Permeable analogues of cGMP promote hepatic calcium inflow induced by the synergistic action of glucagon and vasopressin but inhibit that induced by vasopressin alone

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Treatment of perfused rat liver with the nitric oxide-generating reagent molsidomine led to substantial increases in cGMP without itself affecting basal Ca^{2+} fluxes. Under these conditions the ability of glucagon plus vasopressin to induce Ca^{2+} inflow was greatly enhanced. The permeable analogue of cGMP (8-bromo-cGMP) enhanced glucagon plus vasopressin-induced Ca^{2+} inflow to a similar extent as that with molsidomine. This suggests that the effect of the latter is attributable to the generation of cGMP

which itself enhances the ability of the two hormones to induce synergistic Ca^{2+} inflow. While 8-bromo-cGMP (or molsidomine) did not influence Ca^{2+} fluxes induced by glucagon, these agents strongly inhibited Ca^{2+} inflow induced by vasopressin alone. These data show that while 8-bromo-cGMP has no effect on basal Ca^{2+} fluxes, it is able to modify the Ca^{2+} inflow induced by glucagon and vasopressin action in hepatic tissue.

INTRODUCTION

Changes in the cytosolic calcium ion (Ca^{2+}) concentration are crucial for many metabolic and physiological events in all types of cells [1–3]. These changes can be induced by numerous stimuli. Such ‘ Ca^{2+} -mobilizing’ stimuli act through the generation of intracellular second messengers following activation of stimulus-specific receptors located on the plasma membrane (reviewed in [4]).

In some tissues, different second messengers generated by different agonists are able to ‘cross-talk’ and thereby potentiate Ca^{2+} mobilization. In liver, for example, the synergistic actions of glucagon (generating cyclic AMP) and Ca^{2+} -mobilizing agonists (generating InsP_3) lead to greatly enhanced Ca^{2+} uptake (for reviews see [5–7]).

Another second messenger, cyclic GMP, generated by the action of nitric oxide, has been shown to mobilize Ca^{2+} in non-hepatic tissue (reviewed in [8]). There are few reports however of the effect of this agent on Ca^{2+} mobilization in hepatic tissue. Dufour et al. [9] observed that prior treatment of hepatocytes with 8-bromo-cGMP or with agents that generate cGMP, decreased the ability of vasopressin to increase the cytosolic Ca^{2+} concentration. Their data however did not allow a definitive conclusion to be drawn regarding the effects of cGMP on vasopressin-induced Ca^{2+} fluxes. Recently we suggested some possible sites and mechanisms of action of cGMP in agonist-induced hepatic Ca^{2+} fluxes [10]. In light of this and of our interest on the influence of signalling ‘cross-talk’ on hepatic Ca^{2+} mobilization ([6,7,10,11]), we initiated an investigation of these issues. Use was made of the ability of the long-acting anti-anginal drug molsidomine to generate nitric oxide in liver [12] and, in turn, of the ability of nitric oxide to generate cGMP also in liver [13].

EXPERIMENTAL

Male Wistar-strain albino rats, weighing from 250 to 350 g having free access to food, were anaesthetized with sodium pentobarbitone (50 mg/kg body wt). Livers were perfused with Krebs–Henseleit [14] bicarbonate buffer equilibrated with O_2/CO_2 (19:1) and containing 1.3 mM CaCl_2 ; the final oxygen

concentration was approx. 1 mM. Perfusions were conducted in a non-recirculating mode and the perfusate delivered at a constant volume of 35 ml/min by means of a Gilson Minipuls 3 peristaltic pump. Livers were pre-perfused for at least 20 min before the infusion of any hormone each of which was administered by a pump-driven infusion syringe. When administered, molsidomine was added 1 min after the start of the perfusion. The perfusate Ca^{2+} concentration was monitored continuously with a Radiometer F2112 Ca^{2+} -selective electrode in a flow-through chamber placed on the outflow side of the liver; this is described in detail elsewhere [15,16]. The electrode was coupled to a Radiometer K801 reference electrode via an agarose/KCl salt-bridge, and the combined signals were fed via an Orion Ionalyzer through a MacLab 2e recorder into a Macintosh computer. The calcium data were based on a sample speed of 20 samples per second. Data were then analysed, calculated and graphed using Igor (Wavemetrics, Oregon) software. Ca^{2+} concentration changes are expressed as mmol/min per g of liver. An average value of 0.1 mmol/min per g of liver corresponds to a change of perfusate Ca^{2+} of 40 μM . Each trace was a representative of at least three independent experiments. An upwards deflection in each trace reflects Ca^{2+} efflux and a downwards deflection Ca^{2+} inflow.

The concentration of cGMP in the whole liver was determined using the cyclic GMP[³H] kit supplied by Amersham. Briefly, at the indicated times, the perfusion was terminated and the liver rapidly removed, weighed, diced and homogenized in 50 mM Tris/4 mM EDTA buffer (pH 7.5). Glucagon was from Eli Lilly (Australia), Sydney, Australia. Vasopressin was from Sigma Chemical Co. and molsidomine was a product of Cayman Chemical Co., MI, U.S.A. Ca^{2+} -selective electrode membranes (F2112) and filling solutions (S43316) were obtained from Radiometer, Copenhagen, Denmark. All other chemicals used were of analytical reagent grade.

RESULTS AND DISCUSSION

Preliminary experiments

In preliminary experiments (data not shown) the following points were established: (a) molsidomine alone (25 μM) did not induce

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Figure 1 Effects of treatment of perfused rat livers with molsidomine or 8-bromo-cGMP on Ca^{2+} fluxes induced by glucagon and vasopressin

Livers were perfused as indicated in the Experimental section. In (a) molsidomine was continuously administered (bold trace) for the duration of the experiment commencing 1 min after the onset of the perfusion; 20 min later glucagon (10 nM) was administered and another 4 min later, vasopressin (10 nM) was administered. In control experiments (light, dashed trace), molsidomine was not administered. In (b) 8-bromo-cGMP (100 nM, bold trace) was added in place of molsidomine 7 min prior to glucagon. In control experiments (light, dashed trace), 8-bromo-cGMP was not administered. In (c) 8-bromo-cGMP (50 nM) was infused 7 min prior to vasopressin alone (10 nM; bold trace). In control experiments (light, dashed trace), 8-bromo-cGMP was not administered. The data in (a) and (b) are representative of three individual experiments carried out with separate rat livers and in (c) are the means of three individual experiments carried out with separate rat livers.

Ca^{2+} mobilization (efflux nor influx) in the absence of glucagon plus vasopressin; (b) when testing the effect of molsidomine concentration on inducing optimal increases in glucagon plus vasopressin-induced Ca^{2+} influx, we observed concentrations of 10 and 50 μM led to increases in such Ca^{2+} influx that were less than that produced by 25 μM molsidomine. The latter concentration was therefore used in the experiments described below.

Administration of molsidomine to the perfused rat liver leads to increases in cGMP

Direct measurements were made to confirm previous observations that molsidomine administration to the perfused rat liver leads to the generation of nitric oxide [12] and subsequent elevation of cGMP concentrations [13]. Several protocols in this group of experiments were undertaken taking account of the potential effects of these agents on glucagon plus vasopressin-induced Ca^{2+} mobilization (see below in Figure 1). The data (Table 1) show first that the values we obtained for basal hepatic

Table 1 Effects of molsidomine treatment on cGMP levels in the perfused rat liver

Perfused rat livers were treated with molsidomine (25 μM) for the times indicated below. Glucagon (10 nM) or vasopressin (10 nM) was administered at the times indicated (see also Figure 1). At the conclusion of the individual experiments, livers were diced and homogenized and the levels of cGMP assayed as in the Experimental section. Values are the mean $\pm$ S.E.M. of four individual experiments with each assay performed in duplicate. Less than 10% variation occurred between duplicates.

Treatment	cGMP formed (pmol/g of liver)
Control	3.13 $\pm$ 0.84
Glucagon (6 min)	3.52 $\pm$ 1.29
+ vasopressin (2 min)	
Molsidomine (20 min)	24.87 $\pm$ 6.13*
Molsidomine (20 min)	16.00 $\pm$ 4.24*
+ glucagon (6 min)†	
Molsidomine (20 min)	17.49 $\pm$ 4.49*
+ glucagon (6 min)	
+ vasopressin (2 min)†	

* $P < 0.05$ vs. control.

† Molsidomine infusion was continued during administration of the hormones.

cGMP concentrations were similar to those reported by others [13]. Second, we observed that perfused rat liver treated with this agent for 20 min, raised hepatic cGMP concentrations from 3 to almost 25 pmol/g of liver, i.e. some 8-fold (Table 1). Third, the administration of glucagon plus vasopressin did not significantly affect the extent to which cGMP was elevated following molsidomine action. Also noteworthy is that the degree to which cGMP concentrations were elevated by molsidomine, as seen here, was similar to that observed by Arnold et al. [13] who examined the effects of nitric oxide gas on cGMP formation in liver tissue preparations. We assumed for the purpose of these calculations that 1 g wet wt of liver was equivalent to 120 mg protein (see, e.g. refs in [17]).

We concluded from this information that molsidomine administration to the perfused rat liver for 20 min under the conditions in which we measure basal and hormone-induced Ca^{2+} mobilization, leads to the elevation of significant amounts of cGMP.

Administration of molsidomine to the perfused rat liver leads to increases in Ca^{2+} influx induced by glucagon plus vasopressin

We examined the effects of molsidomine pretreatment of the perfused rat liver on glucagon plus vasopressin-induced Ca^{2+} mobilization. We found previously that the combination of glucagon plus vasopressin, as employed in these experiments, produced optimal Ca^{2+} influx by the perfused rat liver [18]. Figure 1a shows that molsidomine pretreatment (which led to elevated [cGMP], Table 1), modified Ca^{2+} mobilization induced by the synergistic action of glucagon plus vasopressin in at least four ways: (i) the time taken for vasopressin to induce Ca^{2+} inflow following glucagon administration, (ii) the extent of initial Ca^{2+} efflux, (iii) the rate of Ca^{2+} influx, and (iv) the amount of Ca^{2+} flowing into the liver.

(i) The onset of vasopressin-induced Ca^{2+} inflow that follows glucagon administration is prolonged by molsidomine

In a previous study [16] we reported that glucagon administration decreases the time of onset of vasopressin- or

Table 2 Quantitative analysis summarizing the effects of molsidomine treatment on Ca²⁺ fluxes induced by glucagon plus vasopressin

The data were obtained from several experiments identical to those shown in Figure 1a, and were analysed for statistical significance. Values are means $\pm$ S.E.M. of three individual experiments.

	Control	After molsidomine treatment
Onset of vasopressin-induced Ca ²⁺ inflow* (s)	28 $\pm$ 3	34 $\pm$ 2
Vasopressin-induced Ca ²⁺ release* (nmol/g liver)	8 $\pm$ 1	4 $\pm$ 1
Maximum rate of Ca ²⁺ inflow* (μ mol/min per g liver)	0.4 $\pm$ 0.05	0.6 $\pm$ 0.10
Slope (increase in rate of Ca ²⁺ inflow) (μ mol/min ² per g liver)	0.53 $\pm$ 0.05	0.88 $\pm$ 0.10
Amount of Ca ²⁺ taken up*† (μ mol/g liver)	2.72 $\pm$ 0.02	3.76 $\pm$ 0.04

* Vasopressin was administered 4 min after glucagon.
† This value was obtained after 10 min of vasopressin infusion.

phenylephrine-induced Ca²⁺ influx. Figure 1a shows that molsidomine action, by contrast, prolongs the time between vasopressin administration and the onset of Ca²⁺ inflow. In the control this was 28 $\pm$ 3 s (n = 3) and, following molsidomine treatment, it was 34 $\pm$ 2 s (n = 3) (see Table 2).

(ii) Vasopressin-induced Ca²⁺ efflux that follows glucagon administration, is attenuated by molsidomine

The areas under the (upward) curves in the traces in Figure 1a represent the net amount of Ca²⁺ released in response to vasopressin administration. This was 8 $\pm$ 1 and 4 $\pm$ 1 nmol/g of liver (each n = 3) in the control and molsidomine-treated livers, respectively.

(iii) The rate of Ca²⁺ influx is enhanced by molsidomine

Analysis of the downward portion of the trace, which reflects Ca²⁺ influx, reveals several features of the effects of molsidomine. The maximal rates of Ca²⁺ influx approach 0.6 and 0.4 μ mol/min per g of liver with and without molsidomine treatment, respectively. Also there are clear differences in the slope [0.88 $\pm$ 0.1 and 0.53 $\pm$ 0.05 μ mol/min² per g of liver, respectively (n = 3)]. We interpret the initial increase in slope to reflect increases in either or both the rate of Ca²⁺-channel opening and activation induced by the action of molsidomine.

(iv) The amount of Ca²⁺ inflow is enhanced by molsidomine

The magnitude of Ca²⁺ inflow induced by the synergistic actions of glucagon plus vasopressin alone in the perfused rat liver is substantial. Altin and Bygrave [18] have previously calculated 'total' amounts of up to 3.8 μ mol/g of liver, which are comparable with values observed in Figure 1a, as the values presented here represent only the first 10 min of infusion with hormones. Molsidomine treatment clearly enhanced this even further; by up to 40%. This Ca²⁺ inflow was previously shown by us [18] and by others [19,20] to be direct into the mitochondria. This point was not examined in these experiments but we assume the same ultimate residual site of the ion although at this time we cannot exclude its removal in, for example, bile [21,22].

Table 2 contains a summary of the statistical analysis of the data relating to points (i)–(iv) above.

Permeable analogues of cGMP potentiate Ca²⁺ influx induced by the synergistic action of glucagon plus vasopressin

The information gained in Figure 1a and Table 1 led us to conclude that either nitric oxide or cGMP (or both) is the agent responsible for molsidomine's enhancement of Ca²⁺ influx. In this set of experiments we examined the possibility that cGMP was the active agent. Figure 1b shows that administration of the permeable analogue 8-bromo-cGMP (100 nM) for 7 min prior to the administration of glucagon and vasopressin potentiates the rate of Ca²⁺ inflow in a manner similar to that observed with molsidomine.

Actions of cGMP on Ca²⁺ fluxes induced by glucagon or vasopressin alone

Glucagon alone administered to the perfused rat liver induced only a small but detectable efflux of Ca²⁺ (see review in [6]). Control experiments in this work (data not presented) showed that neither the prior administration of molsidomine nor of 8-bromo-cGMP influenced this observation. By contrast however both of these agents altered the pattern of Ca²⁺ fluxes induced by vasopressin. Data in Figure 1c show the typical three phases of Ca²⁺ flux changes; an initial net release of the ion followed shortly by Ca²⁺ influx and finally a prolonged release of the ion. This pattern is well documented [18]. Prior administration of molsidomine (not shown) or of 8-bromo-cGMP (Figure 1c) resulted in a doubling of the initial efflux and approx. 80% inhibition of Ca²⁺ influx. Accordingly the final phase of Ca²⁺ efflux was significantly diminished. By varying the concentration of 8-bromo-cGMP, it was ascertained that near-maximal inhibition of Ca²⁺ influx (80%) was observed at 50 nM (not shown).

CONCLUDING REMARKS

This study shows that the permeable analogue 8-bromo-cGMP, which duplicated the effects of molsidomine, is able to modulate hepatic Ca²⁺ fluxes induced either by glucagon plus vasopressin acting together or vasopressin acting alone. We have extended the work of Dufour et al. [9] who showed that 1 mM 8-bromo-cGMP inhibited the increase in vasopressin-induced cytosolic Ca²⁺.

Consistent with the discussion above, we have shown that 8-bromo-cGMP, by contrast with the permeable analogue of AMP, was unable to promote Ca²⁺ inflow following the artificial emptying of the endoplasmic reticulum with BHQ [22]. It thus seems that the action of cGMP alone does not lead to enhanced Ca²⁺ inflow even when the endoplasmic reticulum pool has been emptied by artificial means.

The mechanism(s) by which cGMP influences agonist-induced hepatic Ca²⁺ fluxes as observed in this work remain unclear. Consideration of our data and those of Dufour et al. [9] would suggest that one action of cGMP is to attenuate the ability of vasopressin to release endoplasmic reticular Ca²⁺. This second messenger may also interfere with the generation of any purported chemical agent involved in inducing Ca²⁺ influx at the plasma membrane [23]. Another action, perhaps at the level of the G-proteins involved [7], would need to be considered to explain the potentiating action of cGMP when cAMP also is generated alongside the actions of vasopressin. Studies investigating these possibilities are in progress.

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