numbers of high and low affinity binding sites in dog renal arteries versus renal veins and increased potassium chloride and norepinephrine induced tension development in renal arteries versus renal veins. Impaired contractile ability in aortae from hypertensive animals has been associated with altered [$^{45}Ca^{2+}$] distribution [15].

The data presented here suggest that alterations in vascular function with increasing age may be due to changes in calcium uptake and binding. There is a significant trend toward an increase in the number of both high and low affinity sites with age. There is also a definite increase in the K_D for low affinity sites and a tendency toward an increase in K_D at high affinity sites. It is not possible to determine if these changes are cause or effect, e.g. increased number as a result of decreased affinity or vice versa. It is possible that the increased number may be a compensatory mechanism for a cellular decrease in affinity.

In summary, the effects of age on uptake and binding of [$^{45}Ca^{2+}$] to rat aorta were studied. Total uptake of [$^{45}Ca^{2+}$] was similar in aortae from Wistar rats aged 4, 12 and 21 months. High affinity binding sites increased at 21 months,

and the apparent affinity seemed to decrease also. Together these account for the lack of changes in total uptake. Low affinity binding sites and dissociation constants increased with age. Functional changes in vascular smooth muscle with increasing age may be due to altered calcium binding.

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Forskolin effects on the beta-adrenergic responsiveness of rat hepatocytes

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Forskolin, a diterpene isolated from the Indian plant *Coleus forskohli*, activates adenylate cyclase in both intact cells and plasma membranes [1]. Furthermore, it has been observed that low concentrations of forskolin can magnify the action of hormones that activate adenylate cyclase [2].

Liver cells from rats contain three types of adrenoceptors, i.e. alpha$_1$, alpha$_2$- and beta-adrenoceptors. In hepatocytes from normal adult rats, the metabolic actions of epinephrine are mediated through a calcium-dependent cyclic AMP-independent alpha$_1$-adrenergic mechanism [3–5]. Beta-adrenoceptors seem to play, if any, a minor role, and the physiological significance of alpha$_2$-adrenoceptors is unknown. However, there are certain conditions in which beta-adrenoceptors play a significant role in the actions of epinephrine in liver cells. Among these conditions are the age of the animals [6], hypothyroidism [7], adrenalectomy [8], cholestasis [9], liver regeneration [9, 10], and dedifferentiation during cell culture [11]. In most of these conditions it has been observed that the increased beta-adrenergic responsiveness is associated with an increased number of beta-adrenoceptors [7–11]. In the present paper, we report that low concentrations of forskolin can enhance the beta-adrenergic responsiveness of liver cells from adult normal rats which indicates that an increase in the number of beta-adrenoceptors is not required to observe beta-adrenergic actions in liver cells.

Materials and methods

l-Epinephrine, *l*-isoproterenol, urease, glutamine and ornithine were obtained from the Sigma Chemical Co. Bovine serum albumin (fraction V) and collagenase (type II) were obtained from the Reheis Chemical Co. and Worthington respectively. Forskolin (7β-acetoxy-8,13-epoxy-12, 6β, 9α-trihydroxylabd-14-ene-11-one) was obtained from Calbiochem. [^{3}H]Cyclic AMP was obtained from the New England Nuclear Corp.

Female Wistar rats weighing between 180 and 200 g were employed. Hepatocytes were isolated by the method of Berry and Friend [12] and incubated in Krebs–Ringer bicarbonate buffer supplemented with 1% albumin, 10 mM glucose, 10 mM glutamine and 2 mM ornithine. Cells (≈40 mg wet weight) were incubated in 1 ml of buffer for 60 min at 37°. Urea was determined by the method of Gutman and Bergmeyer [13]. Cyclic AMP accumulation 2 min after the addition of the agents was determined by the method of Gilman [14].

Results and discussion

In normal rat hepatocytes, activation of beta-adrenoceptors did not result in stimulation of ureogenesis (Ref. 5 and Fig. 1) although a clear 2-fold increase in cyclic AMP levels was produced (Fig. 2). Forskolin (10^{-7} M) did not elevate by itself urea production or cyclic AMP levels (Table 1). However, it clearly magnified the accumulation of cyclic AMP produced by isoproterenol (Fig. 2). This marked increase in cyclic AMP levels resulted in activation of ureogenesis (Fig. 1). The effects of isoproterenol (10^{-6} M) were blocked by the beta-adrenergic antagonist propranolol (10^{-5} M). Forskolin alone at higher concentrations markedly increased cyclic AMP levels and ureogenesis (Table 1).

Our results clearly show that forskolin can markedly magnify the beta-adrenergic responsiveness of adult rat hepatocytes. In addition, the data also show that an increase in the number of beta-adrenoceptors is not an absolute requirement to observe metabolically significant beta-adrenergic responsiveness in hepatocytes. Recently, it has been observed that forskolin can potentiate the action of glucagon on liver adenylate cyclase [15].

The mechanism through which forskolin potentiates hormonal stimulation of adenylate cyclase is obscure. Hormonal modulation of adenylate cyclase activity is mediated through the interaction of the hormone-receptor complexes with the guanine nucleotide regulatory proteins (Ns and Ni for stimulation and inhibition of the enzyme respectively) and the catalytic subunit of adenylate cyclase. We have shown previously that administration of pertussis toxin to