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Interactions between receptors that increase cytosolic calcium and cyclic AMP in guinea-pig liver cells

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1 The action of agonists which increase the K^+ permeability of liver cells was studied by using a K^+-sensitive electrode to record the net movement of K^+ between guinea-pig isolated hepatocytes and their suspension medium.

2 Two types of agonist were examined. Type 1 comprised angiotensin II, ATP, noradrenaline and amidephrine, all of which are thought to raise cytosolic Ca^{2+} in hepatocytes. The Type 2 agonists were isoprenaline and glucagon, which activate adenylate cyclase.

3 Each type of agonist initiated K^+ loss from the hepatocytes though the response to Type 2 agonists was more variable than that to Type 1, and sometimes absent.

4 Simultaneous application of a small concentration of an agonist from each class caused a loss of K^+ which was much larger than the sum of that seen with each agonist alone, i.e. potentiation occurred.

5 The α-adrenoceptor antagonist, WB 4101, abolished potentiation if applied after an α-agonist, and before a Type 2 agonist, showing that both receptors have to be active for potentiation to occur.

6 Simultaneous application of a maximal concentration of each type of agonist caused a larger loss of K^+ (~17% of the cell total within 45 s) than did a maximal concentration of a Type 1 agonist alone (~10%).

7 Since the K^+ loss caused by these agonists is thought to be a consequence of a rise in cytosolic Ca^{2+}, the influence of both types of agonist on ^{45}Ca and ^{42}K efflux from guinea-pig liver slices was studied.

8 The effect of isoprenaline on ^{45}Ca and ^{42}K efflux became much greater following a previous application of the α-adrenoceptor agonist, amidephrine.

9 In the presence of apamin, the potentiated effect of isoprenaline on ^{42}K efflux was greatly reduced whereas that on ^{45}Ca efflux was little affected.

10 The effects of Type 1 and Type 2 agonists separately and together on the cyclic AMP content of isolated hepatocytes were examined. Type 2 agonists increased cyclic AMP in the expected way. The increase became slightly smaller, if anything, when a Type 1 agonist was applied at the same time. Hence potentiation could not be ascribed to changes in cyclic AMP formation.

11 Possible mechanisms for potentiation are discussed. Our evidence suggests, albeit indirectly, that it is a consequence of an interaction between the effects of the two types of agonist on cytosolic Ca^{2+}.

Introduction

Guinea-pig hepatocytes possess at least five distinct receptors which can initiate rapid increases in metabolic activity and membrane permeability. These receptors can be subdivided on the basis of their mechanism of action. One group (Type 1) increases cytosolic Ca^{2+}, but not adenosine 3′:5′-cyclic monophosphate (cyclic AMP). It comprises receptors responsive to angiotensin II (Weiss & Putney, 1978: de Witt & Putney, 1984) adenosine triphosphate (ATP; see Jenkinson & Koller, 1977; Burgess *et al.*, 1981) and α-adrenoceptor agonists (Haylett & Jenkinson, 1972a,b; Osborn, 1978; Egashira, 1980; Burgess *et al.*, 1979, 1981). The second group (Type 2) activates adenylate cyclase, hence increasing the cyclic AMP content of the hepatocyte, and is exemp-

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lified by the receptors for glucagon and the β actions of catecholamines (for a review see Exton, 1982).

It is now clear that activation of either type of receptor can accelerate hepatic glycogenolysis (see Haylett, 1979; Ellis, 1980; Exton, 1982, for reviews). Exton and his colleagues have shown that for this response the sequence of events initiated by Type 1 and Type 2 receptors converge at the stage of activation of phosphorylase *b* kinase (see Exton, 1982).

Less is known about the way in which the various membrane receptors initiate the increase in K^+ permeability which in the liver of most species (though not the rat) accompanies the increase in glycogenolysis but can be dissociated from it, for example, by the action of the K^+ channel blocker apamin (Banks *et al.*, 1979; Burgess *et al.*, 1981). This response is probably a consequence of an increase in cytosolic Ca^{2+}, and is always seen when Type 1 receptors are activated (Haylett, 1976; Weiss & Putney, 1978; Egashira, 1980; Burgess *et al.*, 1981; de Witt & Putney, 1984). In contrast, activation of Type 2 receptors (e.g. β-adrenoceptors) elicits an increase in K^+ permeability in only a proportion of guinea-pig liver preparations (Haylett & Jenkinson, 1972b). However, the response always occurs if a β-agonist is applied up to 20 min or so after the tissue has been exposed to certain α-agonists, for example, amidephrine and oxymetazoline. Moreover, when an α- and a β-agonist are given together in low concentrations, the effect on membrane permeability is much greater than the sum of that to each agent alone (Jenkinson & Koller, 1977). This interaction has been termed potentiation and is the subject of the present work. We had three main aims: (1) To determine whether potentiation, which had been detected and hitherto studied only in liver slices, also occurs in dispersed hepatocytes. (2) To test whether the same interaction occurs with other receptor pairs, one from each of the groups mentioned above, and (3) to study the underlying mechanisms, and, in particular, the roles of cyclic AMP and Ca^{2+} ions.

Methods

Isolation of hepatocytes

The collagenase method was used to isolate hepatocytes from the livers of male Hartley guinea-pigs (200–300 g) essentially as described by Seglen (1972, 1973; see also Burgess *et al.*, 1981). The animals were anaesthetized with sodium pentobarbitone (Sagatal: 36 mg kg^{-1} body wt. i.p.) supplemented if needed with ether. The portal vein was cannulated and the liver flushed gently with a solution containing (mM): NaCl 116, KCl 5.4, $MgSO_4$ 0.819 NaH_2PO_4 0.96, $NaHCO_3$ 25, glucose 5.6 and EGTA 0.52. The liver was removed from the animal, mounted in a perfusion chamber and perfused with the same solution for 10 min at 37°C under constant pressure (flow rate 30 ± 3 ml min^{-1}: mean ± s.e.mean from 10 experiments) and then for a further 10 min with an EGTA-free solution of similar composition but containing Ca^{2+} (5.1 mM) and collagenase (50 mg 150 ml^{-1}). The liver was next minced finely with a razor-blade and incubated at 37° in the high Ca^{2+}-collagenase solution for a further 6–8 min with continual agitation and gassing. Following addition of new-born calf serum (10 ml 60 ml^{-1} solution), the crude hepatocyte suspension was filtered through a coarse nylon sieve, centrifuged (60*g* for 2 min) and the collected cells washed three times in normal physiological medium (composition as above except that EGTA was omitted and Ca^{2+} was 1.8 mM) before finally being suspended in Eagle's medium (Wellcome) containing (mM): NaCl 116, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.81, NaH_2PO_4 0.96, $NaHCO_3$ 25 and (mg ml^{-1}): amino acids 805, vitamins 8.1, glucose 1000, L-glutamine 292, phenol red 10 and 2% (w/v) bovine serum albumin. All physiological salt solutions were oxygenated and maintained at pH 7.4 by equilibration with 95% O_2, 5% CO_2. The cells were resuspended in fresh medium 30 min after preparation and every 60 min thereafter. Their viability was assessed by the Trypan Blue exclusion test and was between 80 and 95%.

Measurement of ion movements

(a) *K^+* Agonist-induced release of K^+ from continuously stirred suspensions of hepatocytes (2 ml, containing ca. 20 mg tissue, dry weight) was studied by using a K^+-sensitive electrode to record the concomitant increase in the K^+ concentration of the suspension medium (see Burgess *et al.*, 1981). The amount lost was expressed as a percentage of total cell K^+, as measured by flame photometry on another cell sample taken at the same time. In 37 experiments, the K^+ content of these control cells was 259 ± 6 mmol kg^{-1} tissue (dry weight, mean ± s.e.mean). This is close to the value of 269 ± 4 ($n = 52$) observed in our earlier work (see Burgess *et al.*, 1981, for a more detailed account of the method, and of the errors involved).

(b) *Efflux of ^{42}K and ^{45}Ca from guinea-pig liver slices* The influence of drugs on the efflux of ^{42}K and ^{45}Ca from guinea-pig liver slices, prepared and incubated as described by Haylett & Jenkinson (1972a), was studied by the method of Haylett (1976; see also Burgess *et al.*, 1981).

Cyclic AMP content of hepatocytes

The cyclic 3',5'-adenosine monophosphate (cyclic AMP) content of the hepatocytes was determined essentially as described by Osborn (1978) for guinea-pig liver slices: 0.5 ml aliquots of hepatocytes (30.4 ± 1.5 mg dry wt. 0.5 ml^{-1}: mean ± s.e.mean from 8 experiments) were placed in pre-warmed (37°C) 1.5 ml Eppendorf centrifuge tubes (t = 0 min) and continually stirred for 3 min at which time 0.25 ml of 10% (w/v) trichloroacetic acid (TCA) at 0°C was added with vigorous mixing to ensure rapid protein denaturation. Drugs were always introduced 1 min after the start of incubation so that the contact time with cells was 2 min. After addition of TCA, the tubes were cooled to 0°C for 10 min, centrifuged quickly (2 min) and 0.5 ml aliquots of the supernatant loaded onto 3.5×0.5 cm columns of Dowex 50×8 (H^+ form) and eluted with distilled water. This procedure removed the TCA (eluted in the first to fourth ml as determined by pH and u.v. absorption measurements) and most of the ATP present in the sample. Cyclic AMP eluted in the fourth to ninth ml inclusive. Recovery from the column of a small amount of [^{3}H]-cyclic AMP added to the sample was $77.3 \pm 1.2\%$ (mean ± s.e.mean from 7 experiments). The eluate containing the cyclic AMP was then freeze-dried. Later it was reconstituted in 0.5–2.0 ml of cyclic AMP assay buffer and assayed for cyclic AMP by the method of Tovey *et al.* (1974). Reagent blank 100 μl (i.e. Eagle's medium processed in the same manner as that for Eagle's plus cells) was added to both the 'adsorption efficiency control' and standards. The overall recovery of cyclic AMP added to control samples of cells was $88 \pm 5\%$ (mean ± s.e.mean from 7 experiments).

Materials and drugs

All inorganic salts used in the bathing solutions were of Analar quality. ^{45}Ca, ^{42}K and the cyclic AMP assay reagents were obtained from the Radiochemical Centre, Amersham. Dowex (50×8 H^+ form) and EGTA (ethyleneglycol-*bis*-(β-aminoethyl ether) N,N'-tetraacetic acid) from Sigma, valinomycin and collagenase from Boehringer, Eagle's medium (MEM) from Wellcome Reagents Ltd., albumin (fraction V) from Miles Laboratories, newborn calf serum from Gibco Biocult and trichloroacetic acid from BDH.

Drugs used were: adenosine (hemi-sulphate salt), adenosine 5'-triphosphate (ATP: disodium salt), isoleucine 5-angiotensin II (human form-synthetic), glucagon (bovine and porcine mixture), S-(*p*-nitrobenzyl)-6-thioguanosine, propranolol hydrochloride, arginine- and lysine-vasopressin (Sigma); (–)-amidephrine hydrochloride (a gift from Mead Johnson); (–)-isoprenaline bitartrate and WB 4101 (2-N([2',6'-dimethoxyphenoxyethyl] aminomethyl)-1,4-benzodioxan) (gifts from Ward Blenkinsop); (–)-noradrenaline bitartrate (Koch-Light); phentolamine mesylate (Ciba). Apamin was a kind gift from Dr Barbara Banks, University College London. It is now available from Serva and Sigma.

Statistical procedures

Students's *t*-statistic was used in estimating confidence limits and the significances of differences between means.

Results

Receptor-mediated K^+ loss from dispersed hepatocytes

Type 1 receptors Both noradrenaline and ATP cause dispersed guinea-pig hepatocytes to lose K^+ (Burgess *et al.*, 1981), and this was confirmed in the present work. The loss is rapid, being complete within 1 min, and can be attributed to a transient increase in the K^+ permeability of the cell membrane. As already briefly reported (Cocks *et al.*, 1981), the same response is seen with angiotensin II, as was to be expected from the work of Weiss & Putney (1978) with liver slices (see also de Witt & Putney, 1984).

The concentration-response relation for angiotensin is shown in Figure 1, together with that for noradrenaline and ATP. It is noteworthy that the K^+ losses induced by maximal concentrations of the three agents are of the same order (ca. 9–11% of the cell content).

A second polypeptide, 8-arginine vasopressin, was examined since, like angiotensin II and noradrenaline, it has been reported to increase cytosolic Ca^{2+} in rat hepatocytes (Assimacopoulos-Jeannet *et al.*, 1977; Chen *et al.*, 1978; see also Putney, 1978 and Hems & Whitton, 1980). However, in each of 8 experiments, no effect of arginine vasopressin (up to 0.1 iu ml^{-1}: 0.26 μM) could be detected: lysine vasopressin (up to 0.1 iu ml^{-1}: 0.47 μM) was similarly ineffective. The lack of responsiveness to vasopressin was not due to the collagenase treatment during cell isolation since both arginine- and lysine-vasopressin also failed to release K^+ from suspensions of guinea-pig liver slices which were, however, responsive to ATP, angiotensin II and the α-adrenoceptor agonist amidephrine. Guinea-pig hepatocytes do not therefore appear to possess vasopressin receptors mediating changes in K^+ permeability (and, by inference, increases in cytosolic Ca^{2+}). This confirms the conclusions of de Witt & Putney (1984).

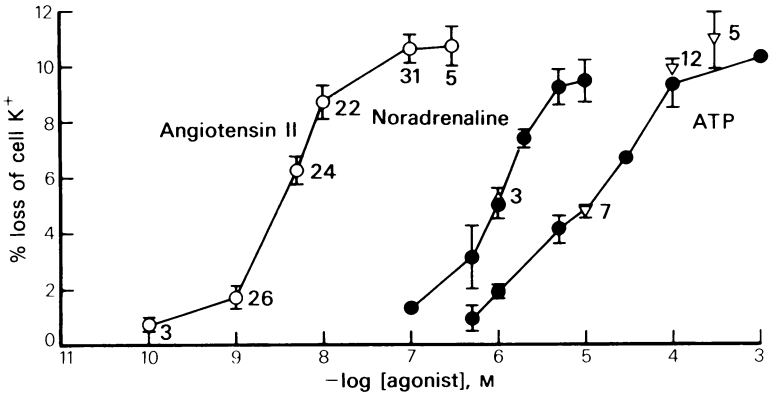

Figure 1 Concentration-response relationship (○) for the action of angiotensin II in causing K^+ loss from guinea-pig hepatocytes. The net movement of K^+ was measured using a K^+-sensitive electrode placed in the cell suspension (see also Methods, and Figure 7). The values plotted are the means for the numbers of observation indicated with vertical lines showing s.e.means. Results previously obtained for noradrenaline (in the presence of propranolol, 5 μM) and ATP have been included for comparison (● from Burgess *et al.*, 1981), together with equivalent values from the present work (△, noradrenaline: ▽, ATP).

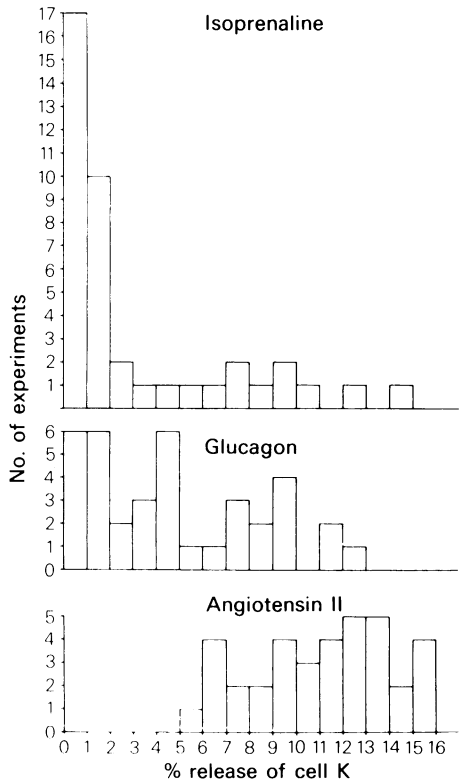

Figure 2 Histograms showing the variation in the losses of K^+ observed on exposing guinea-pig hepatocytes to isoprenaline (50–100 nM), glucagon (10–100 nM) or angiotensin II (100–300 nM). These concentrations of isoprenaline and glucagon were maximal or near maximal in causing K^+ loss from those cell preparations which responded to Type 2 receptor agonists (see Text).

Type 2 receptors In contrast to the actions of noradrenaline, ATP and angiotensin II, the effects of isoprenaline and glucagon on the K^+ content of guinea-pig hepatocytes varied greatly from preparation to preparation (though not within samples of cells taken from the same animal). Some preparations showed a striking loss of K^+, at least as large as that seen with Type 1 agonists in the same preparations, and of similar time course. Others did not respond (<1% K^+ loss) even when exposed to a concentration of isoprenaline (100 nM) sufficient to cause a maximum loss of K^+ from responsive cells. This variation is illustrated in Figure 2, and is of the same order as seen in earlier experiments with liver slices (Haylett & Jenkinson, 1972b; Jenkinson & Koller, 1977). It could not be related to differences either in the K^+ content of the cells, or in the size of the response to Type 1 agonists, though we gained the impression that the proportion of responsive preparations rose in the winter months.

The variation in the response to isoprenaline was almost matched by that to glucagon, though a smaller proportion (16%, as compared with 41% for isoprenaline) of the preparations tested were unresponsive (<1% K^+ loss). Hepatocytes which did not respond to glucagon also failed to lose K^+ when isoprenaline was applied. They were however fully responsive to angiotensin II, ATP and noradrenaline. Furthermore, the loss of K^+ from them became much greater when either isoprenaline or glucagon was applied together with angiotensin II (see below). Also, both glucagon and isoprenaline caused dose-dependent increases in the cyclic AMP content of such cells (see later). The lack of K^+ loss in response to isoprenaline or glucagon given alone could not

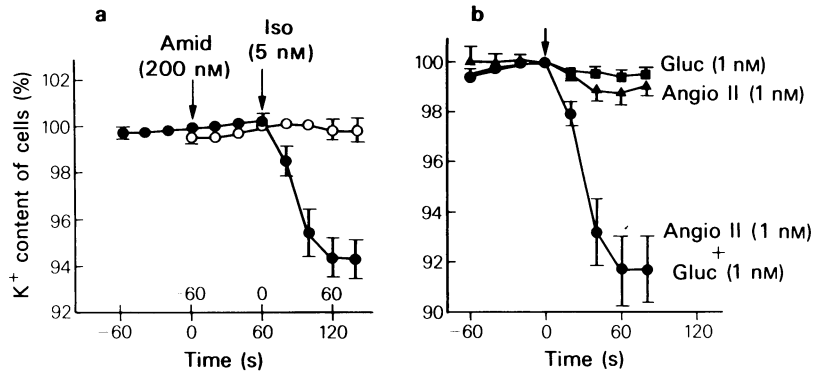

Figure 3 (a) Influence of the α-adrenoceptor agonist amidephrine on the effect of isoprenaline on the K^+ content of guinea-pig hepatocytes in suspension: (○) cells treated with (−)-isoprenaline (5 nM) alone, applied at 0 time (upper abscissa scale); (●) cells treated first with (−)-amidephrine (200 nM, at 0 time, lower time scale) and then 60 s later, with (−)-isoprenaline (5 nM), as before. All values are the means of at least 5 observations, each from a separate experiment, and have been expressed in terms of the K^+ content of the cells at 0 time; vertical lines indicate s.e.means. (b) K^+ loss from guinea-pig hepatocytes exposed (at arrow) to glucagon (1 nM) and angiotensin II (1 nM) either separately (■ ▲) or together (●). Other details as in (a).

therefore be attributed to impaired receptor function.

Other agonists tested Adenosine has been reported to have complex effects on adenylate cyclase in rat hepatocytes: both stimulation and inhibition have been described (e.g., Cooper & Londos, 1979; Londos *et al.*, 1980). However, in each of 4 experiments, guinea-pig hepatocytes neither gained nor lost K^+ in response to adenosine (up to 500 μM) even in the presence of the adenosine uptake inhibitor *p*-nitrobenzyithioguanosine (10 μM). Nor did adenosine (100 μM) affect the K^+ release caused by either angiotensin II or glucagon.

Potentiated release of K^+

Combined application of a Type 1 receptor agonist (angiotensin II, ATP, amidephrine or noradrenaline in the presence of propranolol) with one of Type 2 (glucagon or isoprenaline) at concentrations which when tested separately caused little release of K^+, resulted in a much greater than additive K^+ loss, i.e., potentiation occurred. Figure 3a shows the effect of a low concentration of amidephrine (200 nM) on the response to isoprenaline (5 nM). Alone each caused little K^+ loss, whereas addition of isoprenaline in the presence of amidephrine initiated a substantial response with a time course similar to that seen with a Type 1 agonist on its own (cf. Figure 2 in Burgess *et al.*, 1981). A similar interaction was seen if the agonists were added simultaneously, as is illustrated for the pair angiotensin II and glucagon in Figure 3b.

Figure 4 depicts concentration-response curves for the agonists angiotensin II and glucagon applied either separately or together in experiments in which high concentrations of glucagon caused less than 2% release of cell K^+. Potentiated increases in K^+ release were observed at each concentration above 0.1 nM. For example, angiotensin II and glucagon together (each at 1 nM) caused 8.22 ± 1.55% (s.e.mean, $n = 6$) release, whereas separate applications gave releases of 1.15 ± 0.80 and 0.27 ± 0.42% ($n = 6$) respectively. The difference between the 'combined' response and the sum of the 'separate' responses was 6.8%. with 95% confidence limits of 11.4 and 2.2%. Since these do not include 0, it can be concluded that potentiation has occurred. Further, the maximum K^+ release for angiotensin II plus glucagon (each at 100 nM)

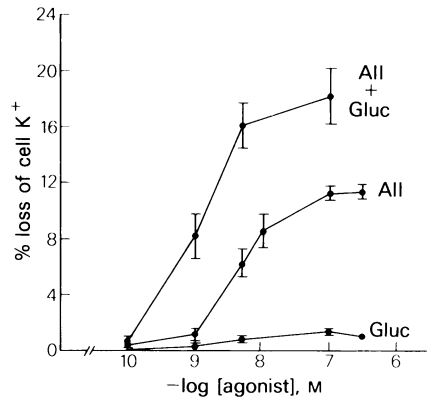

Figure 4 Concentration-response relationships for K^+ loss from guinea-pig hepatocytes exposed to angiotensin II and glucagon applied either separately or together at the same concentration. Values represent means ($n = 6$) with s.e.means shown by vertical lines.

Table 1 Loss of K^+ from dispersed guinea-pig hepatocytes exposed to Type 1 (angiotensin II and ATP) and Type 2 receptor agonists (glucagon) separately and together

Agonist	*Concentration (μM)*	*% release of cell K^+*
Angiotensin II	0.1	10.5 ± 0.6
	0.3	10.6 ± 0.7
ATP	100	9.8 ± 0.6
	300	10.9 ± 1.0
Angiotensin II + ATP	0.1 + 100	10.5 ± 1.0
Glucagon	0.1	6.2 ± 1.4
Angiotensin II + glucagon	0.1 + 0.1	16.1 ± 0.8

Values are expressed as means ± s.e.mean from 6 experiments.

was, at 18.1%, significantly greater ($P < 0.01$) than that for angiotensin II alone (11.3%).

In another group of experiments (Table 1) in which 100 nM glucagon caused 6.2 ± 1.4% release of cell K^+ (mean ± s.e.mean, $n = 6$) the maximum K^+ release increased to 16.1% when angiotensin II (100 nM) was added together with glucagon. This is significantly greater ($P < 0.001$) than the maximum value for angiotensin alone (10.6 ± 0.7%). In this case, i.e. when the cells were already responsive to Type 2 agonists, the maximum K^+ release for combined angiotensin II plus glucagon was approximately additive, although 'potentiation' was evident at lower agonist concentrations. Table 1 also shows that combining maximal doses of Type 1 agonists (e.g. ATP and angiotensin II) did not result in a substantial further release of K^+.

The mechanism of potentiation

(1) *Do changes in intracellular Ca^{2+} underlie potentiation?* Because the increase in P_K initiated by Type 1 agonists seems likely to be triggered by a rise in cytosolic Ca^{2+} concentration, the possibility arises that potentiation of the effect on P_K reflects a correspondingly greater change in $[Ca^{2+}]_i$. The best way to test this would be to measure $[Ca^{2+}]_i$ directly. Suitable methods applicable to hepatocytes have only recently become available (see e.g. Charest *et al.*, 1983), and in the present work the problem was approached indirectly, by measuring the rate of loss of labelled Ca^{2+} from guinea-pig liver slices. Should $[Ca^{2+}]_i$ rise, the efflux of ^{45}Ca would be expected to increase. Figures 5 and 6 show the results obtained. Figure 5 illustrates that, as expected, isoprenaline caused a considerably larger increase in ^{45}Ca (as well as in ^{42}K) efflux when applied after the α-adrenoceptor agonist, amidephrine. Further evi-

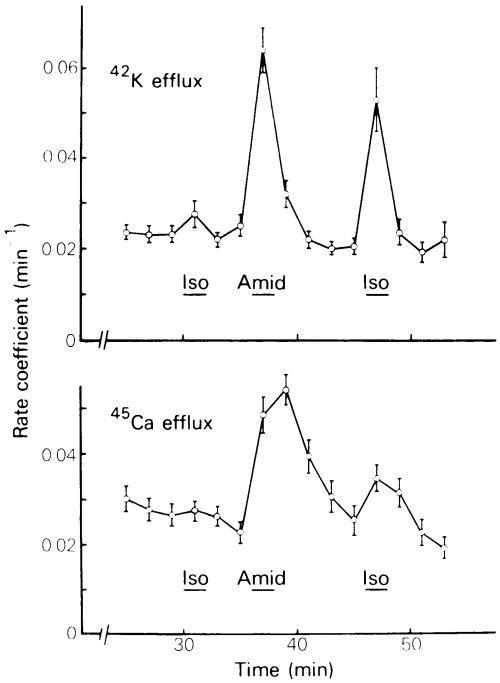

Figure 5 Potentiation by (−)-amidephrine (10 μM, Amid) of the effect of (−)-isoprenaline (50 nM, Iso) on the efflux of labelled K^+ and Ca^{2+} from guinea-pig liver slices. Ordinates: rate coefficient (i.e., fractional loss of tracer per min) for ^{42}K (upper) and ^{45}Ca (lower) efflux. Abscissae: time after removal of the slices from the 'load' solution containing ^{42}K and ^{45}Ca. Each point is the mean of 7 values from separate experiments; s.e.means shown by vertical lines.

dence came from a variation of the experiment, in which a K^+-channel blocking agent, apamin, was included in the bathing fluid from 4 min before the second application of isoprenaline onwards. After amidephrine, isoprenaline in the presence of apamin still caused a larger increase in ^{45}Ca efflux though the effect on ^{42}K efflux was much reduced (Figure 6). This is consistent with our working hypotheses that increases in cytosolic Ca^{2+} underlie the elevation in P_K and that potentiation of the effect on P_K is a consequence of a greater rise in $[Ca^{2+}]_i$.

That the relative increase in the effect on ^{45}Ca efflux of isoprenaline applied after, as compared with before, amidephrine in the experiments of Figures 5 and 6 is not as great as that on ^{42}K efflux may be a consequence of the large effect of amidephrine on the ^{45}Ca content of the tissue. There is good evidence that Type 1 agonists cause Ca^{2+} release from intracellular stores in guinea-pig hepatocytes (Weiss & Putney, 1978; Karashima, 1981; de Witt & Putney, 1984) and the specific activity of ^{45}Ca in these stores

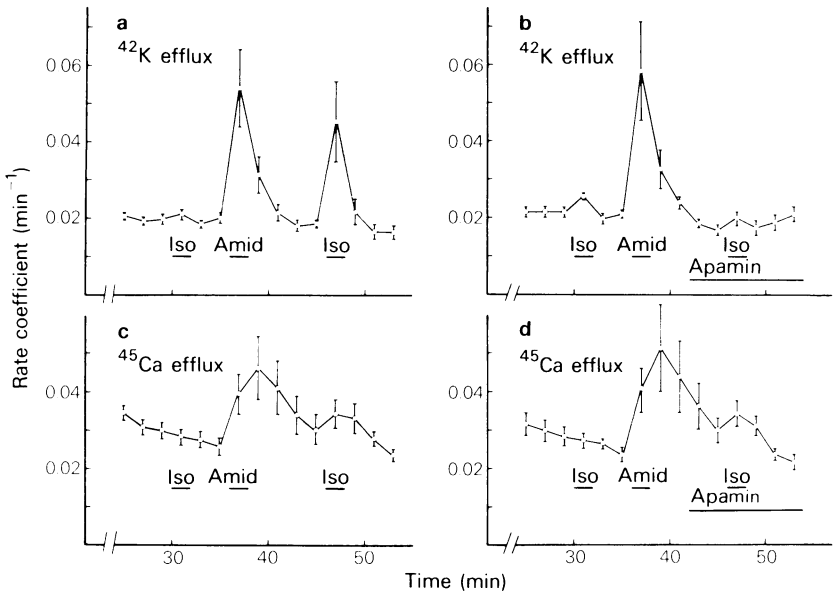

Figure 6 As Figure 5, but showing the effect of apamin (10 nM, present for the time indicated by the lower horizontal bars in b and d) on the response to the second application of isoprenaline. Mean of 4 paired experiments; s.e.mean indicated by vertical lines.

can be expected to fall considerably following the action of a large concentration of a Type 1 receptor agonist such as amidephrine.

(2) *Does potentiation require simultaneous activity of Type 1 and Type 2 receptors?* This point was examined using a suspension of dispersed hepatocytes. The K^+ losses caused by separate applications of relatively low concentrations of (−)-amidephrine (200 nM) and glucagon (10 nM) were first tested, using K^+-sensitive electrodes as before. The losses observed were less than 1% with amidephrine and

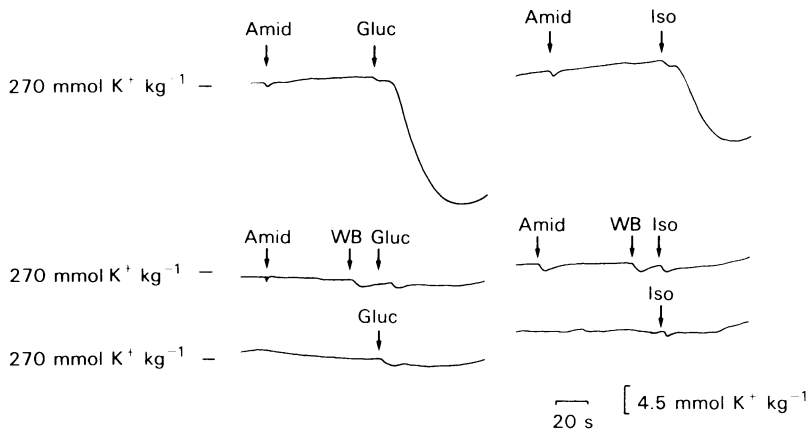

Figure 7 Records from experiments in which a K^+-sensitive electrode placed in the cell suspension was used to follow agonist induced changes in the K^+ content of guinea-pig hepatocytes. The tracings have been calibrated in terms of the amounts of K^+ in the cells (~ 270 mmol kg^{-1} dry weight: see calibrations at left, and lower right). Left: The lowest trace shows the response to glucagon alone (Gluc, 10 nM, at arrow). The top trace shows that prior application of (−)-amidephrine (Amid, 200 nM, at first arrow) greatly enhances the K^+ loss caused by glucagon (again at 10 nM, at second arrow). The middle trace illustrates that the potentiating action of amidephrine is abolished by the α-adrenoceptor antagonist WB 4101 (WB, 10 μM, added to the cell suspension at the second arrow). Right: As left, but with (−)-isoprenaline (Iso, 10 nM) rather than glucagon.

2.1 ± 0.8% (s.e.mean, $n = 6$) with glucagon. However, when the same concentration of glucagon was added to the bath 75–80 s after the addition of amidephrine, the cells lost 9.9 ± 1.0% ($n = 4$) of their K^+ content, i.e. there was considerable potentiation (Figure 7). The experiment was next repeated, but for the application of the α-adrenoceptor antagonist WB 4101 (10 μM) 10–20 s prior to glucagon. The release of K^+ after glucagon was now only 1.3 ± 0.8% ($n = 6$), differing little from the response to glucagon alone. Hence, potentiation had been abolished. Similar results were obtained with isoprenaline rather than glucagon as the Type 2 agonist (Figure 7, right). In the same cells, WB 4101 (again at 10 μM) did not affect either the responses to glucagon, isoprenaline and angiotensin II, or potentiation between angiotensin II and glucagon. In another set of experiments of the same kind, phentolamine (10 μM) abolished potentiation between amidephrine and isoprenaline without affecting the responses to the latter. We conclude that potentiation requires the continued activation, if at low level, of both types of receptor.

(3) *Has cyclic AMP a role in potentiation?* Our final aim was to test whether potentiation could be explained in terms of an influence of Type 1 receptors on the formation of cyclic AMP in response to Type 2 receptor activation. Angiotensin II was chosen as a representative Type 1 agonist and was applied at a concentration (10 nM) sufficient to cause 8.7% K^+ loss, approximately 80% of the maximum response (see Figure 1). There was no corresponding rise in the cyclic AMP content of the hepatocytes (see also Osborn, 1978); the control cells contained 0.83 ± 0.15 (s.e.mean) pmol cyclic AMP mg^{-1} dry tissue, as compared with 0.84 ± 0.09 pmol mg^{-1} ($n = 6$) for those that had been exposed to angiotensin II for 2 min.

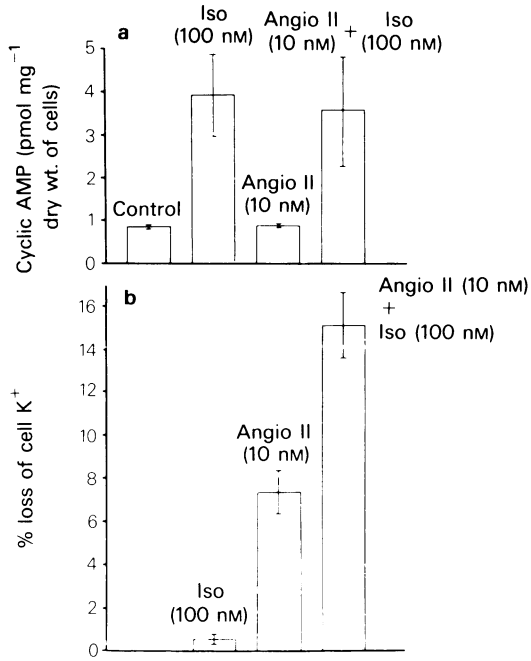

Figure 9 Comparison of two responses ((a) cyclic AMP production: (b) K^+ loss) of the same guinea-pig hepatocytes exposed to (−)-isoprenaline (Iso) and angiotensin II (Angio II) either separately or together. Note that combined application of the two agonists considerably increases the loss of K^+ but not the rise in cyclic AMP. Means of $n = 4$; s.e.mean shown by vertical lines.

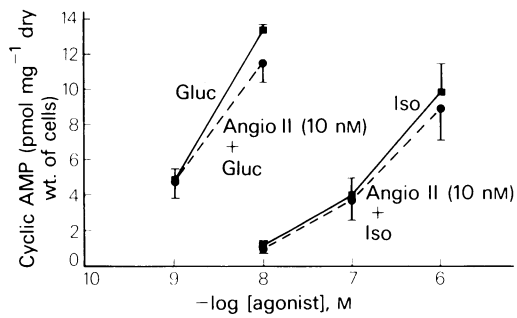

Figure 8 Concentration-response relationships for cyclic AMP production in guinea-pig hepatocytes exposed for 2 min to either glucagon (Gluc) or (−)-isoprenaline (Iso). Each agonist was tested either alone (continuous lines) or in the presence of angiotensin II (Angio II, 10 nM), applied at the same time (dashed lines). Each point shows the mean of at least 4 values, each from a separate experiment; s.e.means indicated by vertical lines.

Figure 8 illustrates the influence of the same concentration of angiotensin on the increases in cyclic AMP observed when the cells were exposed to the Type 2 receptor agonists glucagon and isoprenaline. Unlike the effect on K^+ loss, the rise in cyclic AMP became, if anything, slightly smaller in the presence of angiotensin. The contrast between the potentiation of the effect on K^+ content and the lack of potentiation of the increase in cyclic AMP is evident in Figure 9 which combines the results of 4 experiments in which both responses were measured in the same cell preparations. Though a full study would require cyclic AMP measurements at a range of Type 1 agonist concentrations and exposure times, our results suggest that potentiation is unlikely to be attributable to an increase in cyclic AMP formation.

Discussion

We have found that potentiation, as originally described in guinea-pig liver slices, also occurs in isolated hepatocytes. As in slices (Jenkinson & Koller, 1977), it is not restricted to α- and β-adrenoceptor pairs, but appears to be a more general interaction between agonists that raise cytosolic Ca^{2+} and those that increase intracellular cyclic AMP. Thus, potentiation is as readily seen with pairs of peptides, one from each class (e.g. angiotensin II and glucagon) as with amidephrine and isoprenaline.

Our earlier study of the phenomenon in slices (Jenkinson & Koller, 1977) had shown that some α-adrenoceptor agonists (e.g. amidephrine, oxymetazoline) but not others could potentiate β-agonists (and cyclic AMP) applied up to 20 min later, as indeed the experiments of Figures 5 and 6 illustrate. In discussing this, we noted that the difference could be that the former α-agonists acted for longer so that some α-adrenoceptors were still active when the β-agonist was subsequently applied. The present finding (Figure 7) that an adequate concentration of an α-adrenoceptor antagonist will abolish the potentiating action of a prior application of an α-agonist to isolated cells, is in keeping with such an explanation. It also makes unlikely an alternative possibility considered in our earlier paper, namely, that the prolonged potentiation seen in slices reflected a cellular change which outlasted α-adrenoceptor activation. It seems instead that both types of receptor need to be active. The same conclusion has been reached independently by Dr G.M. Burgess, using the agonist pair angiotensin and isoprenaline (personal communication).

The new findings also allow us to exclude a second possible mechanism for potentiation raised in our previous paper. This was that the application of a Type 1 receptor agonist could result in phosphodiesterase inhibition, and hence in a greater rise in cytosolic cyclic AMP in response to a Type 2 agonist (or externally applied cyclic AMP). Our measurements of the cyclic AMP content of isolated liver cells have shown that, on the contrary, simultaneous activation of Type 1 receptors (by angiotensin II) caused, if anything, a slight reduction in the amount of additional cyclic AMP formed in response to glucagon or isoprenaline.

A third possibility we can now discard is that potentiation relates only to the changes in K^+ permeability and movement that follow receptor activation. As Figure 6 shows, the efflux of ^{45}Ca is also increased when potentiation occurs and, moreover, the increase is still seen when the effect on K^+ movement has been abolished by apamin. This is in keeping with the idea that changes in cytosolic Ca^{2+} underlie the rise in P_K.

How then is potentiation to be explained? Two main findings have to be accounted for. (1) When low concentrations of a Type 1 and a Type 2 receptor agonist are applied together, the ensuing increase in P_K (as assessed from changes in either membrane potential, ^{42}K efflux or K^+ content) is considerably larger than the sum of those to each agonist alone. This potentiated effect on P_K is probably a consequence of either a greater rise in $[Ca^{2+}]_i$ or an increase in the Ca^{2+}-sensitivity of the mechanism responsible for the rise in P_K. (2) Combined application of a Type 1 and a Type 2 agonist produced a greater loss of K^+ (and, by inference, a greater rise in $[Ca^{2+}]_i$) than can be elicited by maximal concentrations of a Type 1 agonist (compare Figures 1 and 4).

In seeking to explain these findings, we are hampered by the present uncertainty about the ways in which membrane receptors can increase cytosolic Ca^{2+} in hepatocytes. Two quite different hypotheses are under consideration. One view is that Ca^{2+} is released from stores associated with the cell membrane, and perhaps also the associated endoplasmic reticulum (Althaus-Saltzmann *et al.*, 1980; Poggioli *et al.*, 1980; Burgess *et al.*, 1983). Others (see Williamson *et al.*, 1981 and Exton, 1981 for references and further discussion) have concluded that the mitochondria are an important primary source from which Type 1 receptors, acting through an as yet unidentified intermediate, initiate the release of Ca^{2+}. This issue, and the role of an increase in the Ca^{2+} permeability of the membrane (see de Witt & Putney, 1984), will have to be settled before the interactions between Type 1 and Type 2 receptors can be fully understoood. The following schemes are accordingly tentative.

We suppose first that Type 2 agonists, acting through cyclic AMP, can increase $[Ca^{2+}]_i$ (and hence P_K) by releasing Ca^{2+} from stores within the cells (Karashima, 1981). This Ca^{2+}-releasing action must vary from preparation to preparation in order to explain the large variations in the membrane response to Type 2 receptor activation (Figure 2). Potentiation could then be accounted for in at least two ways. One possibility is that Type 1 and Type 2 receptor agonists can each release Ca^{2+} from the same cellular store, but via separate pathways with the following properties. (a) Simultaneous activation of the pathways at a low level causes a more than additive loss of Ca^{2+}. (b) The pathway linked to Type 1 receptors has a limited capacity. This is to account for our finding that the three Type 1 receptors studied (those for α-agonists, ATP and angiotensin II) produce the same maximum response (a net loss of 9–11% of cell K^+) which is considerably smaller than that attainable (18–20%) either when Type 1 and Type 2 receptors are activated together (see Table 1 and Figure 4) or when the divalent cation

ionophore A23187 is applied (Burgess *et al.*, 1981).

A second, and admittedly equally speculative, possibility is as follows (see also a similar proposal by Putney, 1978). Type 2 receptor agonists, acting through cyclic AMP, may reduce the affinity and/or capacity of one or more of the intracellular storage sites for Ca^{2+}. In a proportion of guinea-pig liver preparations (perhaps those in which these stores are either unusually full, or labile) this leads to a substantial rise in cytosolic Ca^{2+}, and, hence, in P_K. In every preparation, however, the Ca^{2+}-buffering capacity of the cell would be reduced. Simultaneous application of a Type 1 receptor agonist would then be expected to cause a greater increase in $[Ca^{2+}]_i$, and hence in P_K, as was observed.

Potentiation could also be explained as a consequence of a positive influence of cyclic AMP on the Ca^{2+} sensitivity of P_K and of the mechanism controlling Ca^{2+} efflux (to explain the findings with ^{45}Ca in the experiments of Figures 5 and 6).

Which, if any, of these schemes is correct can only be decided when more is known about the control of cytosolic Ca^{2+} in liver cells.

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