Biochimica et Biophysica Acta, 469 (1977) 52—60

BBA 77781

QUERCETIN: A NOVEL INHIBITOR OF Ca^{2+} INFLUX AND EXOCYTOSIS IN RAT PERITONEAL MAST CELLS

C.M.S. FEWTRELL and B.D. GOMPERTS

Department of Experimental Pathology, University College Hospital Medical School, University Street, London WC1 E 6JJ (U.K.)

(Received February 2nd, 1977)

Summary

The effect of the transport ATPase inhibitor, quercetin on histamine secretion from antigen sensitized mast cells was examined. At micromolar concentrations, quercetin had an immediate inhibitory effect on histamine secretion mediated by antigen, concanavalin A and ATP but it had little effect on release induced by the ionophores A23187 and X537A. Quercetin exerts its effect after the binding of the releasing ligands and the distinction between its effect on ligand induced and A23187 induced secretion suggests that it affects the normal path of Ca^{2+} entry into the cell.

The inhibitory effects of quercetin were compared with those of the structurally related anti-allergic drugs cromoglycate and AH7725.

Introduction

Mast cell degranulation and the release of histamine by a variety of agents such as antigen [1], anti-immunoglobulin [2], lectins [3] and high molecular weight dextrans [4] (all of which crosslink relevant cell surface receptors), ATP [5] and the carboxylate ionophore A23187 [6] are dependent on the presence of extracellular Ca^{2+}. There is good evidence for the belief that it is the entry of Ca^{2+} into the cytosol which triggers exocytosis in many secretory cells including the mast cell, but except in the case of the ionophore-mediated process, the mechanism whereby membrane permeability to Ca^{2+} is increased is unknown. A number of ideas have been aired [7]. These include an involvement of the phosphatidylinositol cycle [8,9], a reaction involving limited proteolysis [10,11] and generation of transmembrane channels due to aggregation of cell surface receptors by a mechanism similar to that proposed for alamethicin [12]. Since exocytosis can be initiated by application of extracellular ATP [5], there is also the possibility of an involvement of a membrane ATPase.

Quercetin and a number of other flavones of plant origin have been shown to

interfere with the activity of membrane transport ATPases [13—15] and recently we showed that four flavones (fisetin, quercetin, myricetin and kaempferol) inhibit antigen-induced histamine release from sensitised mast cells and the (Ca^{2+}) ATPase of rabbit sarcoplasmic reticulum [16]. In addition the flavones (i.e. 2-phenyl cromones) are structurally related to the anti-allergic drug cromoglycate [17] which may affect the Ca^{2+} entry pathway [23], and so a more detailed study of the effect of quercetin was undertaken.

Methods

Mast cell preparation

Male Wistar rats were sensitized to egg white protein by intramuscular injections into each hind leg of 0.25 ml of a suspension of pertussis vaccine BP ($8 \cdot 10^8$ organisms/ml) in a solution containing 50 mg dried egg white/ml. 15—30 days later, a cell suspension containing 2—5% of mast cells was obtained by peritoneal lavage with 25 ml of saline solution (NaCl 0.15 M) containing heparin (25 I.U./ml). Mast cells were purified to better than 80% by centrifugation through a discontinuous density gradient of human serum albumin as previously described [18]. The lower albumin layer containing the mast cells was removed and washed in about 40 ml of a Tyrode's solution having the following composition: NaCl 137 mM/KCl 2.7 mM/NaH_2PO_4 0.4 mM/$MgCl_2$ 1.0 mM/$CaCl_2$ 1.8 mM/glucose 5.6 mM/HEPES (4-(2-hydroxy-ethyl)-1-piperazine ethane sulphonic acid) 20 mM adjusted to pH 7.4 with NaOH. In experiments where the effect of varying calcium concentration was to be studied, $CaCl_2$ was omitted from the Tyrode's solution at this stage.

Histamine release

The cells were suspended in a suitable volume (25—50 ml) of Tyrode's solution containing a suspension of phosphatidylserine (20 μM) and maintained at 37°C. (The purpose of adding phosphatidylserine to the incubation medium was to elevate the degree of ligand-induced histamine secretion [19]). After 5 min preincubation 0.5 ml of cell suspension was added to 0.5 ml of Tyrode's solution containing the releasing agent and inhibitor at 37°C. Concentrations of releasing agents were those described in Fig. 1 unless otherwise indicated. The reaction was terminated after 2—10 min by transferring the tubes to ice and adding 2 ml of ice-cold saline. The cell suspensions were centrifuged at 6500 × *g* for 5 min and the supernatants assayed for histamine. A separate sample of the cell suspension was suitably diluted and heated at 100°C for 5 min to release the total cell histamine.

Assay of histamine

Histamine was measured fluorimetrically essentially according to the method of Shore et al. [20] but omitting the organic extractions except when the ionophores A23187 and X537A were used to release the cells. These ionophores are intensely fluorescent, and interfered with the measurement of histamine, so they were removed from acidified histamine-containing solutions by a single extraction with hexane (3 vols).

Histamine release is expressed as a percentage of the total cell content after

correcting for the spontaneous release which occurs in the absence of releasing agents. None of the inhibitors had a significant effect on spontaneous histamine release.

Materials

Quercetin (Sigma) was dissolved as a stock solution (25 mM) in dimethyl sulphoxide without further purification. The ionophores A23187 and X537A were the generous gifts of Dr. R. Hamill of Eli Lilly & Co. and of Dr. J. Berger of Hoffman La Roche. These were made up as stock solutions (5 mM) in ethanol. There was no effect of the organic solvents at the concentrations used (<0.4% by volume) on the response of the cells. Cromoglycate (gift of Fisons Ltd.) and AH 7725 (gift of Allen and Hanbury Ltd.) were made up as aqueous solutions.

Results and Discussion

The effect of quercetin on histamine secretion induced by a number of different releasing agents is shown in Fig. 1. Quercetin is a potent inhibitor of

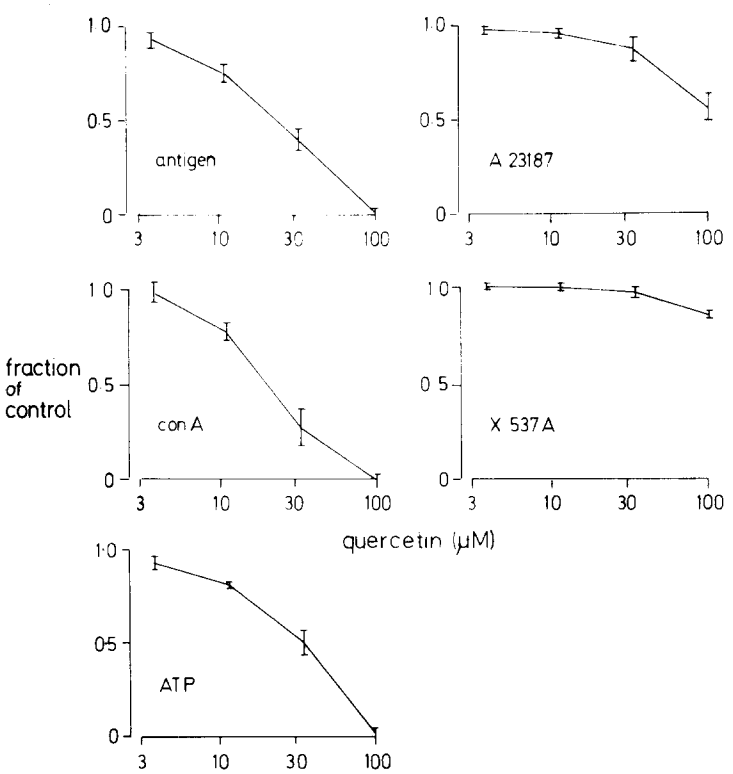

Fig. 1. Dose-response curves for inhibition by quercetin of histamine secretion induced by antigen (egg white proteins 10 μg/ml), concanavalin A (10 μg/ml), ATP (100 μM), A23187 (6 μM) and X537A (20 μM). Results are expressed as a fraction of the secretion observed in the absence of quercetin. Bars show S.E.M.

release induced by antigen, concanavalin A and ATP. The possibility that quercetin could be acting as a competitive inhibitor of ligand binding to mast cells seems unlikely as the site of action of ATP must be rather different from that of the crosslinking ligands. This conclusion is reinforced by the experiments shown in Fig. 2 which demonstrate that in no case is it possible to overcome quercetin inhibition by increasing the concentration of releasing agent. These results also exclude the possibility that quercetin is inactivating the releasers in some way before they affect the cell.

The dose response curves for quercetin inhibition of secretion induced by antigen, concanavalin A and ATP are remarkably similar (Fig. 1) and so it is probable that quercetin acts on a stage subsequent to ligand binding, which is common to all three releasers. Since all are dependent on extracellular Ca^{2+} for their effect, a common Ca^{2+} entry pathway is a plausible site of action for quercetin.

In contrast, histamine release induced by the ionophores X537A and A23187 was virtually unaffected by quercetin. The absence of an effect of quercetin on X537A mediated release was not unexpected since release induced by this rather non-specific ionophore is not a metabolically dependent process and will

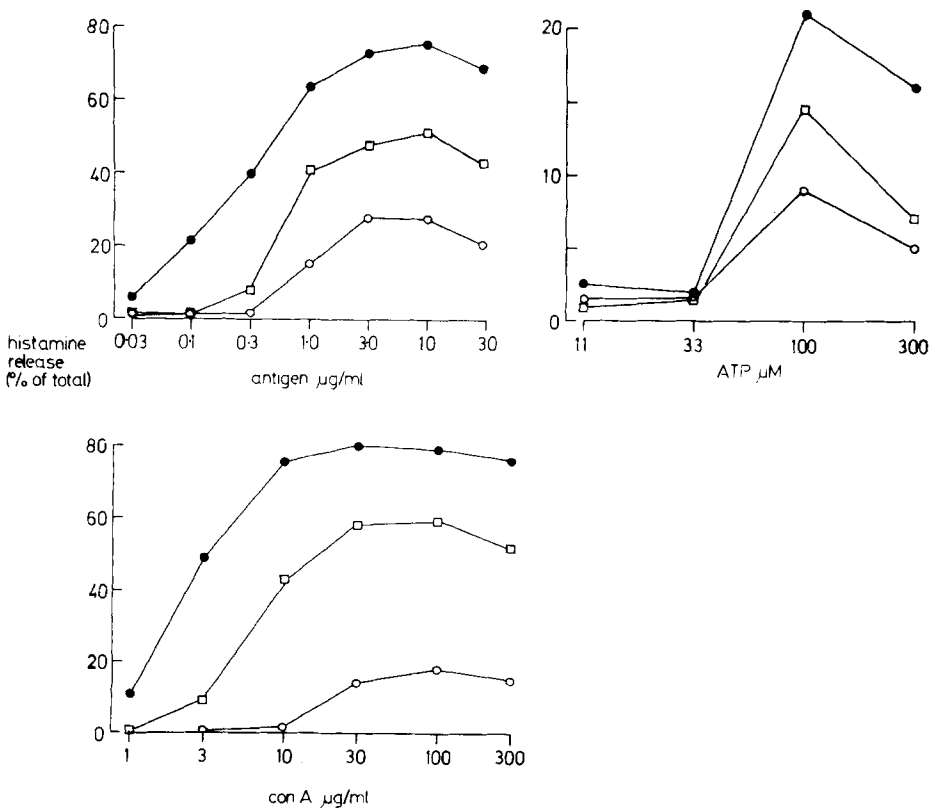

Fig. 2. Dose-response curves for histamine secretion induced by antigen, ATP and concanavalin A in the presence and absence of quercetin. ●———●, no quercetin; □———□, quercetin 33 μM; ○———○, quercetin 100 μM.

occur in the absence of calcium and so bears little relation to antigen-induced secretion [6].

The small degree of inhibition of A23187-induced secretion observed with 100 μM quercetin is probably due to a direct interaction between A23187 and quercetin and it is unlikely that the flavone has any significant effect on the cellular response to A23187. In support of this we found that (1) mixtures of ionophore and quercetin form precipitates on standing; (2) there is a red shift in the fluorescence emission spectrum of A23187 in the presence of quercetin (Fig. 3a); (3) quercetin is able to prevent movement of ions across membranes made permeable with A23187 (Fig. 3b).

From the above results it would appear that quercetin has little effect on histamine release from mast cells rendered permeable to Ca^{2+} with A23187, which suggests that its effect on the ligand mediated process involves blockade of the normal calcium influx pathway. The possibility that quercetin may act

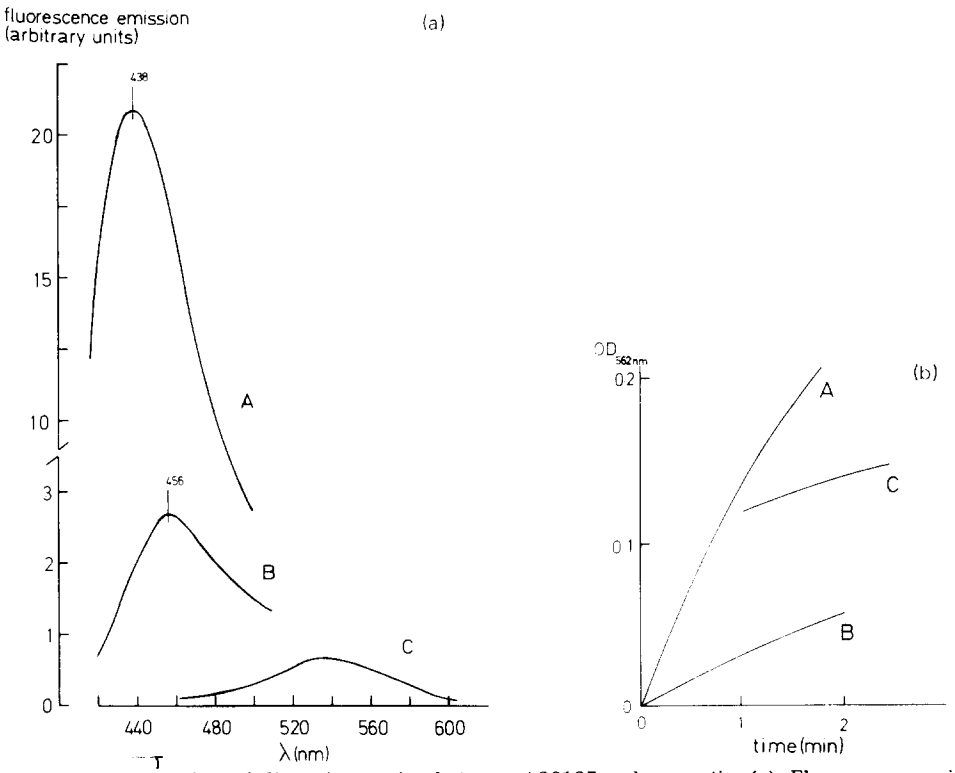

Fig. 3. Demonstration of direct interaction between A23187 and quercetin. (a). Fluorescence emission spectra of A23187 (366 nm excitation). The ionophore was suspended at a concentration of 5 μM in normal Tyrode's solution. Curve A, A23187 alone; curve B, A23187 plus quercetin 100 μM; curve C, quercetin 100 μM alone. (b). Effect of quercetin (100 μM) on release of Fe^{2+} from liposomes treated with A23187, 5 μM. Liposomes were generated and maintained in ascorbate (0.1 M) solution as previously described [21]. The appearance of extra-liposomal Fe^{2+} was detected with ferrozine (0.5 mM). Curve A, release of Fe^{2+} due to the addition of A23187; curve B, effect of adding quercetin to the liposomal suspension together with the ionophore; curve C, effect of adding quercetin 1 min after A23187. For the purpose of these experiments we used Fe^{2+} for which A23187 is an effective ionophore [21]; Fe^{2+} has the advantage over Ca^{2+} of being more readily detected by optical methods. With the use of a calcium electrode we were able to get essentially similar results for Ca^{2+} flux.

by sequestering or displacing Ca^{2+} can be excluded for a number of reasons. Firstly, the A23187 mediated process requires calcium and yet it is virtually unaffected by quercetin. In addition there is a considerable molar excess of calcium over quercetin even at the highest concentration of the inhibitor used, and as Fig. 4 shows, it is not possible to overcome the inhibition of antigen-induced secretion by raising the calcium concentration.

The inhibitory effect of quercetin on antigen-induced histamine secretion is reversible. Cells treated with 100 μM quercetin for 5 min at 37°C and then washed with Tyrode's solution containing human albumin (0.2% to prevent spontaneous histamine release) secreted as much histamine in response to antigen challenge as similarly washed control cells. In addition the inhibition observed with a subsequent exposure to quercetin (30 μM) was unaffected by pretreatment with quercetin.

Unlike many other inhibitors applied to intact cells (e.g. metabolic inhibitors [22] and dibutyryl cyclic AMP [23]) the effect of quercetin is manifest when it is added to the cells at the same time as the releasing agent and prior incubation with quercetin does not significantly augment its inhibitory effect on antigen mediated release (Fig. 5). This is in contrast to the anti-allergic drug cromoglycate whose inhibitory effect is reduced by incubation as is shown by the dashed line in Fig. 5 (data from ref. 24). However, pretreatment of the cells with cromoglycate (30 μM) for 30 min completely abolished the inhibition normally observed with a subsequent dose of 30 μM quercetin added together

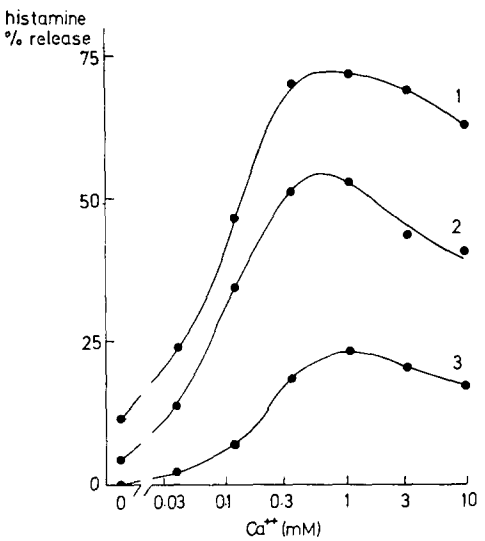

Fig. 4. Ca^{2+} dose-response curves for antigen-mediated histamine secretion in the presence and absence of quercetin. Curve 1, no quercetin; curve 2, quercetin 33 μM; curve 3, quercetin 100 μM.

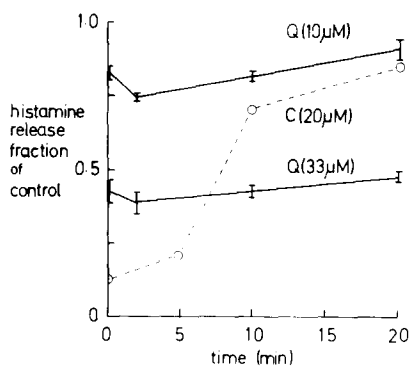

Fig. 5. Time course of onset of inhibition of antigen-stimulated histamine release in the presence of 10 and 33 μM quercetin (Q, ———) and 20 μM cromoglycate (C, ○- - - - -○). (Data from Kusner et al. [24]). Mast cells were incubated in Tyrode's solution containing the inhibitors for various times (as indicated by the points on the curves) before adding the antigen. The reaction was stopped as described, after a further 2 min. Results are expressed as a fraction of the release observed in the absence of inhibitor, and the bars show S.E.M.

with the antigen (Table I). This suggests that cromoglycate and quercetin are acting at the same site [25] which seems reasonable in view of their structural similarities and their ability to block the normal ligand mediated entry of Ca^{2+} into the cell [23]. Other experiments show that they differ in a number of respects:

(1) cromoglycate and the related compound AH7725 [26] have little effect on histamine release in the presence of phosphatidylserine while inhibition due to quercetin is unaffected (Fig. 6).

(2) the inhibitory effect of cromoglycate decreases with time [24]; quercetin inhibition is maintained (see above)

(3) cromoglycate and AH7725 are not inhibitors of (Ca^{2+})ATPase of rabbit muscle sarcoplasmic reticulum (unpublished results).

Recent work has shown that quercetin and certain other flavones are inhibitors of ion transport ATPases, and so we examined the possibility that the effect of quercetin on histamine secretion might be expressed through its inhibitory effect on one of the membrane ATPases of the mast cell. We tried to discover whether the stimulation by ATP requires its hydrolysis by an ecto-ATPase [27] on the mast cell surface. Such an enzyme, activated by high concentrations of Ca^{2+} has been described [28] although doubts about its actual association with mast cells have been expressed [29]. However the structurally related but non-hydrolysable ATP-analogue adenylylimidodiphosphate [30] both failed to stimulate release over the range 10—300 μM, and also failed (at 500 μM) to inhibit release induced by ATP (50 and 100 μM). This suggests that the ATP receptor site on the membrane surface is unusually selective, even to the extent of not recognising the structural analogue. An alternative approach was to see whether the impermeant polyanion suramin which has been shown to be a potent ATPase inhibitor [31] could prevent ATP induced histamine secretion. However, suramin even at 100 μM had no effect. This substance which inhibits all membrane ATPases so far tested is only active on the substrate side [31]. Since we saw no inhibition by suramin, it is unlikely that an ecto-ATPase is involved in ATP mediated secretion.

Ouabain at concentrations of between $5 \cdot 10^{-7}$ M and 10^{-3} M with 0—15 min pre-incubation was without effect on histamine release stimulated by antigen, which excludes the possibility that quercetin acts by way of the ($Na^+ + K^+$)-ATPase. It has already been shown that the mitochondrial ATPase inhibitor oligomycin prevents both antigen and A23187 mediated secretion [22]. Since ionophore-mediated secretion was insensitive to quercetin, it is unlikely that

TABLE I

Effect of pretreatment with cromoglycate on the inhibition of antigen induced histamine release by quercetin. Cells were incubated at 37°C for 30 min with or without cromoglycate. They were then added to tubes containing antigen with or without quercetin and incubated for a further two min at 37°C.

Pretreatment	% Histamine release		Fraction of control
	Control	30 μm quercetin	
None	19.0	3.8	0.22
30 μM cromoglycate	16.8	13.2	0.79

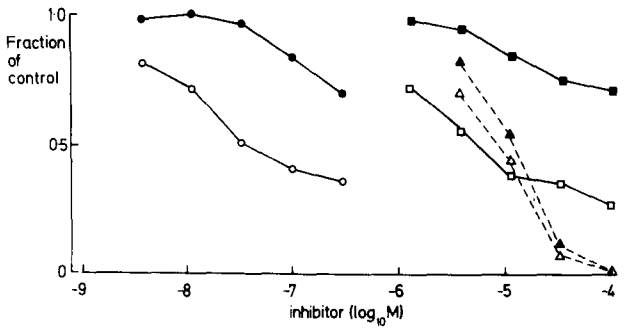

Fig. 6. Effect of phosphatidylserine on the inhibition of antigen-mediated histamine secretion by AH7725 (○———○, ●———●), cromoglycate (□———□, ■———■), and quercetin (△———△, ▲———▲). Open symbols indicate inhibition in the absence of phosphatidylserine, closed symbols indicate inhibition in the presence of phosphatidylserine. Uninhibited release was 20% in the absence of phosphatidylserine and 46% in its presence.

the inhibitory flavones exert their effect at the level of mitochondrial oxidative phosphorylation.

We have already shown that quercetin and the other inhibitory flavones (fisetin, myricetin and kaempferol) are inhibitors of the (Ca^{2+}) ATPase of rabbit muscle sarcoplasmic reticulum, while rutin and morin are without effect on either system [16] so it is possible that quercetin may be exerting its effect via a membrane transport (Ca^{2+})ATPase. Although such an enzyme has yet to be demonstrated in the plasma membrane of the mast cell, there can be little doubt about its universality and its role in the maintenance of low Ca^{2+} levels in the cytosol of resting cells (approx. 10^{-7} M in all tissues where this has been measured [32]). In any process involving ion transport, the possibility that both the inward and the outward movements of ions occur by a common pathway must be examined. Racker has suggested that quercetin may act by increasing the pumping efficiency (i.e. ion translocation/ATP hydrolysis ratio) of the partially uncoupled transport ATPases which have been described in certain tumour cells [14].

Whilst we cannot exclude the possibility that quercetin acts at a site which is quite unrelated to Ca^{2+} transport ATPase, the following mechanism is an attractive idea. Binding of signalling ligands to receptors on the mast cell surface permits the passive influx of Ca^{2+} into the cell by transiently uncoupling the Ca^{2+} transport pathway from the (Ca^{2+})ATPase which is normally responsible for the ejection of Ca^{2+} from the resting cell. The inhibitory action of quercetin could be directed at the postulated uncoupling process by ensuring that the Ca^{2+} pathway remains fully coupled to the (Ca^{2+})ATPase even when stimulating ligands are applied to the cell. In this way the passive influx of Ca^{2+} would be prevented and exocytosis would not take place.

Acknowledgements

We thank Steve Young who carried out the Fe^{2+} flux experiment illustrated in Fig. 3. This work was supported by the Medical Research Council.

References

1 Foreman, J.C. and Mongar, J.L. (1972) J. Physiol. 224, 753—769
2 Humphrey, J.H., Austen, K.F. and Rapp, H.J. (1963) Immunology 6, 226—245
3 Siraganian, P.A. and Siraganian, R.P. (1974) J. Immunol. 112, 2117—2125
4 Foreman, J.C. and Mongar, J.L. (1972) Brit. J. Pharmacol. 46, 767—769
5 Diamant, B. and Kruger, P.G. (1967) Acta Physiol. Scand. 71, 291—302
6 Foreman, J.C., Mongar, J.L. and Gomperts, B.D. (1973) Nature 245, 249—251
7 Gomperts, B.D. (1976) in Receptors and Recognition (Cuatrecasas, P. and Greaves, M.F., eds.), Vol. 2, pp. 45—102, Chapman and Hall, London
8 Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81—147
9 Michell, R.H., Jafferji, S.S. and Jones, L.M. (1976) FEBS Lett. 69, 1—5
10 Becker, E.L. and Austen, K.F. (1966) J. Exp. Med. 124, 379—395
11 Kaliner, M. and Austen, K.F. (1973) J. Exp. Med. 138, 1077—1094
12 Allison, A.C. (1974) in Control of Proliferation in Animal Cells (Clarkson, B. and Baserga, R., eds.) Vol. 1, pp. 447—459, Cold Spring Harbor Conferences on Cell Proliferation
13 Carpenedo, F., Bortignon, C., Bruni, A. and Santi, R. (1969) Biochem. Pharmacol. 18, 1495—1500
14 Suolinna, E.-M., Buchsbaum, R.N. and Racker, E. (1975) Cancer Res. 35, 1865—1872
15 Kuriki, Y. and Racker, E. (1976) Biochemistry 15, 4951—4956
16 Fewtrell, C.M.S. and Gomperts, B.D. (1977) Nature 265, 635—636
17 Cox, J.S.G. (1967) Nature 216, 1328—1329
18 Lawson, D., Fewtrell, C., Gomperts, B. and Raff, M.C. (1975) J. Exp. Med. 142, 391—402
19 Goth, A., Adams, H.R. and Knoohuizen, M. (1971) Science 173, 1034—1035
20 Shore, P.A., Burkhalter, A. and Cohn, V.H. (1959) J. Pharmacol. Exp. Ther. 127, 182—186
21 Young, S.D. and Gomperts, B.D. Submitted for publication
22 Garland, L.G. and Mongar, J.L. (1976) Int. Arch. Allergy Appl. Immunol. 50, 27—42
23 Foreman, J.C., Mongar, J.L., Gomperts, B.D. and Garland, L.G. (1975) Biochem. Pharmacol. 24, 538—540
24 Kusner, E.J., Dubnick, B. and Herzig, D.G. (1973) J. Pharmacol. Exp. Ther. 184, 41—46
25 Marshall, P.W., Thomson, D.S. and Evans, D.P. (1976) Int. Arch. Allergy Appl. Immunol. 51, 274—283
26 Fullarton, J., Martin, L.E. and Vardey, C. (1973) Int. Arch. Allergy Appl. Immunol. 45, 84—86
27 de Pierre, J.W. and Karnovsky, M.L. (1974) J. Biol. Chem. 249, 7111—7120
28 Cooper, P.H. and Stanworth, D.R. (1976) Biochem. J. 156, 691—700
29 Sugiyama, K. (1971) Jap. J. Pharmacol. 21, 531—539
30 Yount, R.G., Babcock, D., Ballantyne, W. and Ojala, D. (1971) Biochemistry 10, 2484—2489
31 Fortes, P.A.G., Ellory, J.C. and Lew, V.I. (1973) Biochim. Biophys. Acta 318, 262—272
32 Baker, P.F. (1972) Prog. Biophys. 24, 177—223