Vasoconstrictor agonists activate G-protein-dependent receptor-operated calcium channels in pig aortic microsomes

Lynda M. BLAYNEY, Peter W. GAPPER and Andrew C. NEWBY

Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Receptor-operated Ca^{2+} channels were characterized by their ability to decrease steady-state ATP-dependent Ca^{2+} accumulation into pig aortic microsomes. The vasoconstrictor agents noradrenaline, angiotensin II and adenosine 5'-[$\alpha\beta$ -methylene]triphosphate (pp[CH₂]pA) all decreased Ca^{2+} accumulation only when sonicated into vesicles (to allow access to receptor sites) and in the presence of guanosine 5'-[$\beta\gamma$ -imido]triphosphate to activate transducing G-proteins. The effect of noradrenaline was inhibited by the α_2 antagonist yohimbine, but not by the α_1 antagonist prazosin. The effect of none of the agonists was reversed by diltiazem. SK&F 96365 (an inhibitor of receptor-mediated Ca^{2+} influx into intact cells) reversed the effect of noradrenaline, but not that of pp[CH₂]pA, which suggests that at least two receptor-operated channels may be present in this preparation.

INTRODUCTION

Vasoconstrictor agonists invoke contractions in vascular smooth muscle by raising intracellular Ca^{2+} [1]. In the absence of extracellular Ca^{2+} , contraction is transient and mediated by mobilization of intracellular Ca^{2+} stores through activation of phosphoinositidase C [2–6]. When extracellular Ca^{2+} is present, there is a sustained component to contraction which is maintained by Ca^{2+} influx, in part through receptor-operated Ca^{2+} channels (ROCs) in the plasma membrane [7]. Depolarization caused by influx of Ca^{2+} through ROCs may then open voltage-gated Ca^{2+} channels. Hence receptor-mediated Ca^{2+} entry can be partially inhibited, in some studies of vascular smooth muscle, by inhibitors of voltage-sensitive Ca^{2+} channels such as the dihydropyridine nifedipine [8,9]. The structure and regulatory mechanism of ROCs is uncertain. Studies in some non-excitable cells suggest the existence of a ROC opened from the cytoplasmic side of the plasma membrane by the second messenger Ins(1,4,5) P_3 [10–12] or Ins(1,4,5) P_3 plus Ins(1,3,4,5) P_4 [13]. Electrophysiological studies of rabbit ear artery have shown, on the contrary, a ROC directly opened by ATP [14]. This ATP-operated channel appears not to be inhibited by SK&F 96365, which, however, inhibits receptor-mediated Ca^{2+} influx into platelets, endothelial cells and neutrophils [15]. Taken together these observations suggest that ROCs are heterogeneous with respect to their mechanism of activation and sensitivity to inhibitors.

We have demonstrated, using an indirect method, that pig aortic microsomes contain a ROC directly opened by histamine, which requires a G-protein for activation [16] and which is inhibited by SK&F 96365 [17]. Furthermore, this histamine- and G-protein-linked ROC is inhibited by phosphorylation by cyclic-GMP-dependent protein kinase [17], consistent with studies showing that nitrovasodilators linked to cyclic GMP production inhibit receptor-mediated Ca^{2+} influx in intact vascular smooth muscle [18,19]. Using this technique for measuring the activity of ROCs, we tested here whether similar ROCs could be activated by the other vasoconstrictor agonists, noradrenaline, angiotensin II and adenosine 5'-[$\beta\gamma$ -methylene]triphosphate (pp[CH₂]pA) [20–26]. The dependency of such channels on G-proteins and their sensitivity to SK&F 96365 were also investigated.

MATERIALS AND METHODS

Materials

ATP (disodium salt) was obtained from Boehringer, $^{45}\text{CaCl}_2$ from Amersham, and all other biochemicals were obtained from Sigma. Diltiazem solution was prepared by dissolving 60 mg Tildiem tablets (Lorex Pharmaceuticals) in 20 mM-Tris/maleate (pH 6.8) containing KCl (0.1 M) [16]. SK&F 96365 was a gift from SmithKline Beecham Pharmaceuticals.

Measurement of the activity of ROCs

This was done indirectly according to the following principles. Pig aortic microsomes containing inside-out plasma-membrane vesicles accumulated Ca^{2+} by ATP-dependent Ca^{2+} pumps. A decrease in the steady-state Ca^{2+} accumulation could occur if the permeability of the plasma membrane was altered by the opening of a channel permeable to Ca^{2+} . The buffer used throughout these experiments was 20 mM-Tris/maleate (pH 6.8) containing KCl (0.1 M). To open the ROC, pig aortic plasma membranes were sonicated with agonist so as to occupy cell-surface receptors located on the inside of inside-out plasma-membrane vesicles (controls were sonicated with buffer, and guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) was added to activate G-protein on the outside (cytoplasmic surface) [16].

Pig aortas were obtained fresh from the abattoir, and microsomal vesicles were prepared from them by method B of reference [16]. Vesicles (2–5 mg of protein/ml) were mixed with agonist (noradrenaline, clonidine, phenylephrine or angiotensin II, all 50 μM , or pp[CH₂]pA, 10 μM) or buffer (control), in a total volume of 440 μl , contained in 1.5 ml Eppendorf tubes, and sonicated as reported previously [16]. ATP-dependent steady-state Ca^{2+} accumulation was measured by adding 0.1 ml of the sonicated vesicle mixture to 0.9 ml of Ca^{2+} -accumulation buffer [16] containing the same final concentrations of agonist as during sonication and with the addition, where indicated, of p[NH]ppG (final concn. 0.1 mM). In the concentration/response experiment for noradrenaline the antagonists prazosin (5 μM) or yohimbine (0.5 μM) were added alone or together with noradrenaline (0.5–50 μM) before sonication. Ascorbic acid (100 μM) was added to all the experimental buffers when using noradrenaline, and

Abbreviations used: SK&F 96365, 1[β -[3-(4-methoxyphenol)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; ROC, receptor-operated Ca^{2+} channel; pp[CH₂]pA, adenosine 5'-[$\alpha\beta$ -methylene]triphosphate.

Table 1. Effect of vasoconstrictors sonicated into or added outside vesicles and p[NH]ppG on Ca²⁺ accumulation

Steady-state ATP-dependent Ca²⁺ accumulation (nmol of Ca²⁺/mg of protein) was measured in microsomal vesicles to which vasoconstrictors were added at the concentrations shown, either before (columns 3 and 4) or after (columns 1 and 2) sonication. Vesicles were subsequently added to Ca²⁺-accumulation buffer, and Ca²⁺ accumulation was measured in either the presence (columns 2 and 4) or the absence (columns 1 and 3) of p[NH]ppG (0.1 M). **P* < 0.05 versus column 3.

Agonist	Agonist added after sonication		Agonist added before sonication	
	0	+ p[NH]ppG	0	+ p[NH]ppG
Noradrenaline (50 µM) (<i>n</i> = 16)	75.95 ± 5.97	69.43 ± 10.78	75.42 ± 6.37	61.17 ± 5.4*
Clonidine (50 µM) (<i>n</i> = 23)	58.52 ± 5.92	59.81 ± 6.07	69.73 ± 6.06	57.00 ± 5.49*
Phenylephrine (50 µM) (<i>n</i> = 24)	74.73 ± 7.58	84.06 ± 10.23	86.05 ± 10.98	81.24 ± 8.89
Angiotensin II (50 µM) (<i>n</i> = 20)	84.19 ± 10.49	74.93 ± 8.82	88.11 ± 10.86	63.97 ± 8.39*
pp[CH ₂]pA (10 µM) (<i>n</i> = 24)	86.60 ± 7.15	91.75 ± 10.2	94.02 ± 8.8	72.94 ± 4.55*

the experiments were carried out in dim light to prevent adrenochrome formation [27]. Diltiazem and SK&F 96365 were added to vesicles (in 1% of the final volume) for 2 min, after sonication with agonist, and before starting the Ca²⁺-accumulation experiments. The concentrations of agonists and antagonists used were chosen to give maximal effect.

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 Table 1 illustrate that there was no significant decrease in Ca²⁺ accumulation when p[NH]ppG was added to vesicles that had been sonicated with buffer, even though vasoconstrictor agonists were present in the Ca²⁺-accumulation buffer (cf. columns 1 and 2). Under these conditions the vasoconstrictor agonists were presumably denied access to receptor sites located inside the vesicles. In contrast, when the agonists noradrenaline, clonidine, angiotensin II or pp[CH₂]pA were sonicated into the vesicles, addition of p[NH]ppG caused a significant decrease in Ca²⁺ accumulation (cf. columns 3 and 4). Sonication with or without agonist led to similar steady-state Ca²⁺ accumulation measured in the absence of p[NH]ppG (cf. columns 1 and 3), except for clonidine, which appeared to increase Ca²⁺ accumulation. The results suggest that noradrenaline, clonidine, angiotensin II or pp[CH₂]pA each open a ROC by a mechanism that requires a G-protein. The correct topological arrangement of receptor on the outer plasmalemmal (intravesicular) surface and G-protein on the cytoplasmic side (extravesicular) was also required.

Pharmacological studies of catecholamine-mediated Ca²⁺ influx in rat and rabbit aortic preparations have shown it to be mediated via an α₂-subtype receptor [22–24]. Opening of ROCs in our preparation by clonidine (an α₂ agonist), but not by phenylephrine (an α₁ agonist) (Table 1), is consistent with these data. Further studies (Fig. 1) showed that the response to noradrenaline was inhibited by yohimbine (an α₂-adrenoceptor inhibitor), but unaffected by prazosin (an α₁-adrenoceptor inhibitor), confirming that this ROC is indeed linked to α₂ receptors.

Studies [17] with the histamine-linked ROC in this preparation showed it not to be inhibited by diltiazem, an inhibitor of

Fig. 1. Effect of α-antagonists on the opening of ROCs

Steady-state ATP-dependent Ca²⁺ accumulation was measured, in the presence of p[NH]ppG, in vesicles sonicated with the noradrenaline concentrations shown. Noradrenaline was also present at these concentrations in the Ca²⁺-accumulation incubation medium (●). The α₁-antagonist prazosin (5 µM) (○; *a*) or the α₂-agonist yohimbine (0.5 µM) (■; *b*) were added with noradrenaline or alone, both before sonication and into the Ca²⁺-accumulation incubation medium. **P* < 0.05 versus absence of noradrenaline; †*P* < 0.05 versus absence of yohimbine.

voltage-operated Ca²⁺ channels. It was, however, inhibited by very low concentrations (10 nM) of SK&F 96365, an inhibitor of receptor-mediated Ca²⁺ influx. In the present study, the effects of

Table 2. Effect of diltiazem and SK&F 96365 on the ROCs opened by noradrenaline and $\text{pp}[\text{CH}_2]\text{pA}$

Steady-state ATP-dependent Ca^{2+} accumulation (nmol of Ca^{2+} /mg of protein) was measured in vesicles sonicated with either noradrenaline or $\text{pp}[\text{CH}_2]\text{pA}$. Ca^{2+} accumulation was then measured with $\text{p}[\text{NH}]\text{ppG}$ (0.1 mM), diltiazem (10 μM) or SK&F 96365 (100 nM) present when indicated. Diltiazem and SK&F 96365 were added to the sonicated vesicles and incubated for 2 min at room temperature, before measurement of Ca^{2+} accumulation in medium containing $\text{p}[\text{NH}]\text{ppG}$ and the antagonists at the same final concentration. * $P < 0.05$ versus absence of $\text{p}[\text{NH}]\text{ppG}$; † $P < 0.05$ versus control.

Agonist	No $\text{p}[\text{NH}]\text{ppG}$	+ $\text{p}[\text{NH}]\text{ppG}$		
		Control	+ diltiazem (10 μM)	+ SK&F 96365 (100 nM)
Noradrenaline (50 μM) ($n = 20$)	93.87 $\pm$ 7.14	67.41 $\pm$ 6.49*	66.37 $\pm$ 9.20*	85.10 $\pm$ 7.74†
$\text{pp}[\text{CH}_2]\text{pA}$ (10 μM) ($n = 24$)	106.54 $\pm$ 10.66	80.04 $\pm$ 6.59*	72.08 $\pm$ 7.37*	74.154 $\pm$ 9.5*

diltiazem and SK&F 96365 were examined against the ROCs opened by noradrenaline and $\text{pp}[\text{CH}_2]\text{pA}$. The data in Table 2 demonstrate that $\text{p}[\text{NH}]\text{ppG}$ caused a significant decrease in Ca^{2+} accumulation with either agonist (cf. columns 1 and 2), which confirmed the observations reported above. Diltiazem (10 μM) did not reverse the effect of $\text{p}[\text{NH}]\text{ppG}$ in the presence of either agonist (cf. columns 1 and 3). However, SK&F 96365 (100 nM) reversed the effect of $\text{p}[\text{NH}]\text{ppG}$, but only with noradrenaline and not with $\text{pp}[\text{CH}_2]\text{pA}$ as agonist. These results suggest that the ROC opened by noradrenaline may be related to that opened by histamine, but different from that opened by $\text{pp}[\text{CH}_2]\text{pA}$. The absence of an effect of SK&F 96365 on the $\text{pp}[\text{CH}_2]\text{pA}$ -operated channel is consistent with data for the ATP-operated ROC of rabbit ear-artery smooth muscle [1], although its relationship to the ROC our preparation is uncertain. For example, a role for guanine nucleotide has not been established for the ATP-operated channel in the ear artery. It has, however, been shown that $\text{pp}[\text{CH}_2]\text{pA}$ is an agonist for the $\text{P}_{2\text{x}}$ -subtype ATP receptors in vascular smooth muscle, which mediate vasoconstriction [25,26].

In this and our two previous papers [16,17], we have described an indirect assay to measure the opening of ROCs, based on inhibition of steady-state Ca^{2+} accumulation. In each series of experiments, opening of ROCs required the presence of topologically extracellular agonist and cytoplasmic $\text{p}[\text{NH}]\text{ppG}$. The effects of agonists appeared in each case to be mediated by the receptor subtype which mediates vasoconstriction and, in the case of histamine, to be inhibited by cyclic-GMP-dependent protein kinase, consistent with the effect of nitrovasodilators [18]. Furthermore, the effect of $\text{p}[\text{NH}]\text{ppG}$ was antagonized by guanosine 5'-[β -thio]diphosphate [16], as occurs for other G-protein-mediated processes. Moreover, when Ca^{2+} efflux was measured directly [16], this was shown to be increased by histamine and $\text{p}[\text{NH}]\text{ppG}$. Finally, reversal of the effect of $\text{p}[\text{NH}]\text{ppG}$ by Ni^{2+} [17] and, where found, SK&F 96365 is also consistent with opening of a Ca^{2+} channel.

Inhibition of steady-state Ca^{2+} accumulation might alternatively occur if receptor occupation led to decreased Ca^{2+} -ATPase activity. Ca^{2+} -ATPase activity was, however, increased rather than decreased in response to histamine and $\text{p}[\text{NH}]\text{ppG}$ [16], which is consistent with increased permeability of the vesicles relieving thermodynamic constraints. Production of $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol might also result from vasoconstrictor receptor occupancy and G-protein activation, and $\text{Ins}(1,4,5)\text{P}_3$ might then trigger Ca^{2+} permeability in plasma-membrane or endoplasmic-reticulum vesicles. We showed [16] that $\text{Ins}(1,4,5)\text{P}_3$ could not, however, substitute for $\text{p}[\text{NH}]\text{ppG}$ in decreasing Ca^{2+} accumulation. In the experiments measuring Ca^{2+} release directly,

moreover, a 100-fold dilution step rendered an effect of $\text{Ins}(1,4,5)\text{P}_3$ unlikely. Diacylglycerol is also unlikely to inhibit Ca^{2+} accumulation, because in bovine aortic smooth-muscle preparations protein kinase C activates rather than inhibits plasma-membrane Ca^{2+} -pump activity [28].

Our results are therefore most consistent with the interpretation that inhibition of Ca^{2+} accumulation is caused by opening of G-protein-coupled ROCs. The preparation apparently contains ROCs opened by H_1 -histamine [3,29,30], α_2 -adrenergic [22–24] and $\text{P}_{2\text{x}}$ purinergic receptors [25,26], all of which are known to be associated with vasoconstriction. The $\text{P}_{2\text{x}}$ -associated ROC was not inhibited by SK&F 96365, which suggests there may be at least two classes of ROC in our preparation. If so, there exists the untested possibility that they may be linked to their different receptors by different G-proteins.

We thank Dr. M. J. Lewis and Dr. J. Merritt for helpful discussions. This work was supported by the British Heart Foundation.

REFERENCES

- Somlyo, A. P. (1985) *Circ. Res.* **57**, 497–507
- Matsumoto, T., Kanaide, H., Nishimura, J., Shogakiuchi, Y., Kobayashi, S. & Nakamura, H. (1986) *Biochem. Biophys. Res. Commun.* **140**, 172–177
- Suematsu, E., Hirata, M., Hashimoto, T. & Kuriyama, H. (1984) *Biochem. Biophys. Res. Commun.* **120**, 481–485
- Yamamoto, H. & Van Breeman, C. (1985) *Biochem. Biophys. Res. Commun.* **130**, 270–274
- Somlyo, A. V., Bond, M., Somlyo, A. P. & Scarpa, A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5231–5235
- Watrass, J. & Benevolensky, D. (1987) *Biochim. Biophys. Acta* **931**, 354–363
- Rink, T. J. (1990) *FEBS Lett.* **268**, 381–385
- Reuterghem, C. U., Vigne, P., Barhanin, J., Schmid-Alliana, A., Frelin, C. & Lazdunski, M. (1988) *Biochem. Biophys. Res. Commun.* **157**, 977–985
- Simpson, A. W. M., Strampfl, A. & Ashley, C. C. (1990) *Biochem. J.* **267**, 277–280
- Rengasamy, A. & Feinberg, H. (1988) *Biochem. Biophys. Res. Commun.* **150**, 1021–1026
- Kuno, M. & Gardner, P. (1987) *Nature (London)* **326**, 301–304
- Penner, R. & Neher, E. (1988) *J. Exp. Biol.* **139**, 329–345
- Irvine, R. F. & Moore, R. M. (1986) *Biochem. J.* **240**, 917–920
- Benham, C. D. & Tsien, R. W. (1987) *Nature (London)* **328**, 275–278
- Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chaviec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. & Rink, T. J. (1990) *Biochem. J.* **271**, 515–522
- Blayney, L. M. & Newby, A. C. (1990) *Biochem. J.* **267**, 105–109
- Blayney, L. M., Gapper, P. W. & Newby, A. C. (1991) *Biochem. J.* **273**, 803–806
- Collins, P., Henderson, A. H., Lang, D. & Lewis, M. (1988) *J. Physiol. (London)* **400**, 395–404

19. Griffith, T. M., Lewis, M. J., Newby, A. C. & Henderson, A. H. (1988) *J. Am. Coll. Cardiol.* **12**, 797-806
20. Chardonnens, D., Lang, U., Capponi, A. M. & Vallotton, M. B. (1989) *J. Cardiovasc. Pharmacol.* **14**, S39-S44
21. Dostal, D. E., Murahashi, T. & Peach, M. J. (1990) *Hypertension* **15**, 815-822
22. Van Meel, J. C. A., De Jonge, A., Kalkman, H. O., Wilffert, B., Timmermans, B. M. W. M. & Zwieten, V. (1981) *Eur. J. Pharmacol.* **69**, 205-208
23. Haeusler, G. (1985) *J. Cardiovasc. Pharmacol.* **7** (Suppl. 6), S3-S8
24. Haeusler, G., Etienne de Peyer, J., Yajima, M. & Schultz, G. (1986) *J. Cardiovasc. Pharmacol.* **8**, S107-S110
25. Kennedy, C. & Burnstock, G. (1985) *Blood Vessels* **22**, 145-155
26. Burnstock, G. (1987) *Blood Vessels* **24**, 156-160
27. Hughes, I. E. & Smith, J. A. (1978) *J. Pharm. Pharmacol.* **30**, 124-126
28. Fukuda, T., Ogurusu, T., Furukawa, K. & Shigekawa, M. (1990) *J. Biochem. (Tokyo)* **108**, 629-634
29. Tenner, T. E. & McCully, J. P. (1981) *Eur. J. Pharmacol.* **73**, 293-300
30. Van de Voorde, J. & Leusen, I. (1983) *Eur. J. Pharmacol.* **87**, 113-120

Received 16 July 1991/19 September 1991; accepted 1 October 1991