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Further Studies on the Interactions Between the Calcium Mobilization and Cyclic AMP Pathways in Guinea Pig Hepatocytes

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SUMMARY

Isoproterenol (50 nM) potentiated the effects of angiotensin (1–50 nM) on ^{86}Rb efflux and ^{45}Ca efflux from guinea pig hepatocytes. This effect occurred in the presence or absence of extracellular Ca^{2+} and required the simultaneous presence of both isoproterenol and angiotensin. Neither the divalent cation-ophore, A23187, nor 4β-phorbol dibutyrate could substitute for angiotensin. The effects of isoproterenol were greatest with submaximal concentrations of angiotensin, whereas maximal concentrations of angiotensin were affected little. Isoproterenol did not substantially increase the formation of [^{3}H]inositol trisphosphate or the ratio of isomers [^{3}H]inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,3,4-trisphosphate formed in response to angiotensin. Isoproterenol also enhanced the phase of Ca^{2+} mobilization involving Ca^{2+} entry which is consistent with the previously proposed functional linkage between receptor-regulated Ca^{2+} release and Ca^{2+} entry. These findings suggest that isoproterenol may act by increasing the sensitivity of the endoplasmic reticulum to the Ca^{2+}-releasing action of inositol 1,4,5-trisphosphate.

In the liver, two pathways have been described linking hormone receptor activation to glucose release, one involving cyclic AMP, and the other Ca^{2+} (1–3). Activation of the Ca^{2+} mobilization pathway causes increased efflux of cellular K^+ (in most species) due to the opening of Ca^{2+}-regulated potassium channels (4, 5). When these pathways are activated individually, their signaling mechanisms appear to be completely distinct; the β-adrenoceptor agonist, isoproterenol, increases cellular cyclic AMP without marked effects on Ca^{2+} metabolism, whereas the Ca^{2+}-mobilizing hormones (angiotensin II and α_1-adrenoceptor agonists, for example) cause intracellular Ca^{2+} release and accelerated Ca^{2+} entry without an elevation of cellular cyclic AMP (1, 2). When the pathways are activated simultaneously, however, there is marked potentiation of the Ca^{2+} mobilization pathway (6, 7; see also Ref. 17). For example, in guinea pig liver slices, β-adrenoceptor agonists or exogenous cyclic AMP do not cause substantial K^+ efflux or Ca^{2+} release, but in tissues pretreated with a Ca^{2+}-mobilizing hormone, the agents induce a substantial mobilization of cellular Ca^{2+} and concomitant K^+ flux (6; see also Ref. 17).

Here we report experiments on the nature of this interaction in guinea pig hepatocytes. The results suggest that the cyclic AMP pathway increases either an initial signal of the Ca^{2+}-mobilizing pathway or, more likely, the sensitivity of the Ca^{2+} mobilization pathway to a receptor-regulated signal.

Materials and Methods

Preparation of hepatocytes. Guinea pig hepatocytes were prepared by collagenase perfusion of guinea pig liver as previously described (8). The cells were incubated in Eagle's solution with 2% bovine serum albumin at pH 7.4, 37°, under 5% CO_2 in O_2. Cellular viability was routinely greater than 90% as determined by trypan blue exclusion.

Efflux of $^{86}Rb^+$ and $^{45}Ca^{2+}$. Cells were incubated for 1 hr in Eagle's solution containing 8 μCi/ml of $^{45}Ca^{2+}$ and 0.12 μCi/ml of $^{86}Rb^+$. They were then twice centrifuged and resuspended in minimal essential medium without albumin, drawn into perfusion chambers, and perfused as previously described (9). The perfusate was collected at 1-min intervals for up to 40 min, at which time the chambers were perfused with 5% (w/v) Triton X-100 to release the remaining cellular radioactivity. The contents of $^{86}Rb^+$ and $^{45}Ca^{2+}$ in the perfusate collections and Triton X-100 extract were determined with standard double-isotope techniques.

Determination of [^{3}H]inositol phosphates. Hepatocyte lipids

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ABBREVIATIONS: IP_3, inositol trisphosphate (isomer unknown); (1,4,5)IP_3, inositol 1,4,5-trisphosphate; (1,3,4)IP_3, inositol 1,3,4-trisphosphate; (1,3,4,5)IP_4, inositol 1,3,4,5-tetrakisphosphate; ER, endoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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were prelabeled with [^{3}H]inositol, and, following various experimental protocols, [^{3}H]inositol phosphates were determined as previously described (10). For some samples, IP_3 was resolved into the 1,4,5- and 1,3,4-isomers by high pressure liquid chromatography as previously described (11).

Measurement of ^{45}Ca release from permeable hepatocytes. Hepatocytes were permeabilized with saponin and incubated with 180 nM $^{45}Ca^{2+}$ (buffered with EGTA), 1.5 mM ATP, and ATP-regenerating system as described previously (8). Uptake of $^{45}Ca^{2+}$ and net release of $^{45}Ca^{2+}$ induced by various concentrations of (1,4,5)IP_3 were determined by filtration as described previously (12, 13).

Materials. Inositol (1,4,5)-trisphosphate was generously donated by R. Irvine, Babraham. The $^{86}Rb^+$ and $^{45}Ca^{2+}$ were obtained from New England Nuclear, Boston, MA, and [^{3}H]inositol was obtained from American Radiolabelled Products, St. Louis, MO. Isoproterenol, angiotensin II, 4β-phorbol dibutyrate, ionophore A23187, ATP, cAMP, catalytic subunit of cAMP-dependent protein kinase, and EGTA were obtained from Sigma Chemical Co., St. Louis, MO.

Statistics. Dispersions, where shown, indicate ±1 SE from at least four independent experiments, unless noted otherwise. The SE values were sometimes omitted when less than 10% of the means. Differences were determined by analysis of variance, and significance was attributed to F statistics greater than those tabulated for 0.05.

Results

For these studies, the Ca^{2+}-mobilizing agonist used was angiotensin (II), and the cyclase-activating agonist was isoproterenol (in the presence of 10 μM phentolamine to prevent α_1 activation). As shown previously (9), isoproterenol (50 nM) alone caused little if any increase in efflux of $^{86}Rb^+$ (a marker for K^+ efflux) or $^{45}Ca^{2+}$, although for unknown reasons small but significant responses were noted in the absence of extracellular Ca^{2+} (Fig. 1). Angiotensin II (5 nM) provoked a marked increase in $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux which returned toward baseline by 10 min. When isoproterenol was added, in the presence of angiotensin (once the efflux induced by angiotensin had returned to baseline), a substantial increase in efflux of both nuclides was noted. This increase was similar in the presence or absence of extracellular Ca^{2+} (Fig. 1).

The results summarized in Fig. 2 demonstrate that the continued presence of angiotensin is necessary for the response to isoproterenol. When the angiotensin perfusion was discontinued 2 min before isoproterenol, no response was observed.

The increase in cytosolic Ca^{2+} induced by angiotensin did not seem to be sufficient to produce this interaction since, in the presence or absence of extracellular Ca^{2+}, the Ca^{2+} ionophore A23187 could not substitute for angiotensin in this regard (results in the absence of Ca^{2+} shown in Fig. 3). Since angiotensin also causes activation of C kinase, a supramaximal concentration (20 μM) of 4β-phorbol dibutyrate, which causes Ca^{2+}-independent activation of C-kinase (14, 15), was tested in a similar protocol; however, 4β-phorbol dibutyrate, alone or in combination with A23187, also failed to reproduce the interaction with isoproterenol which angiotensin caused (not shown).

The experimental results shown in Fig. 4 indicate that at

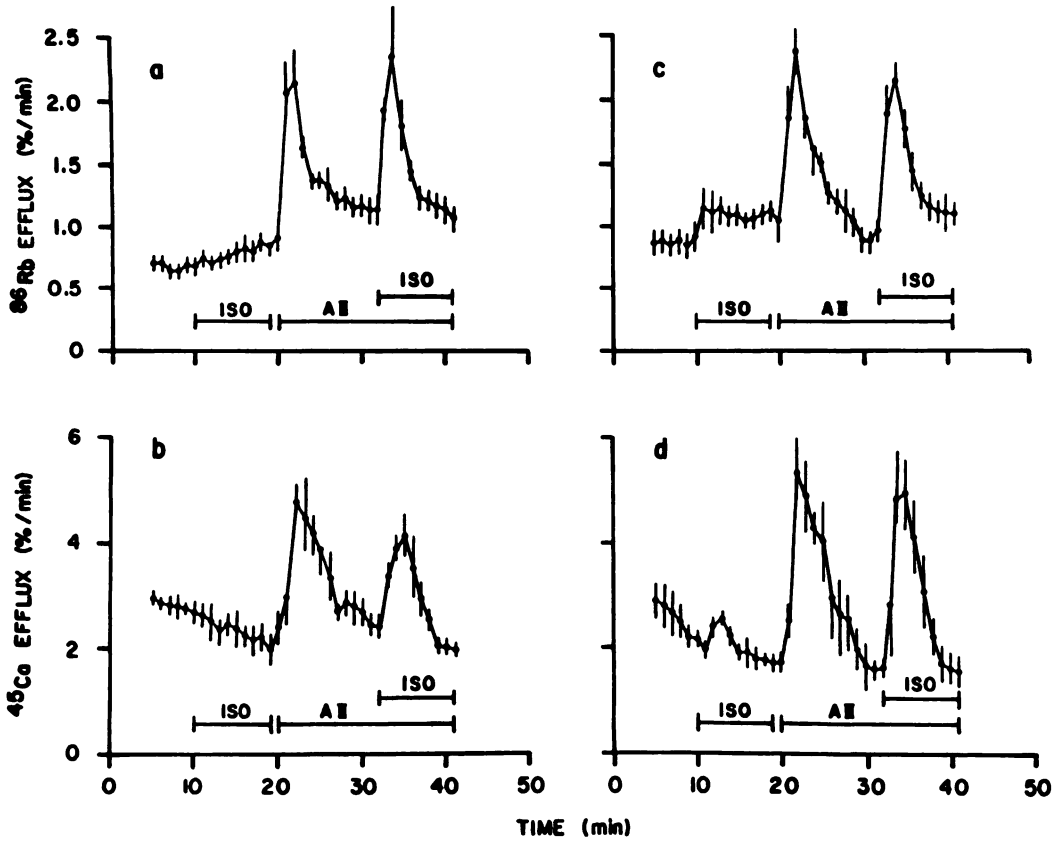

Fig. 1. Efflux of ^{86}Rb (a and c) or ^{45}Ca (b and d) from guinea pig hepatocytes. Hepatocytes were preincubated with ^{86}Rb and ^{45}Ca for approximately 1 hr and injected into perfusion chambers; then, the perfusate was collected with a fraction collector as previously described (9). During the indicated intervals, the perfusate contained either 50 nM isoproterenol (*ISO*), 5 nM angiotensin (*A II*), or both. c and d indicate experiments in which the Eagle's medium contained no added Ca^{2+} and 0.1 mM EGTA.

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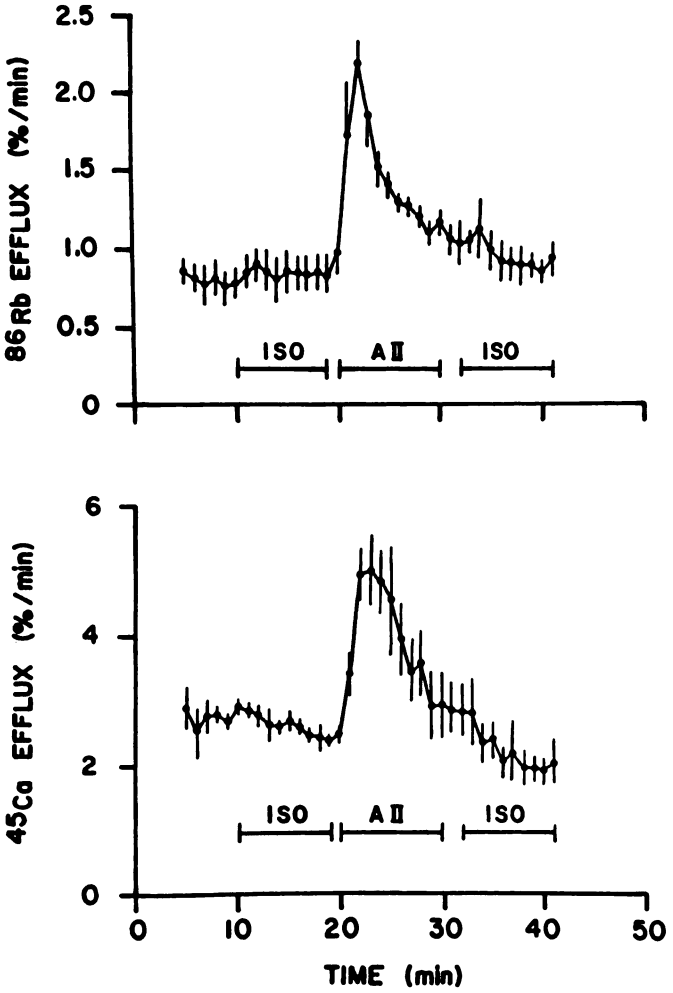

Fig. 2. A protocol similar to that shown in Fig. 1 (a and b) except that perfusion with angiotensin was discontinued 2 min before the second addition of isoproterenol.

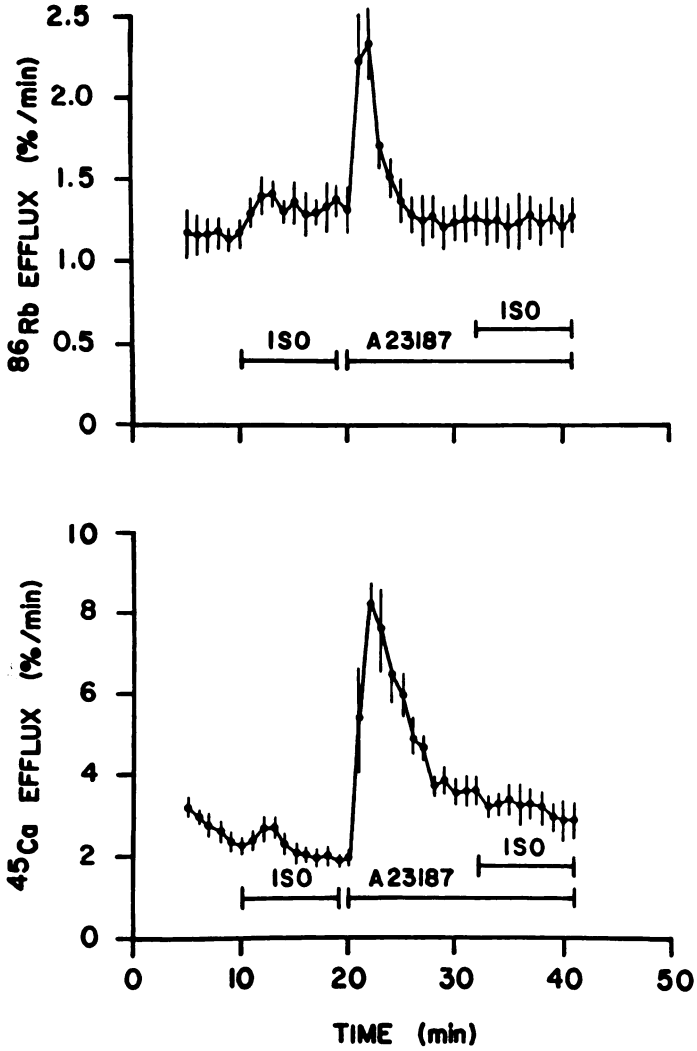

Fig. 3. Lack of interaction of isoproterenol and A23187. The protocol was as in Fig. 1 (c and d) except that, instead of angiotensin, 10 μM A23187 was used.

least some of the Ca^{2+} mobilized by isoproterenol in the presence of angiotensin comes from the cellular Ca^{2+} pool regulated by angiotensin alone. Thus, when submaximal Ca^{2+} release was induced by 5 nM angiotensin, 50 nM isoproterenol actually provoked a slightly greater release of $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ than if preceded by a supramaximal concentration of angiotensin (1 μM). In other words, at least for these two concentrations of angiotensin, the more Ca^{2+} released by angiotensin, the less was subsequently released by isoproterenol.

Similar conclusions were reached by examining the effects of 50 nM isoproterenol applied simultaneously with different concentrations of angiotensin. As shown in Fig. 5, the greatest potentiation was seen at the submaximal concentrations of angiotensin. The net effect of this is that the concentration-effect relationship for angiotensin is shifted leftward along the concentration axis by isoproterenol (Fig. 6).

A possible conclusion from these findings is that isoproterenol somehow amplifies the coupling mechanism between receptor occupation and Ca^{2+} mobilization. Since (1,4,5)IP_3 appears, at least in part, to mediate this coupling, the effect of angiotensin, in the presence and absence of isoproterenol, on formation of IP_3 was examined. On the basis of data from six separate experiments, [^{3}H]IP_3 formation due to 5 nM isoproterenol was increased slightly, but statistically significantly, by 50 nM isoproterenol which, on its own, had no effect on [^{3}H]IP_3 accumulation (not shown). These data, and data from two separate experiments utilizing higher and lower concentrations of angiotensin, are shown in Fig. 6 for comparison with the ^{86}Rb flux data.

Agonists such as angiotensin mobilize cellular Ca^{2+} by activation of Ca^{2+} entry as well as by release of sequestered Ca^{2+}, resulting in Ca^{2+}-dependent and independent phases of ^{86}Rb efflux (9). Fig. 7 summarizes experiments designed to test the effects of isoproterenol on these two phases. Angiotensin (5 nM) was added in the absence of extracellular Ca^{2+} and then, from 20–30 min, [Ca^{2+}] was increased to 3 mM. At this submaximal concentration of angiotensin, the second phase of efflux that follows addition of Ca^{2+} (9) was quite small, but significantly greater than the control efflux [which showed the typical decrease in efflux seen when Ca^{2+} is added in the absence of hormone (9)]. When 50 nM isoproterenol was added with the angiotensin, not only was the initial phase of the response, which is independent of extracellular Ca^{2+}, increased, but the secondary response, on addition of 3 mM Ca^{2+} was also significantly potentiated. This finding suggests that the mechanism of potentiation by isoproterenol of angiotensin-induced Ca^{2+}

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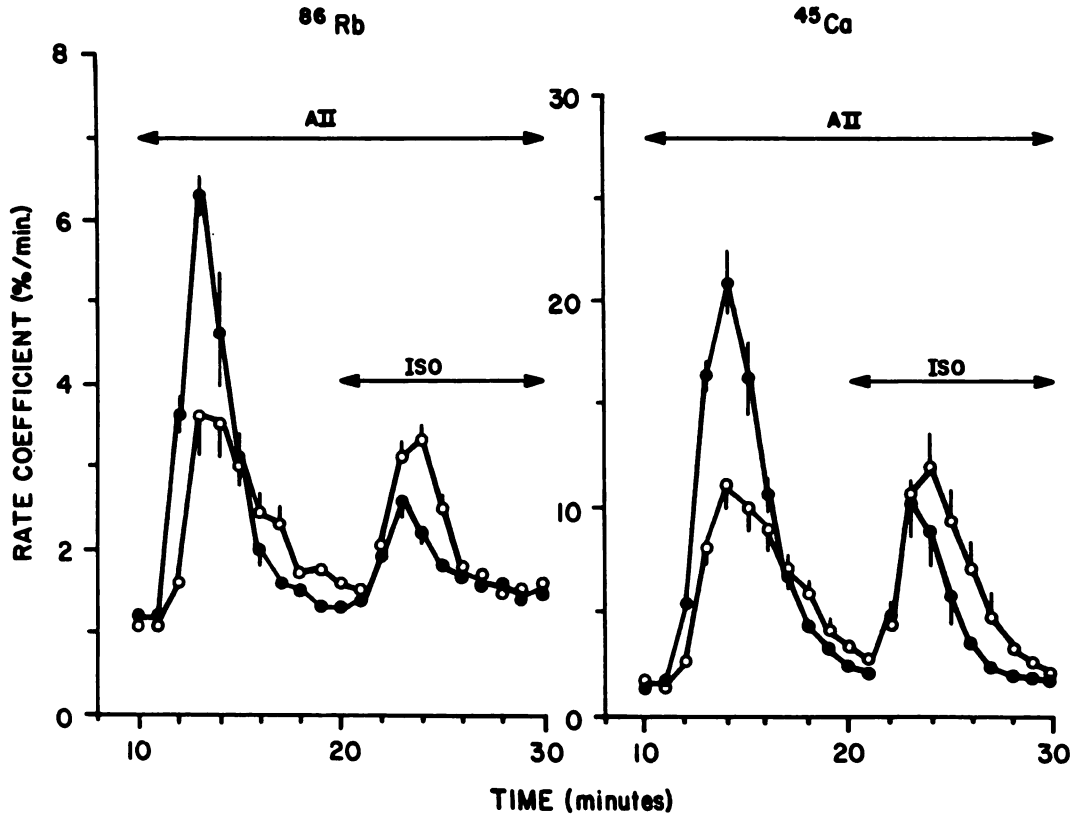

Fig. 4. Effects of submaximal (5 nM, ○) and supramaximal (1 μM, ●) concentrations of angiotensin (*AII*) on ^{86}Rb and ^{45}Ca efflux in response to 50 nM isoproterenol (*ISO*). The protocol was similar to that for Fig. 1 (a and b).

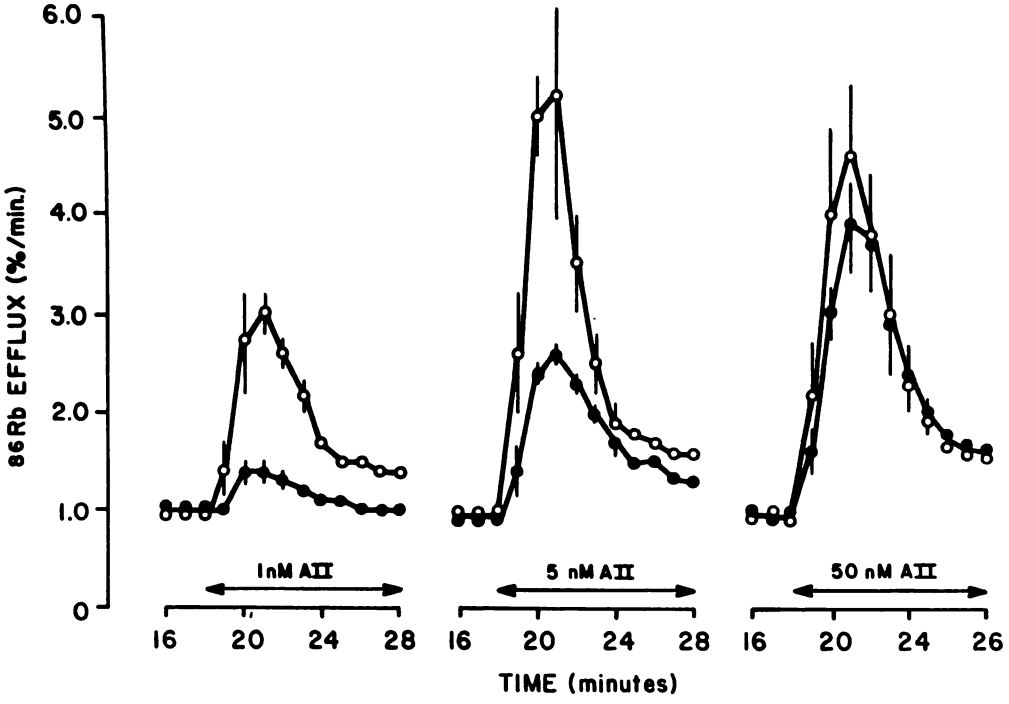

Fig. 5. Potentiation by 50 nM isoproterenol of ^{86}Rb efflux due to 1, 5, or 50 nM angiotensin (*AII*) when both agonists are applied simultaneously. The protocol was otherwise similar to that for Fig. 1a. ●, angiotensin alone; ○, angiotensin plus 50 nM isoproterenol.

mobilization involves the release of intracellular Ca^{2+} as well as Ca^{2+} entry across the plasma membrane. The finding that the Ca^{2+} entry phase of the response is also increased in the presence of isoproterenol is in agreement with the finding (16) that there is a potentiation of the Ca^{2+}-mobilizing hormone-induced increase in the rate of influx of ^{45}Ca into rat hepatocytes in the presence of the adenylate cyclase-activating hormone, glucagon.

Discussion

These studies confirm and extend previous findings from other laboratories demonstrating a potentiation by hormones

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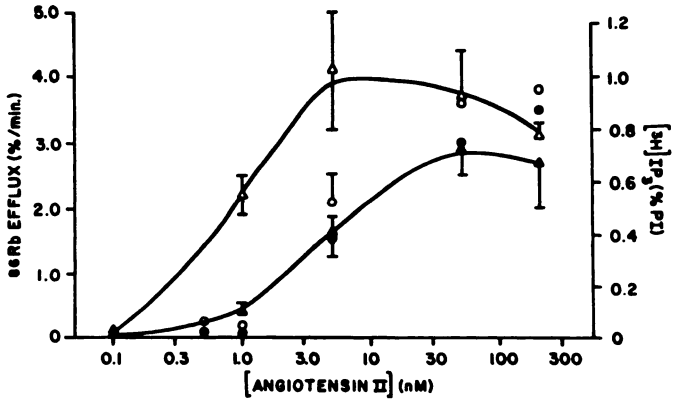

Fig. 6. Concentration-effect relationships for angiotensin on ^{86}Rb efflux(▲, △) and [^{3}H]IP_3 formation (●, ○). The protocol for ^{86}Rb data was as for Fig. 5; the mean maximal increase over basal efflux was plotted: ▲, angiotensin alone; △, angiotensin with 50 nM isoproterenol. For [^{3}H]IP_3, the data indicate the increase over basal [^{3}H]IP_3 5 min after addition of angiotensin alone (●) or with 50 nM isoproterenol (○). Single, independent observations are plotted except for 5 nM angiotensin, where the data indicate mean ± SE from six separate experiments.

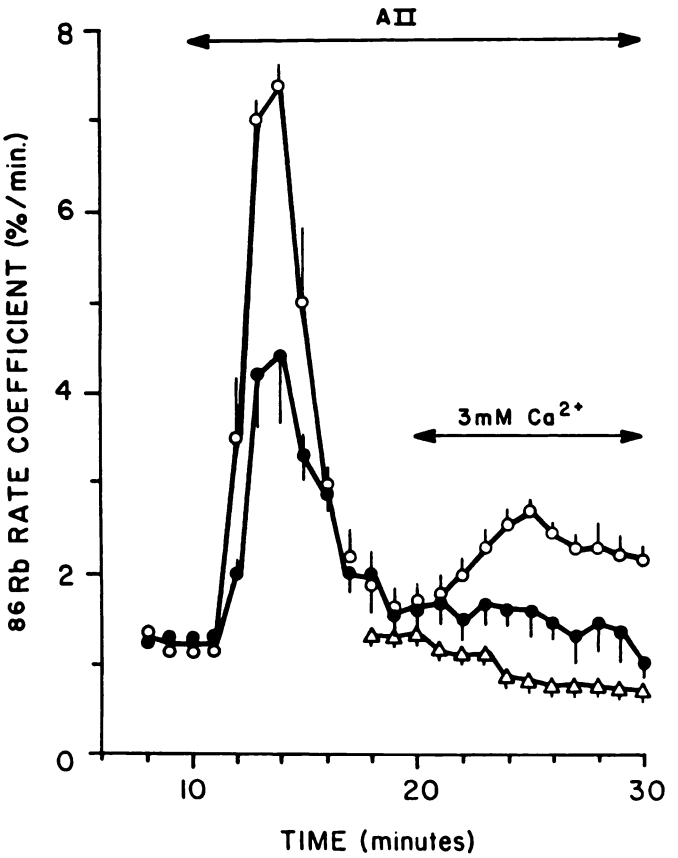

Fig. 7. Effects of 50 nM isoproterenol on Ca^{2+}-independent and Ca^{2+}-dependent phases of ^{86}Rb efflux. Five nM angiotensin (*AII*) was present at 10–30 min. From 0 to 20 min, the perfusate contained no added Ca^{2+} and 0.1 mM EGTA, and from 20 to 30 min it contained 3 mM Ca^{2+}. ●, angiotensin added alone; ○, angiotensin added with 50 nM isoproterenol; △, control (no drugs added, but 3 mM Ca^{2+} added, 20–30 min).

which activate adenylate cyclase of the Ca^{2+}-mobilizing action of agents such as angiotensin (6). The results indicate that the interaction requires the combined presence of angiotensin and isoproterenol (Fig. 2) (see also Ref. 17, where it was shown that an α_1-antagonist applied between an α_1-agonist and either isoproterenol or glucagon prevented the potentiation effect of the latter two hormones). Also, it is likely that isoproterenol potentiates the mechanism regulated by angiotensin (rather than vice versa) because: 1) isoproterenol alone causes little if any Ca^{2+} mobilization, 2) the synergism is greatest at low concentrations of angiotensin, and 3) angiotensin either has no effect (16) or actually inhibits the effects of isoproterenol, epinephrine and glucagon on cAMP accumulation and the resulting metabolic effects (18). However, the interaction does not appear to involve steps subsequent to the generation of signals by angiotensin; neither phorbol dibutyrate, an activator of C-kinase (15), nor the divalent cationophore A23187 mimicked the effects of angiotensin in its ability to interact with isoproterenol (Fig. 3). Furthermore, the effects of angiotensin did not depend on the presence of extracellular Ca^{2+}. The negative results with A23187, as well as the demonstrated effect of isoproterenol in potentiating $^{45}Ca^{2+}$ release as well as ^{86}Rb efflux, indicate that the sensitivity of K^+ channels to Ca^{2+} is not increased.

Thus, there appear to be three possible explanations for the potentiation of the angiotensin response by isoproterenol. These include: 1) potentiation by isoproterenol of the binding of angiotensin to its receptor, 2) an increased generation of Ca^{2+}-mobilizing second messenger(s), and 3) an increased sensitivity of the Ca^{2+} mobilization mechanism(s) to second messenger(s). These possibilities will be considered within the context of the hypothesis that Ca^{2+} mobilization (at least the release phase) due to angiotensin results from receptor activation of phosphatidylinositol 4,5-bisphosphate hydrolysis liberating (1,4,5)IP_3 which, in turn, binds to a specific receptor on some, but not all, of the endoplasmic reticulum, opening Ca^{2+} channels such that the sequestered Ca^{2+} is released to the cytosol (3, 12, 13, 19–21).

Isoproterenol caused only a slight increase in [^{3}H]IP_3 formation in response to angiotensin, in contrast to the marked increase in ^{86}Rb efflux seen, for example, with 1 and 5 nM angiotensin (Fig. 6), a finding inconsistent with alternatives 1 and 2. The data in Fig. 6 for [^{3}H]IP_3 were obtained using the ion exchange technique described by Berridge *et al.* (10), and, thus, the material contains both (1,4,5)IP_3 and the (presumably) less active (1,3,4)IP_3 (the proportion of (1,3,4,5)IP_4 in this fraction in liver is quite small).[4] However, in three experiments in which [^{3}H]IP_3 formed due to 5 nM angiotensin, with and without 50 nM isoproterenol, was separated into its isomers by high pressure liquid chromatography, the relative proportions of (1,4,5)IP_3 and (1,3,4)IP_3 were not altered by isoproterenol treatment (not shown). Furthermore, 50 nM isoproterenol did not change the ^{32}P-labeling of polyphosphoinositides in the presence or absence of 5 nM angiotensin. Collectively, these observations suggest that in guinea pig hepatocytes the effects of isoproterenol and (by inference) cyclic AMP are not primarily due to modification of the hydrolysis of polyphosphoinositides by angiotensin or the cellular metabolism of the putative Ca^{2+}-mobilizing messenger, (1,4,5)IP_3 [see also Ref. 22, although in rat hepatocytes (23) it was reported that a combination of glucagon and vasopressin caused a greater increase in diacylglycerol levels than vasopressin alone, which would imply either an increase in phospholipase C activation or an inhibition of diacylglycerol kinase]. However, the possibility

[4] Unpublished observation.

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that there are actions on other, as yet undiscovered, messengers linking angiotensin receptors to Ca^{2+} mobilization cannot be ruled out.

For the present, the most reasonable hypothesis seems to be that isoproterenol somehow amplifies the extent of Ca^{2+} mobilization due to the formation of (1,4,5)IP_3. This could be accomplished either by an increase in the size of the ER pool which is sensitive to (1,4,5)IP_3, or an increase in the sensitivity of this pool to (1,4,5)IP_3. The data in Figs. 5 and 6 indicate that the maximum response to angiotensin is not markedly increased by isoproterenol, suggesting that isoproterenol does not increase the total pool size. The results in Fig. 4 support this conclusion by suggesting that the extent of depletion of the pool by angiotensin can limit the degree of subsequent Ca^{2+} mobilization by isoproterenol plus angiotensin.

It seems reasonable to suggest, therefore, that isoproterenol may have increased the sensitivity of the ER pool to (1,4,5)IP_3. Since this effect would presumably be due to activation by cAMP of its protein kinase, we examined the effects of the catalytic subunit of cAMP-dependent protein kinase and of cAMP itself on (1,4,5)IP_3-induced release of Ca^{2+} from saponin-permeabilized hepatocytes. The results of these experiments (not shown) were essentially negative; i.e., treatment with cAMP (50 μM–2 mM) and/or catalytic subunit of cAMP-dependent protein kinase (up to 200 μg/ml) did not increase release of $^{45}Ca^{2+}$ by maximal (10 μM) or submaximal (0.05 μM) concentrations of (1,4,5)IP_3 (in the presence or absence of 20 μM phorbol dibutyrate). However, it is possible that cAMP does not regulate ER sensitivity to (1,4,5)IP_3 by direct phosphorylation by the cAMP-dependent kinase but, rather, through the initiation of a cascade, components of which may have been lost due to cellular permeabilization.

In contrast to the release of intracellular Ca^{2+} by (1,4,5)IP_3, the mechanism by which receptors regulate Ca^{2+} entry is not well understood. The results in Fig. 7 suggest the isoproterenol potentiates both of these phases of Ca^{2+} mobilization, which may indicate a common regulatory step or mechanism. Recently, it has been suggested (24) that Ca^{2+} entry may be directly coupled to Ca^{2+} release from ER. These findings are consistent with that idea, in that a potentiation by isoproterenol of (1,4,5)IP_3-induced Ca^{2+} release would be expected to result in a similar potentiation of Ca^{2+} entry, as seen in Fig. 7.

In conclusion, these findings indicate that isoproterenol, probably through cAMP, potentiates the mechanism of Ca^{2+} mobilization due to angiotensin. Since the formation of the putative mediator of Ca^{2+} mobilization, (1,4,5)IP_3, is little affected, it is suggested that cAMP directly or indirectly modifies the sensitivity of the ER to (1,4,5)IP_3. This effect of cAMP could not be reproduced in a permeable cell system, however, which may indicate the loss of soluble factors, perhaps components of a phosphorylation cascade, necessary for this effect. Experiments are now in progress to determine whether such factors exist and, if so, their nature and function.

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