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Inhibition of a receptor-operated calcium channel in pig aortic microsomes by cyclic GMP-dependent protein kinase

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We have further characterized a putative receptor-operated Ca^{2+} channel that is activated by histamine and guanosine 5′-[$\beta\gamma$-imido]triphosphate. Insensitivity to verapamil, diltiazem or nicardipine, but inhibition by Ni^{2+} and SK&F 96365, further identify the channel with receptor-mediated Ca^{2+} entry in intact cells. Inhibition of the channel by cyclic-GMP-dependent protein kinase may contribute to vascular relaxation in response to nitrovasodilators.

INTRODUCTION

Endothelium-derived relaxing factor, which is now known to be NO [1] or a ready source of it [2], mediates vasodilator responses to flow and to a range of circulating and locally released agents [3]. NO, nitrovasodilator drugs and the atrial natriuretic peptides all cause vasodilatation by elevating intracellular cGMP concentration [3] and by lowering intracellular Ca^{2+} concentration [4]. This occurs as a result of inhibition of both Ca^{2+} mobilization from intracellular stores [5–7] and Ca^{2+} influx [8–11], particularly when this is mediated by agonists rather than depolarization. Evidence has accumulated that cGMP elevation inhibits phosphoinositide formation in vascular smooth muscle [5–7], possibly by preventing the coupling between receptors and transducing G-proteins [7]. This mechanism readily accounts for the inhibition of Ca^{2+} mobilization from intracellular stores, but may not explain inhibition of Ca^{2+} influx. In general, at least three different mechanisms of receptor-mediated Ca^{2+} entry into cells have been described. These differ depending on whether the channel is opened directly by agonist, as shown for the action of ATP in rabbit ear artery [12], or whether a second messenger opens the channel from the cytoplasmic face [13]. Influx of Ca^{2+} may also be stimulated indirectly by opening of voltage-operated channels [14,15]. We [16] and Simon *et al.* [17] have obtained evidence in blood platelets that inhibition of Ca^{2+} influx occurs with lower concentrations of nitrovasodilators than those required to inhibit Ca^{2+} release from intracellular stores. This is most easily accounted for by a separate action of cGMP elevation on receptor-operated Ca^{2+} channels. To test this hypothesis, we [18] first established a biochemical assay for a receptor-operated Ca^{2+} channel. We sonicated pig aortic microsomes with histamine so as to occupy cell-surface receptors on inside-out plasma-membrane vesicles. Addition of p[NH]ppG so as to activate G-proteins increased Ca^{2+} permeability, consistent with the opening of a channel. The action of histamine required sonication and was reversed by the H_1 antagonist mepyramine, but not by the H_2 antagonist cimetidine, which was consistent with action at the receptor responsible for vasoconstriction. The effect of p[NH]ppG was inhibited by guanosine 5′-[β-thio]-diphosphate, confirming the involvement of a G-protein. The action of p[NH]ppG was not mimicked by Ins(1,4,5)P_3 [18], which implied that the putative channel was opened directly by histamine and p[NH]ppG. We sought here further evidence to identify the putative channel with the Ca^{2+}-entry mechanism of intact cells, by investigating its inhibition by antagonists of voltage-sensitive and receptor-operated Ca^{2+} channels. We then directly tested the hypothesis that Ca^{2+}-channel activation might be inhibited by cGMP and cGMP-dependent protein kinase.

MATERIALS AND METHODS

Materials

ATP (disodium salt) was obtained from Boehringer, $^{45}CaCl_2$ from Amersham, and all other biochemicals were obtained from Sigma. Diltiazem solution was prepared by dissolving 60 mg Tildiem tablets (Lorex Pharmaceuticals) in buffer. Nifedipine, nicardipine, verapamil and SK&F 96365 were gifts from Bayer U.K. Ltd., Syntex Pharmaceuticals, Abbot Laboratories and SmithKline Beecham Pharmaceuticals respectively.

Measurement of Ca^{2+} accumulation into microsomes

Pig aortas were obtained fresh from the abattoir, and microsomal vesicles were prepared from them by method B [18]. Vesicle preparations (approx. 4 mg of protein/ml) were sonicated with 5.5 mM-histamine, and steady-state ATP-dependent Ca^{2+} accumulation was measured, at 37 °C, 3 min after addition of 0.1 ml of sonicated vesicles to 0.9 ml of reaction buffer, as reported previously [18]. p[NH]ppG (final concn. 0.1 mM) was added to the reaction buffer where indicated. Ni^{2+} was added with histamine before sonication and into the reaction mixture at the same final concentration. When diltiazem, verapamil, nifedipine and nicardipine were used, vesicles were first sonicated with histamine, and then the antagonists were added in 1% of the final volume for 2 min before starting the Ca^{2+}-accumulation experiment. Control experiments had the appropriate quantity of vehicle (instead of antagonist), which was 100 mM-KCl/20 mM-Tris/maleate buffer, pH 6.8, for diltiazem and verapamil, 70% ethanol for nifedipine and ethanol for nicardipine. The experiments with nifedipine were carried out in a dark-room under a sodium lamp. For measuring the effect of cGMP and cGMP-dependent protein kinase on ATP-dependent Ca^{2+} accumulation, vesicles (400 μl) were mixed with 40 μl of storage buffer containing 100 μM-isobutylmethylxanthine and 1 μM-cGMP and no cGMP-dependent protein kinase (control) or the various activities shown in Fig. 1. Histamine was then added, the vesicles were sonicated, and Ca^{2+} accumulation was measured, as above, in medium containing 0.1 mM-p[NH]ppG and 1 mM-ATP.

Abbreviations used: cGMP, cyclic GMP; SK&F 96365, 1-{β-[3-(4-methoxyphenol)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate.

Preparation of cGMP-dependent protein kinase

The cGMP-dependent protein kinase was prepared from pig lung by ion-exchange chromatography [19] and affinity chromatography [20]. Its activity (17.6±3.4 munits/mg of protein; n = 12) was measured [21] with histone as substrate at 30 °C and there was a 1.7 (±0.1; n = 12)-fold activation in the presence of 50 nM-cGMP. One unit of enzyme transferred 1 μmol of organic phosphate to histone under these conditions. The enzyme was stored at 4 °C in a buffer containing 10 mM-KH_2PO_4, 50 mM-2-mercaptoethanol, 2 mM-EDTA and 10% (v/v) glycerol, pH 7.0, and was used within 14 days.

Statistical methods

Values are expressed as means±S.E.M. throughout and were compared by Student's t test with paired data.

RESULTS AND DISCUSSION

The results in Fig. 1 illustrate that addition of p[NH]ppG to pig aortic microsomes sonicated with histamine significantly decreased ATP-dependent steady-state Ca^{2+} accumulation, consistent with our previous work [18]. As we showed [18], the difference in Ca^{2+} accumulation with and without p[NH]ppG results from increased permeability to Ca^{2+} and represents the opening of a putative Ca^{2+} channel. If so, then antagonists of receptor-operated Ca^{2+} channels should reverse the effect of p[NH]ppG on Ca^{2+} accumulation. On the other hand, inhibition of Ca^{2+} accumulation in the absence of p[NH]ppG would indicate non-specific effects of the agents on Ca^{2+}-ATPase activity. The organic Ca^{2+} antagonists verapamil, ditiazem and the dihydropyridines nifedipine and nicardipine are membrane-permeant and relatively selective inhibitors of L-type voltage-operated Ca^{2+} channels [22], although, at concentrations capable of maximally inhibiting such channels, some effects on agonist-induced Ca^{2+} entry have also been reported in intact cells [14,15]. Cations such as Ni^{2+} block Ca^{2+} channels non-selectively [22]. SK&F 96365 is a novel compound reported to inhibit receptor-mediated Ca^{2+} transport into platelets, neutrophils and endothelium [23], cell types which lack L-type channels, although in vascular smooth-muscle cells SK&F 96365 does inhibit L-type channels also [23]. The data in Fig. 1 show that none of the Ca^{2+} antagonists tested decreased Ca^{2+} accumulation in the absence of p[NH]ppG; indeed, verapamil and nicardipine significantly increased Ca^{2+} accumulation. In vesicles sonicated with and then incubated with Ni^{2+}, the inhibition by p[NH]ppG of Ca^{2+} accumulation was reversed (Fig. 1), which implies that the proposed channel was sensitive to Ni^{2+}. In vesicles pretreated with verapamil and diltiazem at the high concentration of 10 μM, the effect of p[NH]ppG to decrease Ca^{2+} accumulation was still observed, which implies that opening of L-type Ca^{2+} channels did not account for the activation of Ca^{2+} permeability by histamine and p[NH]ppG. The data obtained with dihydropyridine Ca^{2+}-channel antagonists was less clear-cut. In the presence of nicardipine (10 μM), a significant decrease in Ca^{2+} accumulation still occurred in response to p[NH]ppG (Fig. 1), as it did with verapamil and diltiazem. In the present of nifedipine (10 μM), by contrast with all the other organic antagonists, the effect of p[NH]ppG on Ca^{2+} accumulation was reversed by approx. 60%. With this exception, the data do not favour a role for voltage-sensitive Ca^{2+} channels, although further experiments using lower concentrations of antagonists may be needed to confirm this. Partial inhibition of receptor-mediated Ca^{2+} entry into intact cells by such high concentrations of nifedipine has, for example, been reported previously [14,15], consistent with an effect on receptor-operated Ca^{2+} channels.

The results in Table 1 show the effect on Ca^{2+} accumulation of SK&F 96365. Since data for membrane permeability of SK&F 96365 are not available, SK&F 96365 was initially sonicated into

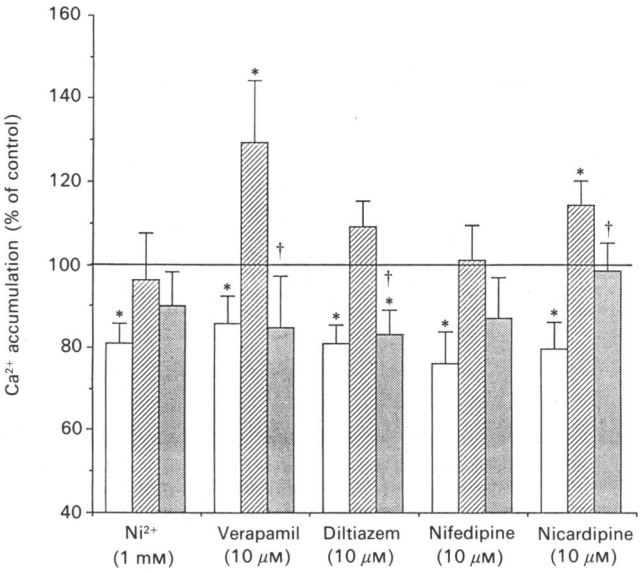

Fig. 1. Effect of Ca^{2+} antagonists on ATP-dependent Ca^{2+} accumulation

Steady-state ATP-dependent Ca^{2+} accumulation was measured. Values are expressed as a percentage of the Ca^{2+} accumulation measured with the same batch of microsomes in the absence of p[NH]ppG and antagonist (control). The number of microsomal preparations used was 16, 22, 27, 14 and 16 for Ni^{2+}, verapamil, diltiazem, nifedipine and nicardipine, respectively. Key: □, +p[NH]ppG, no antagonist; ▨, no p[NH]ppG, +antagonist; ▩, +p[NH]ppG+antagonist. *$P < 0.01$ versus absence of p[NH]ppG and antagonist (control); †$P < 0.01$ versus absence of p[NH]ppG and presence of antagonist.

Table 1. Concentration-dependency of the effect of SK&F 96365 sonicated into or added outside vesicles on ATP-dependent Ca^{2+} accumulation

Steady-state ATP-dependent Ca^{2+} accumulation was measured. Values are expressed as a percentage of the Ca^{2+} accumulation measured with the same batch of microsomes in the absence of p[NH]ppG. In the first series of experiments (n = 10 microsomal preparations), SK&F 96365 was added both before sonication and into the Ca^{2+}-accumulation assay medium at the same final concentration. In the second series of experiments (n = 12 microsomal preparations), SK&F 96365 was added either before sonication or into the Ca^{2+}-accumulation assay medium, but not both. *$P < 0.02$ versus absence of p[NH]ppG.

	Concn. of SK&F 96365 (μM)		Concn. of p[NH]ppG (mM)	
Experiment	Outside	Inside	0	0.1
1	0	0	100	64±8*
	1	1	103±23	120±16
	5	5	95±13	97±15
	10	10	95±11	110±14
	50	50	114±17	117±15
2	0	0	100	84±5*
	0.001	0.01	102±7	84±8*
	0.01	0.1	95±14	112±10
	0.01	0	107±10	106±17
	0.1	0	110±17	108±13

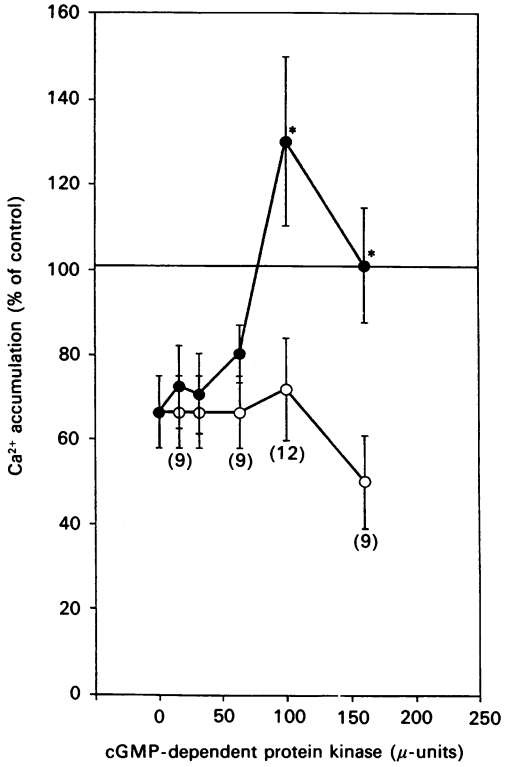

Fig. 2. Effect of cGMP and cGMP-dependent protein kinase on Ca^{2+} accumulation in the presence of p[NH]ppG

Ca^{2+} accumulation was measured into vesicles incubated with cGMP-dependent protein kinase at the concentrations shown without (○) or with (●) 1 μM-cGMP. The activity is expressed as a percentage of that measured in the same batch of microsomes in the absence of p[NH]ppG, cGMP and cGMP-dependent protein kinase (control). The numbers in parentheses indicate the number of microsomal fractions investigated. *$P < 0.05$ versus cGMP-dependent protein kinase alone.

the vesicles at concentrations shown to be effective in intact cells [23] to allow access to the extracellular face of the plasma membrane, and was also added at the same concentrations outside the vesicles to expose the cytosolic face. SK&F 96365 had no effect on Ca^{2+} accumulation in the absence of p[NH]ppG, but reversed the effect of p[NH]ppG at all concentrations between 1 and 50 μM. SK&F 96365 was subsequently used at lower concentrations and was either sonicated into vesicles or added outside only. As shown in Table 1, 10 nM SK&F 96365 was effective when added outside the vesicles, but not when sonicated into the vesicles, when there was a carry-over outside concentration of 1 nM. Sonicating with 100 nM SK&F 96365, when there was a carry-over concentration of 10 nM outside, completely reversed the effect of p[NH]ppG. Addition of 100 nM SK&F 96365 outside was also effective, as expected. The experiments suggest that SK&F 96365 interacts preferentially with the cytoplasmic face of the putative Ca^{2+} channel and was effective at 10 nM and above. Reversal of the effect of p[NH]ppG on ATP-dependent Ca^{2+} accumulation by Ni^{2+} and SK&F 96365 supports the conclusion that we are measuring opening of an ion channel related to the Ca^{2+} influx mechanism of intact cells. In intact cells, the IC_{50} value (concn. giving 50% inhibition) for SK&F 96365 was reported to be approx. 10 μM [23], whereas we obtained an effect at 10 nM in our experiments. Since we found SK&F 96365 to be more potent when exposed to the cytoplasmic face of the membrane, its lower potency in intact cells may reflect impermeability, or formation of a concentration gradient across the plasma membrane. Alternatively, the histamine- and p[NH]ppG-activated channel may be intrinsically more sensitive to inhibition by SK&F 96365 than are other similar channels. An inhibitor of this affinity may be useful for ligand binding and affinity chromatography of the channel.

Fig. 2 shows the effect on Ca^{2+} accumulation in the presence of p[NH]ppG of cGMP and cGMP-dependent protein kinase, either singly or in combination. Neither cGMP alone nor a range of activities of the protein kinase alone reversed the effect of p[NH]ppG on Ca^{2+} accumulation. A combination of cGMP with 100 or 150 μ-units of cGMP-dependent protein kinase, however, completely reversed the effect of p[NH]ppG (Fig. 2). With cGMP and 100 μ-units of the kinase, Ca^{2+} accumulation in the absence of p[NH]ppG (62 ± 11 nmol/mg of protein; $n = 12$) was not affected (62 ± 8 nmol/mg of protein).

These data identify an inhibitory effect of cGMP and cGMP-dependent protein kinase on the putative receptor-mediated Ca^{2+} channel. Since the substrate of the kinase, ATP, was present only outside the vesicles, inhibition appeared to result from phosphorylation at the cytoplasmic face of inside-out plasma-membrane vesicles. Such a mechanism for inhibition of Ca^{2+} influx is consistent with data from intact cells [8–11], and is likely to contribute to vascular relaxation, especially in those parts of the vascular tree which are relatively dependent on extracellular Ca^{2+} for contraction [3]. We do not yet know, however, whether this occurs at the level of receptor, G-protein or the putative ion channel itself. Inhibition by cGMP of coupling between receptors and G-proteins, as described by Hirata *et al.* [7], is an attractive possibility, since it provides a common mechanism for inhibition of Ca^{2+} influx and phosphoinositidase C. Differential sensitivity of influx and mobilization to inhibition by cGMP might then result from coupling of different G-proteins to the various effector mechanisms. Alternatively, differential inhibition might be explained by shared G-proteins interacting with different relative concentrations of the individual effector mechanisms, in which case a greater excess of G-proteins over effector proteins would be expected to lead to lesser sensitivity to inhibition.

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