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Epinephrine-Stimulated Maintained Rubidium Efflux from Guinea Pig Hepatocytes May Involve α_1- and α_2-Adrenoceptors

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SUMMARY

The epinephrine-stimulated maintained increase in ^{86}Rb efflux from guinea pig hepatocytes may consist of both α_1- and α_2-adrenoceptor-mediated components. Both the α_1-selective adrenoceptor agonist phenylephrine and the α_2-selective agonist clonidine evoked a maintained increase in ^{86}Rb efflux. Prazosin, an α_1-selective antagonist, did not inhibit the maintained increase in ^{86}Rb efflux elicited by epinephrine, whereas yohimbine, an α_2-selective antagonist, did. In the absence of external Ca, no maintained increase in ^{86}Rb efflux was observed. The results suggest that there may be two separate α-adrenergic sensitive Ca influx pathways into guinea pig hepatocytes, one mediated by α_1- and the other by α_2-adrenoceptor activation.

INTRODUCTION

Both α_1- and α_2-adrenoceptors have been implicated in alterations of cellular Ca distribution (1–4). Activation of α_1-adrenoceptors is thought to cause Ca mobilization from intracellular stores and may also mediate Ca influx across the plasma membrane (5, 6). α_2-Adrenoceptor activation can inhibit adenyl cyclase activity (7), and inferences have been made that it may raise cytosolic free calcium levels ($[Ca^{2+}]_i$) (8, 9) by a mechanism which is, as yet, unclear.

The response of guinea pig hepatocytes to epinephrine involves both a transient and a maintained increase in Ca-activated K efflux (6).[2] It has been established for a number of years that epinephrine stimulates K efflux from hepatocytes by its action on α-adrenoceptors (10) causing an increase in $[Ca^{2+}]_i$ which in turn appears to activate a Ca^{2+}-dependent K permeability. More recent evidence has suggested that the biphasic ^{86}Rb efflux response may be specifically due to α_1-adrenoceptor activation (6).

In this study, ^{86}Rb was used as a convenient tracer for potassium movements. The alpha adrenoceptor subtype specificity of the maintained ^{86}Rb efflux response was investigated with relatively selective α_1- and α_2-agonists and -antagonists.

MATERIALS AND METHODS

Materials. Salines were freshly made up with Analar grade chemicals obtained from BDH Ltd., Poole, England. ^{86}Rb was from Amersham International Ltd., Amersham, England. Collagenase (type IV) and adrenergic agonists and antagonists were from Sigma Chemical Company, St Louis, MO.

Hepatocyte isolation. Hepatocytes were isolated using a modification ot the technique described by Berry and Friend (11). Male Hartley guinea pigs between 250 and 450 g were anesthetized with urethane, the liver was exposed, and the hepatic portal vein was cannulated with a Venisystems 14G × 51 mm Abbocath-T cannula. The liver was removed and transferred to a retaining tray. EGTA[3] perfusate, at 37°, was passed through the liver for 10 min and then replaced with Ca perfusate, containing 0.5 mg·ml^{-1} collagenase, which was recirculated through the liver until the tissue became soft (10–20 min). The saline compositions were: EGTA saline (NaCl, 116 mM, KCl, 5.4 mM; $MgSO_4$, 0.81 mM; NaH_2PO_4, 0.96 mM; $NaHCO_3$, 25 mM; glucose, 5.5 mM; EGTA, 0.52 mM, pH 7.4, with NaOH) and calcium saline (as above but with 1.8 mM $CaCl_2$ replacing the EGTA). Salines were gassed with saturated 95% O_2/5% CO_2 at 37° through Silastic gaseous exchange tubing immersed in water.

The liver was removed from the perfusion system and disaggregated with a razor blade; then, the tissue was resuspended in the collagenase saline which was agitated at 120 strokes/min in a shaking water bath at 37°. The suspension was then filtered through three layers of medical gauze and washed three times by centrifugation (1 min at 50 × *g*); then, the cells were resuspended in Ca-Hepes saline (NaCl, 144 mM; KCl, 5.4 mM; $MgCl_2$, 1.8 mM; $CaCl_2$, 1.8 mM; Hepes, 10 mM; glucose, 5.5 mM, pH 7.4, with NaOH) supplemented with 1 mg·ml^{-1} bovine serum albumin. Hepes pH-buffered salines were used for convenience rather than the more generally used bicarbonate-buffered salines after control experiments indicated that the ^{86}Rb efflux response was identical in both saline types. Cell viability was estimated to be in excess of 85–95% with 0.4% (w/v) trypan blue, and the isolated hepatocytes had intracellular ion concentrations (determined by atomic absorption spectrophotometry) similar to those reported by other workers (see Table 1).

Cells were loaded with ^{86}Rb (2–5 μCi/ml) for 1 hr in bovine-serum

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[2] J. M. H., unpublished observations.

[3] The abbreviation used is: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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TABLE 1

Intracellular ion concentrations

Cation concentrations were measured by atomic absorption spectrophotometry. Known volumes of hepatocytes (determined with a Coulter counter) were washed twice in ice-cold Ca-free Hepes saline (no EGTA) in pre-weighed tubes. The pellet volume was determined by weight and the cells were lysed in 0.1% Triton in water. Cell fragments were sedimented by centrifugation, and the cation concentration in the supernatant was measured after appropriate dilution. The contribution of the extracellular space was calculated by subtracting the hepatocyte pellet volume from the total pellet volume and making the necessary correction to the measured pellet ion concentration. Values are expressed as mmol·l^{-1} cell volume.

Ion	Concentration	SE	No.
	mM		
K	114.5	6.4	38
Na	30.8	1.8	40
Ca	1.1	0.1	38
Mg	9.8	0.6	22

albumin-supplemented Ca-Hepes saline at 37° in a shaking waterbath (120 strokes/min) with water-saturated O_2 blown over the suspension. The loaded cells were washed twice by centrifugation (1 min at 50 × *g*) and the loosely packed pellets were resuspended in O_2-saturated Ca-Hepes saline. The suspension was divided into 25-ml conical flasks and incubated at 37° in a shaking waterbath (120 strokes/min). Additions were made to each flask as necessary with appropriate volumes of saline added to control conditions. The final experimental suspension cytocrit was approximately 1% (v/v) determined with a Coulter counter.

Samples (0.5 ml), taken in triplicate at each sample time, were centrifuged for 2 min in an Eppendorf 5412 centrifuge. An aliquot of supernatant was removed from each tube and the radioactivity was determined. The time course of experiments varied from 40 min to 2 hr with replicate samples taken every 5 to 15 min. Aliquots of whole suspensions (0.5 ml) were taken as totals.

Maintained ^{86}Rb efflux data are expressed as the efflux rate constant min^{-1}. This is the gradient of the line described by the data points when plotted as:

$$y = -\ln [1 - (\text{sample count/total count})]$$

against time (see Fig. 1). The theory and proof of this function are described by Hope (12). It assumes a two-compartment system, *i.e.*, a cellular and an extracellular compartment in which all of the isotope is available to diffuse across the membrane. The "goodness of fit" of the calculated line to the experimental data is given by the standard deviation of the regression coefficient. The rate coefficients from replicate experiments were averaged with a weighting of the square root of the standard deviation (weighted mean).

RESULTS

The ^{86}Rb efflux time course and effects of agonist addition to hepatocytes in suspension are shown in Fig. 1. The transient increase in ^{86}Rb efflux following epinephrine addition described by DeWitt and Putney (6) is seen as a "jump" in the proportion of counts present in the supernatant before the first samples were taken (2 min). Following the jump, the ^{86}Rb efflux rate remains raised compared to control rates: the maintained increase in ^{86}Rb efflux. The efflux rate constant min^{-1} for this phenomenon was calculated as described. Weighted mean maintained efflux rates min^{-1} were: 1.8 mM Ca saline, $5.14 \times 10^{-3} \pm 8 \times 10^{-5}$ ($n = 29$); 0.1 mM EGTA saline, $7.01 \times 10^{-3} \pm 4.5 \times 10^{-4}$ ($n = 8$); 10^{-4} M epinephrine in Ca saline, $7.94 \times 10^{-3} \pm 2.3 \times 10^{-4}$ ($n = 7$); 10^{-4} M epinephrine in EGTA saline, $6.93 \times 10^{-3} \pm 7.2 \times 10^{-4}$ ($n = 4$).

Fig. 2 shows the effects of various ^{86}Rb efflux agonists in the presence and absence of extracellular Ca. No ^{86}Rb efflux response occurred in the absence of external Ca, suggesting that influx of Ca into the cytosol mediates the maintained Ca-activated K permeability.

The actions of the known inhibitors of the Ca-activated K permeability, quinidine (13) and apamin (14), on the epinephrine-evoked maintained increase in ^{86}Rb efflux are shown in Fig. 3. Both inhibitors substantially reduced the increase in ^{86}Rb efflux.

The action of the two relatively selective adrenoceptor subtype antagonists, yohimbine (α_2) and prazosin (α_1), on the epinephrine-elicited maintained ^{86}Rb efflux are shown in Fig. 4. The maintained ^{86}Rb efflux, mediated by the entry of external Ca into the cytosol, was totally inhibited by yohimbine (IC_{50} = ~40 nM) but was not reduced by prazosin. The efflux was not inhibited by propranolol, a β-adrenergic selective antagonist, and was not stimulated by concentrations of isoprenaline, a preferential β-adrenergic agonist, of up to 0.1 mM (data not shown).

Maintained ^{86}Rb efflux dose response curves constructed for clonidine, phenylephrine and epinephrine are shown in Fig. 5. All of the agonists elicited a maintained increase in ^{86}Rb efflux, but phenylephrine was considerably less effective, maximally increasing the basal efflux rate by approximately 20% as opposed to approximately 60% for clonidine and epinephrine. This phenylephrine effect may be due to some α_2 activity of the predominantly α_1-adrenoceptor-selective agonist (also see Fig. 6). The approximate EC_{50} for clonidine and epinephrine was 2 μM and for phenylephrine, 4 μM. In this system clonidine appears to be as effective an agonist as epinephrine.

The effects of yohimbine and prazosin on the maintained increase in ^{86}Rb efflux elicited by phenylephrine or clonidine are shown in Fig. 6. Prazosin failed to inhibit the raised ^{86}Rb efflux evoked by either phenylephrine or clonidine. Yohimbine, in contrast, reduced both, suggesting that the predominantly α_1-selective agonist phenylephrine may also display some α_2 activity in this system (also see Fig. 5).

DISCUSSION

In guinea pig hepatocytes, DeWitt and Putney (6) have previously demonstrated an α_1-stimulated biphasic increase in ^{86}Rb efflux. The results of DeWitt and Putney (6) and those of this investigation[4] indicate that, after an initial transient ^{86}Rb efflux in Ca-free perfusate, the addition of Ca to perfusate with the agonist still present caused a "slowly falling" second phase of ^{86}Rb release. This second phase was blocked by prazosin but not by yohimbine.

In the present study external Ca was present throughout the experiments. The results suggest that, in addition to the α_1-sensitive, "slowly falling" phase there may be an α_2-mediated maintained increase in ^{86}Rb release

[4] J. M. H., unpublished observations.

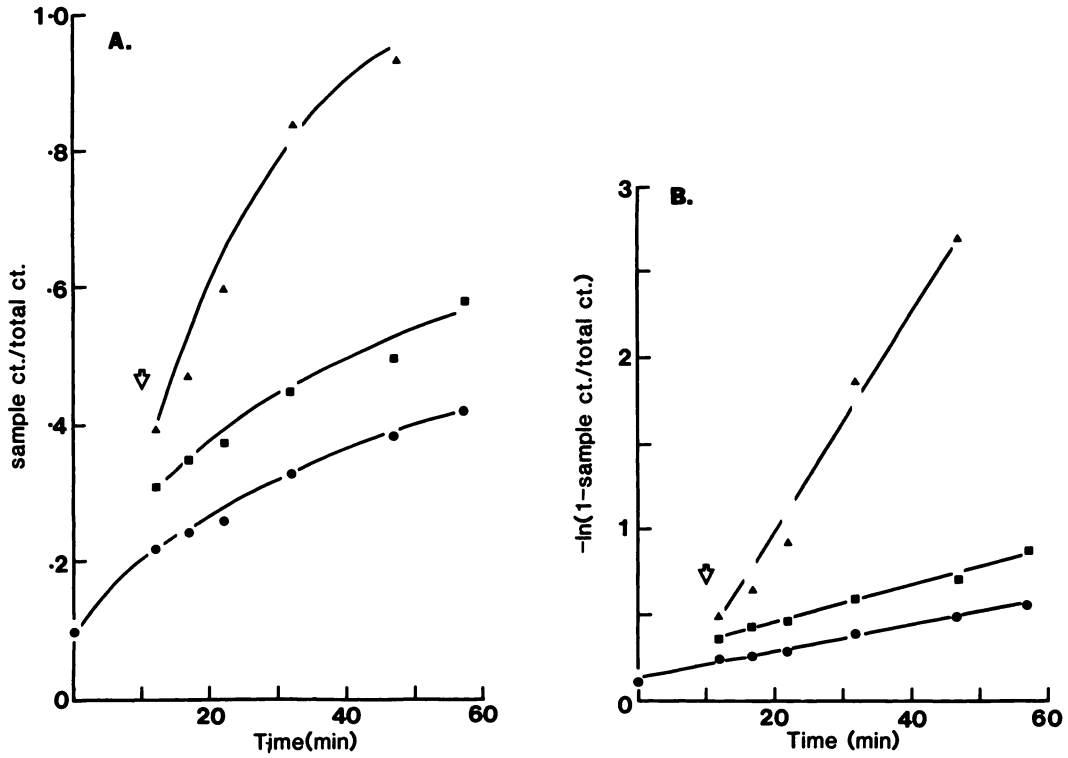

FIG. 1. *Experiments showing (A) the time course of ^{86}Rb efflux from hepatocyte suspensions and (B) the transformed data from which efflux rate constants were determined*

A, ^{86}Rb counts in the supernatant expressed as a fraction of the total suspension isotope counts against time. Additions were made at t = 10 min and were: control (●), 100 μM epinephrine (■), and 7.5 μM valinomycin (▲). Time 0 refers to the first sample point. The washed cells were resuspended in 1.8 mM Ca-Hepes saline at t = −5 min. B, the data transform calculated with the function:

$$y = -\ln(1 - \text{sample count/total count})$$

When plotted against time, the gradient of the line through the data points represents the efflux rate constant. Lines were routinely fitted to the data by using a computer to calculate least mean squares regression fit. Mean efflux rate constants are given in the text. The "jump" immediately following agonist addition is the transient increase in ^{86}Rb efflux, the rate of which could not easily be determined using the suspension sampling technique.

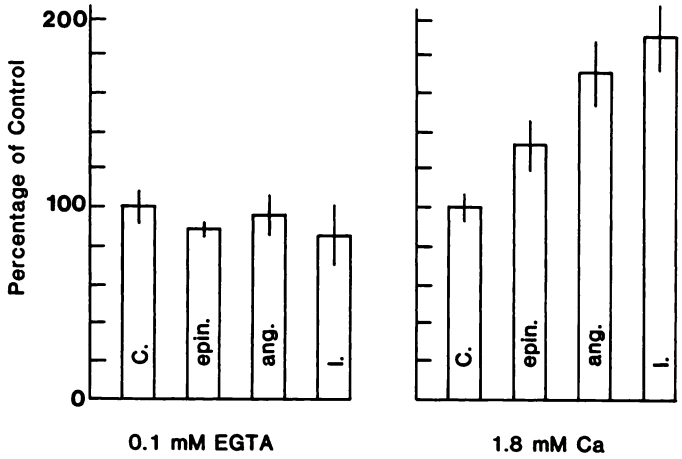

FIG. 2. *Effect of 100 μM epinephrine (epin.), 100 nM angiotensin II (ang.), and 100 μmol·l^{-1} cells A23187 (I.) in the presence and absence of external Ca*

The salines either contained 1.8 mM Ca or 0.1 mM EGTA. Bars show weighted means and errors from two hepatocyte preparations, each sampled in triplicate.

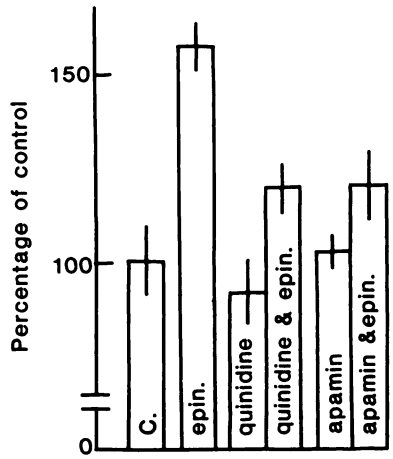

FIG. 3. *Action of inhibitors of the Ca-activated K permeability on the maintained ^{86}Rb efflux elicited by 100 μM epinephrine*

The inhibitors, 100 nM apamin or 100 μM quinidine, were added 5 min before the epinephrine challenge. Bars show weighted means and errors using cells from two hepatocyte preparations, each sampled in triplicate.

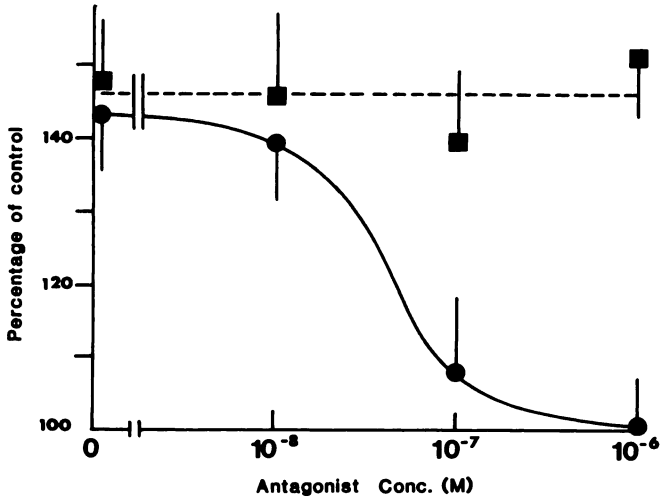

FIG. 4. *Inhibition curves for yohimbine (●) and prazosin (■) on the maintained ^{86}Rb efflux elicited with 3 μM epinephrine*

Antagonists were added 5 min before epinephrine addition. Symbols show weighted means and errors from experiments with cells from four animal preparations, each sampled in triplicate.

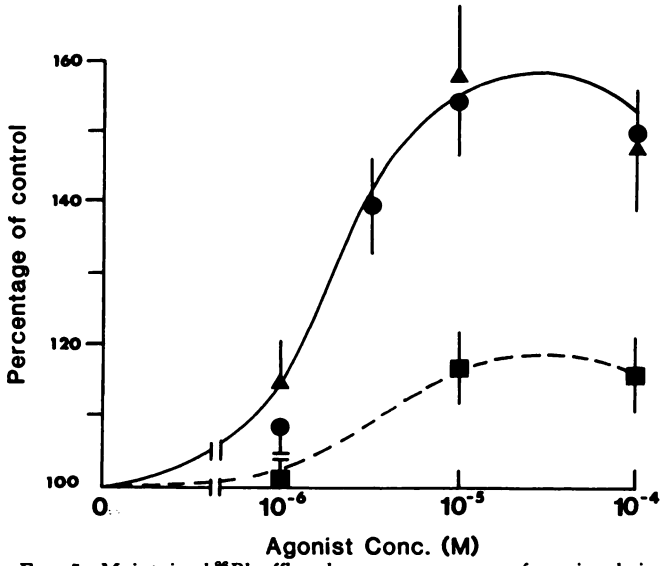

FIG. 5. *Maintained ^{86}Rb efflux dose response curves for epinephrine (●), clonidine (▲), and phenylephrine (■)*

Symbols show weighted means and errors from experiments with cells from three animal preparations, each sampled in triplicate.

which is due exclusively to the entry of external Ca into the cytosol stimulating a Ca-activated K permeability.

With the experimental paradigm used in this study, the maintained ^{86}Rb efflux evoked by epinephrine was not reduced by α_1 blockade over the concentration range investigated but was totally inhibited by yohimbine. The α_1-agonist phenylephrine elicited a maintained increase in ^{86}Rb efflux but was less effective than clonidine or epinephrine, and its effect appeared to be due to some α_2 activity of the preferential α_1-agonist.

One possible explanation for the results may be that the α_1-sensitive Ca influx pathway described by DeWitt and Putney (6) serves as a mechanism for the replenishment of intracellular Ca stores depleted during the transient ^{86}Rb efflux phase (due to the α_1-mediated mobilization of intracellular Ca stores). When Ca is added to the external media it enters the cell via an α_1-adrenoceptor-sensitive pathway, possibly directly into internal storage sites. If the "gates" from these storage sites remain open in the absence of extracellular Ca, the newly added Ca may pass immediately through into the cytosol and stimulate the second phase of the biphasic response. The α_2-adrenoceptor-sensitive Ca influx pathway suggested by the experiments in this report appear to represent a separate intracellular Ca^{2+} control mechanism.

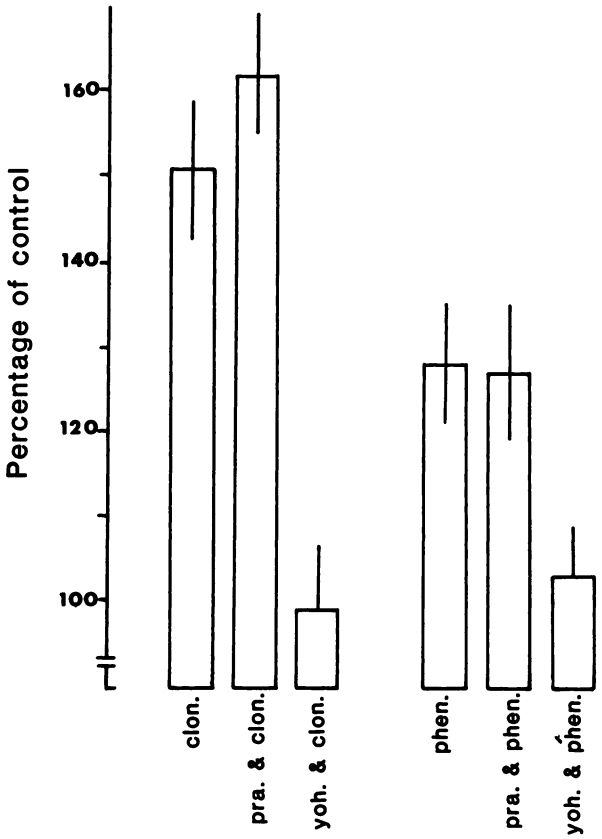

FIG. 6. *Effects of 100 nM prazosin (pra.) and 100 nM yohimbine (yoh.) on the maintained ^{86}Rb effluxes elicited by 10 μM clonidine (clon.) or 10 μM phenylephrine (phen.)*

The antagonists were added 5 min prior to agonist addition. Bars show weighted means and errors from four animal preparations, each sampled in triplicate.

In conclusion, the data presented suggest that there may be two distinct Ca influx pathways, one sensitive to α_2-adrenoceptor blockade, the other being part of the biphasic ^{86}Rb efflux described by DeWitt and Putney (6), sensitive only to α_1 adrenoceptor blockade.

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