Histamine and a guanine nucleotide increase calcium permeability in pig aortic microsomal fractions

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ATP-dependent Ca^{2+} accumulation was measured in pig aortic microsomal fractions containing plasmalemma and endoplasmic reticulum. In vesicles sonicated with histamine, to allow access to internally located receptor sites, guanosine 5'-[β -imido]triphosphate (p[NH]ppG), added to activate externally located guanine-nucleotide-transducing proteins, caused a concentration-dependent decrease in steady-state Ca^{2+} accumulation that was reversed by guanosine 5'-[β -thio]diphosphate. In the presence of p[NH]ppG, sonication with histamine produced a concentration-dependent inhibition of Ca^{2+} accumulation that could be antagonized by the H_1 antagonist mepyramine, but not by the H_2 antagonist cimetidine. The inhibition of steady-state Ca^{2+} accumulation could have resulted from an inhibition of ATP-dependent Ca^{2+} uptake or a stimulation of Ca^{2+} release. We observed, however, that p[NH]ppG plus histamine stimulated, rather than inhibited, Ca^{2+} -ATPase activity. We concluded that p[NH]ppG and histamine acted together to increase Ca^{2+} permeability. In support of this, p[NH]ppG accelerated efflux of Ca^{2+} from passively loaded vesicles sonicated with, but not without, histamine. The effect of p[NH]ppG was unlikely to be due to $\text{Ins}(1,4,5)\text{P}_3$ (and hence release from endoplasmic-reticulum vesicles), since addition of $\text{Ins}(1,4,5)\text{P}_3$ to vesicles sonicated with histamine did not alter steady-state Ca^{2+} accumulation. Our results therefore suggest that histamine and p[NH]ppG increased the permeability of the plasmalemma vesicles and may thus model the process of receptor-mediated Ca^{2+} entry into intact cells.

INTRODUCTION

Histamine [1] and other vasoconstrictor agonists [2] invoke contractions in strips of rabbit aortic smooth muscle, the initial phasic component of which is independent of extracellular Ca^{2+} concentration. These contractions are thought to be mediated by Ca^{2+} released from internal stores, presumably endoplasmic reticulum [3]. Mobilization of intracellular Ca^{2+} in vascular smooth muscle involves activation of phosphoinositidase C to produce $\text{Ins}(1,4,5)\text{P}_3$ [4–7], and this activation is known to require a guanine-nucleotide-transducing protein (G-protein) [8,9]. The endoplasmic-reticulum Ca^{2+} -release mechanism has been characterized in permeabilized cells [4–6] and in broken-cell preparations [7]. The sustained phase of contractions induced by histamine and other agonists depends on the presence of extracellular Ca^{2+} , and therefore is thought to involve Ca^{2+} influx [2]. This occurs through voltage-sensitive Ca^{2+} channels [2] and through putative 'receptor-activated Ca^{2+} channels', also thought to be present in many other cells, including endothelial cells and blood platelets [10,11]. Activation of Ca^{2+} influx may also require a guanine nucleotide, since incubation of rat aortic smooth-muscle cells in culture with pertussis toxin decreases both Ca^{2+} mobilization and Ca^{2+} influx induced by histamine and noradrenaline [12]. G-proteins have also been implicated in Ca^{2+} influx in mast cells [13], in opening of voltage-dependent Ca^{2+} channels in neurons [14] and in gating other ion channels in heart [15] and neurons [16]. The nature of the receptor-operated Ca^{2+} channel is uncertain. Studies conducted in cell types other than vascular smooth muscle suggest the existence of a channel opened from the cytoplasmic side by $\text{Ins}(1,4,5)\text{P}_3$ alone [17–19] or $\text{Ins}(1,4,5)\text{P}_3$ plus $\text{Ins}(1,3,4,5)\text{P}_4$ [20]. Electrophysiological studies with rabbit ear artery smooth-muscle cells provide evidence, in contrast, for a Ca^{2+} channel directly opened by agonist [21].

Further biochemical characterization of the mechanisms

involved in Ca^{2+} influx should be aided by the development of experimental models using broken-cell preparations. To achieve this, we argued that it would be necessary to mimic the normal topological arrangement of agonist (extracellular) and guanine nucleotide (intracellular). Hence microsomal fractions (microsomes) from pig aorta, which contained plasmalemmal vesicles, were sonicated with histamine and then incubated with p[NH]ppG, a non-hydrolysable analogue of GTP. To detect an increase in membrane permeability to Ca^{2+} , we measured either the steady-state level of ATP-dependent Ca^{2+} accumulation or Ca^{2+} efflux from passively loaded vesicles.

MATERIALS AND METHODS

Materials

ATP (disodium salt) was obtained from Boehringer, Percoll from Pharmacia, and $^{45}\text{CaCl}_2$ from New England Nuclear. All other biochemicals were obtained from Sigma.

Preparation of microsomes

Pig aortas were obtained fresh from the abattoir and transported in cold 154 mM-NaCl solution. The endothelium was removed from the opened aorta with a scalpel blade, and the tunica media (consisting solely of vascular smooth muscle) was then stripped from the adventitia.

Preparation A. The buffer used throughout was 20 mM-Tris/maleate (pH 6.8), containing 0.25 M-sorbitol (buffer A). The smooth muscle was minced and then suspended 1:1 (w/v) with buffer and homogenized for 15 s with a Polytron PT 20 at speed setting 3. The homogenate was centrifuged at 2000 *g* for 10 min, and then the fatty surface layer and pellet of debris were discarded. A suspension of Percoll (50%, v/v) in buffer was added to the remaining fraction to a final concentration of 5%

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -activated Mg^{2+} -ATPase; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate.

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Percoll, and the mixture was centrifuged at 8000 *g* for 10 min in an angle-head rotor. The upper half of the resulting suspension was then removed and re-centrifuged at 100000 *g* for 1 h. This yielded a visible pellet on top of a solid layer of Percoll. This pellet, termed the microsomal fraction, was resuspended in Buffer A to a protein concentration of about 3 mg/ml measured by the method of Bradford [22].

Preparation B. The buffer used throughout was 20 mM-Tris/maleate (pH 6.8), containing 0.1 M-KCl (buffer B) [4]. Other procedures were the same as in preparation A, except that the initial homogenizing buffer was supplemented with 1 mM-ATP and 5 mM-MgCl₂, and the final centrifugation was performed at 140000 *g* for 1 h.

Skeletal-muscle microsomes were prepared in the same way as pig aortic microsomes (Preparation A). Red-blood-cell ghosts were prepared by the methods of Steck & Kant [23].

SDS/polyacrylamide-gel electrophoresis and autoradiography

Acyl phosphate derivatives (labelled with ³²P) of the plasmalemma and endoplasmic-reticulum ATPases were prepared from [³²P]ATP and detected by electrophoresis and autoradiography by the method of Wuytack *et al.* [24]. The resolving gel contained 6% acrylamide.

Measurement of Ca²⁺ accumulation

A portion (40 μ l) of buffer A or B as appropriate either without histamine or containing 10 μ M–10 mM-histamine was added to a portion of microsomal fraction (400 μ l, protein concn. 3–5 mg/ml), and the mixture was sonicated for 10 s (MSE Sonicator model PG 100, with a 2 mm probe and set to amplitude 12 μ m). For microsomes sonicated without histamine, histamine was then added at 1 μ M–1 mM final concentration.

ATP-dependent Ca²⁺ accumulation was measured by using ⁴⁵Ca²⁺ and membrane filtration [24]. The reaction was started by the addition of microsomes (final concn. 0.3–0.5 mg of protein/ml) to a total volume of 1 ml of buffer A or B containing 1 mM-ATP, 1 mM-MgCl₂, 0.001–1 mM-p[NH]ppG, 0.5 μ M–0.5 mM-histamine, 1 mM-EGTA and 0.85 mM-CaCl₂ + ⁴⁵Ca²⁺ (3.57 Ci/mol) (calculated to give a free Ca²⁺ concn. of 5 μ M) [24]. After incubation for periods between 0 and 4 min at 37 °C, samples of the mixture (0.4 ml) were withdrawn and loaded on to cellulose nitrate filters (Gelman; 25 mm diam., 0.45 μ m pore size, mixed ester), which were then washed with 5 ml of homogenizing buffer containing 1 mM-EGTA. Filters were then dissolved with 1 ml of 2-methoxyethanol, 10 ml of scintillation fluid was added, and radioactivity was determined in a scintillation counter. The total radioactivity bound minus that bound at 0 min was used to estimate the ⁴⁵Ca²⁺ accumulated.

Measurement of ATPase activity

ATPase activity was determined spectrophotometrically by the pyruvate kinase/lactate dehydrogenase coupled assay [25], with microsomal fractions that had been sonicated with 0.5 mM-histamine. The final concentrations of reagents in a 1 ml cuvette were: 5 mM-MgCl₂, 5 mM-ATP, 1 mM-EGTA with or without addition of 0.85 mM-CaCl₂ (to give an estimated free concentration of 5 μ M-Ca²⁺), 0.26 mM-NADH, 0.1 mM-ouabain, 1.5 mM-phosphoenolpyruvate, 40 units of pyruvate kinase and of lactate dehydrogenase/ml, 0.03–0.05 mg of microsomal protein, 50 μ M-histamine and either 0 or 1 mM-p[NH]ppG, all in buffer B. The reaction was started by addition of ATP, and the rate was determined at 37 °C. The rate was measured after 1 min of incubation to allow time for contaminating ADP to be used by the coupling enzymes. ATPase activity measured with EGTA alone is termed 'basic ATPase', and that measured in the presence

of added Ca²⁺ is termed 'total ATPase'. The difference between total and basic ATPase is taken to be Ca²⁺-ATPase.

Measurement of ⁴⁵Ca²⁺ efflux

Vesicles (420 μ l, containing 4–8 mg of protein/ml) were passively loaded for 2 h at 22 °C in buffer B containing 5 mM-CaCl₂, 0.1 mCi of ⁴⁵Ca²⁺/ml [26] with or without 0.5 mM-histamine. The vesicles were then sonicated as described above. Samples of the mixture (50 μ l) were diluted into 4.95 ml of buffer B containing 1 mM-EGTA, 5 mM-Mg²⁺ and either 200 μ g of saponin/ml, 1 mM-p[NH]ppG or no additions. Samples of the diluted suspension (1 ml) were removed at 10, 20 and 60 s and passed through filters, followed by 5 ml of buffer B containing 1 mM-EGTA and 5 mM-Mg²⁺. The filters were dissolved and counted for radioactivity as described above.

Statistical methods

Values are expressed as means $\pm$ S.E.M. throughout and were compared by Student's *t* test with paired data unless stated otherwise.

RESULTS AND DISCUSSION

Presence of plasmalemma and endoplasmic-reticulum Ca²⁺-ATPase in the microsomal fraction

To study the mechanism of Ca²⁺ influx into intact cells, a broken-cell preparation containing plasmalemmal vesicles was needed. To confirm the presence of plasmalemmal vesicles in the microsomal fraction from pig aorta used here, we labelled the acyl phosphate intermediate of the Ca²⁺-ATPase. Incubation of pig aortic microsomal fractions with [³²P]ATP under appropriate conditions yielded a diffuse band between molecular masses of

Fig. 1. Ca²⁺-ATPases present in pig aortic microsomes

Autoradiographs of ³²P-labelled acyl phosphate derivatives of Ca²⁺-ATPases from pig aortic microsomes (Preparation A) (AM), pig skeletal muscle microsomes (SKM) and human red-blood-cell ghosts (RBC) are shown.

Fig. 2. Time course of $^{45}\text{Ca}^{2+}$ accumulation into microsomes

Microsomes (Preparation A) were sonicated with 0.5 mM-histamine and then incubated with $^{45}\text{Ca}^{2+}$ and either without (●) or with (○) 1 mM-p[NH]ppG. Values are means $\pm$ S.E.M. for 10 microsomal preparations: * $P < 0.025$ versus absence of p[NH]ppG.

130 and 100 kDa on SDS/polyacrylamide-gel electrophoresis (Fig. 1). This size range encompassed those of the endoplasmic-reticulum Ca^{2+} -ATPase prepared from skeletal muscle (100 kDa) and the plasmalemmal Ca^{2+} -ATPase prepared from human red-blood-cell ghosts (130 kDa) (Fig. 1). These data suggested the presence of both plasmalemma and endoplasmic-reticulum Ca^{2+} -ATPases in the microsomal fraction. This conclusion was supported by measurements of the activity of marker enzymes 5'-nucleotidase and NADPH-cytochrome *c* reductase, both of which were present (results not shown), consistent with previous work using similar preparations [4].

Inhibition of Ca^{2+} accumulation requires both histamine and p[NH]ppG

Only sealed inside-out plasmalemma vesicles and right-side-out endoplasmic-reticulum vesicles have the correctly orientated Ca^{2+} -ATPases to accumulate Ca^{2+} actively. ATP-dependent accumulation of Ca^{2+} was observed into microsomal fractions from pig aorta and rapidly became non-linear (Fig. 2), apparently reaching steady state within 4 min. To gain access by histamine to receptor sites inside plasmalemma vesicles, breaking and re-sealing of vesicles by sonication was necessary. Sonication of vesicles by itself decreased steady-state accumulation by $35 \pm 4\%$ ($n = 12$). p[NH]ppG significantly decreased steady-state Ca^{2+} accumulation in vesicles previously sonicated with histamine (Fig. 2). In a second experiment, steady-state Ca^{2+} accumulation in vesicles sonicated with 0.5 mM-histamine (43 ± 7 nmol/mg of protein) was decreased by p[NH]ppG (1 mM) to 31 ± 6 nmol/mg of protein ($n = 8$, $P < 0.02$). In contrast, Ca^{2+} accumulation in

Fig. 3. Concentration-dependence of inhibition of $^{45}\text{Ca}^{2+}$ accumulation by p[NH]ppG and reversal by GDP[S]

Steady-state Ca^{2+} accumulation was measured into vesicles sonicated with 0.5 mM-histamine, as described in the Materials and methods section. Vesicles were incubated with the concentrations of p[NH]ppG shown in the absence (●) or presence (○) of 0.05 mM-GDP[S]. Values are expressed as percentages of those obtained in the absence of p[NH]ppG and GDP[S]. Values are means $\pm$ S.E.M. for the number of preparations shown in parentheses: * $P < 0.002$ versus absence of p[NH]ppG; † $P < 0.03$ versus 0.01 mM-p[NH]ppG.

sonicated vesicles to which histamine was added afterwards (36 ± 6 nmol/mg of protein) was not affected by p[NH]ppG (39 ± 5 nmol/mg of protein; $n = 8$, not significant). These experiments suggested that p[NH]ppG, which would have access to G-proteins on the external face of inside-out plasmalemma vesicles, only decreased Ca^{2+} accumulation when histamine was given access, by sonication, to its receptor sites on the internal face of the vesicles.

To establish that the effect of p[NH]ppG was mediated via a G-protein, the concentration-dependence and effect of a GDP analogue were investigated. p[NH]ppG decreased Ca^{2+} accumulation when present at above 0.01 mM (Fig. 3), in agreement with work on other G-protein-mediated events [27]. The effect of 0.01 mM-p[NH]ppG was abolished by 0.05 mM-GDP[S] (Fig. 3), which had no effect on Ca^{2+} accumulation in the absence of p[NH]ppG (results not shown).

To establish that the effect of histamine was the result of binding to a receptor, its concentration-dependence and the effects of receptor antagonists were studied. Ca^{2+} accumulation measured in the presence of 1 mM-p[NH]ppG was decreased by histamine at concentrations above 50 μM (Fig. 4). The effect of histamine was prevented by the addition, during sonication, of the H_1 -histamine-receptor antagonist mepyramine (Fig. 4a), but not the H_2 -receptor antagonist cimetidine (Fig. 4b). Mepyramine alone decreased Ca^{2+} accumulation by $14 \pm 5\%$ ($n = 12$, $P < 0.02$).

Fig. 4. Concentration-dependence of inhibition of steady-state $^{45}\text{Ca}^{2+}$ accumulation by histamine

Steady-state accumulation of Ca^{2+} was measured in the presence of 1 mM-p[NH]ppG as described in the Materials and methods section, and the values are expressed as percentages of those obtained in the absence of histamine. (a) Microsomes (Preparation A) were sonicated with the final concentrations of histamine shown, without (●) or together with (○) 5 μM -mepyramine: * $P < 0.05$ versus absence of histamine and mepyramine. (b) Microsomes (Preparation B) were sonicated with the final concentrations of histamine shown without (●) or together with (○) 5 μM -cimetidine: * $P < 0.05$ versus absence of histamine and cimetidine; † $P < 0.05$ versus absence of histamine and presence of cimetidine. Values are mean $\pm$ S.E.M. for 12 microsomal fractions.

Effect of histamine and p[NH]ppG on ATPase activity

The possibility was investigated that the decrease in steady-state Ca^{2+} accumulation that we observed resulted from inhibition of Ca^{2+} -ATPase activity. As shown in Table 1, p[NH]ppG decreased the ATPase activity measured in the absence of Ca^{2+} in microsomal preparations sonicated with histamine. However, it did not affect the total activity in the presence of Ca^{2+} , and hence the difference between these two activities, i.e. the Ca^{2+} -ATPase, was significantly increased. Inhibition of ATP-dependent Ca^{2+} uptake was therefore unlikely to explain the decrease in steady-state Ca^{2+} accumulation that we observed.

Ca^{2+} efflux

The decrease in steady-state Ca^{2+} accumulation could alternatively have resulted from acceleration of Ca^{2+} efflux from

Table 1. Effect of p[NH]ppG on microsomal ATPase activity

Microsomal fractions were sonicated with 0.5 mM-histamine and incubated without (control) or with 1 mM-p[NH]ppG (final concn.). The basic ATPase activity was measured as described in the Materials and methods section with EGTA alone, and total activity with 5 μM free Ca^{2+} . Ca^{2+} -ATPase was calculated as the difference between total and basic ATPase activity. Values are means $\pm$ S.E.M. for 15 preparations: * $P < 0.001$ and † $P < 0.02$ versus control

Conditions	ATPase activity (nmol/min per mg of protein)	
	Control	+ 1 mM-p[NH]ppG
Basic	56.5 $\pm$ 8.0	50.5 $\pm$ 6.9*
Total	62.6 $\pm$ 9.5	60.7 $\pm$ 8.5
Ca^{2+} -activated	6.1 $\pm$ 2.5	10.1 $\pm$ 2.6†

Fig. 5. Efflux of $^{45}\text{Ca}^{2+}$ from passively loaded microsomal vesicles

Microsomal vesicles (Preparation B) were loaded with 5 mM- $^{45}\text{Ca}^{2+}$ and were then sonicated in buffer containing 0.5 mM-histamine (a) or without histamine (b). Vesicles were diluted 100-fold into buffer (□, ○), buffer containing 200 μg of saponin/ml (△) or buffer containing 1 mM-p[NH]ppG (■, ●), and then subjected to filtration after the delays shown. Values are means $\pm$ S.E.M. for 20 preparations sonicated with histamine (12 preparations at 20 s) and 12 preparations sonicated without histamine: * $P < 0.05$ versus sonicated with histamine and diluted in the absence of p[NH]ppG (□) at the same time point.

either plasmalemmal or endoplasmic-reticulum vesicles. To test directly whether p[NH]ppG increased Ca^{2+} efflux, vesicles were passively loaded with Ca^{2+} and then diluted. Rapid efflux of Ca^{2+} was observed (Fig. 5) and was accelerated by including saponin in the dilution medium, which suggested that some Ca^{2+} was initially contained within vesicles. In vesicles sonicated with histamine, the Ca^{2+} remaining after 10 s was decreased by 1 mM-p[NH]ppG (Fig. 5a). In a separate series of similar experiments but with 0.1 mM-p[NH]ppG in the dilution medium, Ca^{2+} remaining after 10 s was decreased from 227 ± 20 to 207 ± 9 nmol of Ca^{2+} /mg of protein ($n = 24$, $P < 0.03$). p[NH]ppG had no effect on efflux from vesicles sonicated without histamine (Fig. 5b). Ca^{2+} efflux might have resulted from formation of $\text{Ins}(1,4,5)\text{P}_3$ in the presence of histamine and p[NH]ppG, followed by stimulation of efflux from the endoplasmic-reticulum component of the microsomal fraction. This possibility is unlikely, however, since any $\text{Ins}(1,4,5)\text{P}_3$ or other soluble mediator would have been diluted 100-fold. To examine this possibility further, however, the effect of adding a high concentration (10 μM) of $\text{Ins}(1,4,5)\text{P}_3$ to microsomes sonicated with 0.5 mM-histamine was compared with that of adding 1 mM-p[NH]ppG. Steady-state Ca^{2+} accumulation was not decreased by $\text{Ins}(1,4,5)\text{P}_3$ ($5 \pm 4\%$, $n = 10$), whereas it was decreased by p[NH]ppG in this experiment by $20 \pm 4\%$ ($n = 10$, $P < 0.001$). It is therefore unlikely that $\text{Ins}(1,4,5)\text{P}_3$ and hence Ca^{2+} efflux from endoplasmic reticulum, contributed to decreasing steady-state Ca^{2+} accumulation. Activation of Ca^{2+} efflux from plasmalemmal vesicles appears therefore to be the likely explanation of our observations.

Conclusions

In the six separate series of experiments reported here, the inhibition of Ca^{2+} accumulation by 1 mM-p[NH]ppG plus 0.5 mM-histamine was consistently significant and between 20 and 30%. Reversal of the effect of p[NH]ppG by GDP[S] suggests the involvement of a G-protein. Reversal of the effect of histamine by mepyramine, but not cimetidine, suggests that the histamine

receptor involved is H_1 subtype, which is that known to be associated with contraction in vascular smooth muscle [3,28,29]. The requirement for histamine and p[NH]ppG suggests that a membrane transducing event was involved. In studies of other membrane-signalling events, non-hydrolysable GTP analogues have been shown to act even in the absence of receptor agonists, albeit more slowly [30]. The apparent need for both histamine and p[NH]ppG in our system might therefore reflect the kinetics of activation rather than an absolute requirement for histamine.

Activation of platelets [31] and smooth muscle [32] by Ca^{2+} -mobilizing hormones before preparation of microsomes has been reported to decrease Ca^{2+} -ATPase activity, perhaps by depletion of inositol phospholipids by phosphoinositidase C, since addition of phosphatidylinositol causes a converse activation of Ca^{2+} -ATPase in erythrocytes [33,34], platelets [34] and brain [33]. By contrast, p[NH]ppG plus histamine increased rather than decreased Ca^{2+} -ATPase activity in our experiments. Such an increase is consistent with an acceleration of Ca^{2+} efflux, since this will lower intravesicular Ca^{2+} concentration and relieve constraints on the pump. A similar decrease in steady-state Ca^{2+} accumulation and increase in Ca^{2+} -ATPase activity has been demonstrated in cardiac endoplasmic-reticulum vesicles on the addition of caffeine, which is known to act by making the vesicles more permeable to Ca^{2+} [35]. A significant approx. 10% increase in Ca^{2+} efflux was demonstrated directly in two separate series of experiments. If the contribution of non-vesicular Ca^{2+} binding (estimated from the experiment with saponin) is deducted, then the effect of p[NH]ppG plus histamine was approx. 20%, similar to the effect on Ca^{2+} accumulation. The small magnitude of these effects is not surprising, in view of the heterogeneity of the vesicle preparation used.

The most plausible explanation of our observations is that histamine and p[NH]ppG increased the Ca^{2+} permeability of the plasmalemma vesicles. If so, it seems likely that this represents the opening of the receptor-operated Ca^{2+} -entry mechanism responsible for Ca^{2+} influx into vascular smooth muscle [10]. Further characterization of the molecular events involved is now necessary to confirm this hypothesis and to elucidate the mechanisms involved.

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