Biochemical Pharmacology, Vol. 34, No. 2, pp. 211–216, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ENHANCED OXYGEN CONSUMPTION IN ADRENAL MEDULLA ON STIMULATION WITH ACETYLCHOLINE

ALAN BEVINGTON* and GEORGE K. RADDA
Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU, U.K.

(*Received* 10 *May* 1984; *accepted* 10 *August* 1984)

Abstract—When perfused cortex-free ox adrenal medulla was stimulated to secrete catecholamine by infusion of 0.1 mM acetylcholine for 4 min, the oxygen consumption increased to a value which was 0.15 ± 0.07 μmole O_2/min/g wet weight (±S.D., N = 12) above the pre-stimulation value of 0.49 ± 0.15 ($P < 0.001$). 1.4 ± 0.9 (±S.D., N = 12) mole of catecholamine was secreted per mole of enhanced O_2 consumption in the 16 min following the start of the stimulation. The rate of ATP hydrolysis by the proton-translocating Mg-ATPase of the chromaffin granule may increase on fusing with the plasma membrane of the chromaffin cell during exocytosis. However, from the amount of catecholamine secreted, this was estimated to account for <17% of the oxygen consumption increase.

The amount of catecholamine secreted by 4 min 0.1 mM acetylcholine stimulations correlated with the enhancement of oxygen consumption ($r = 0.82$, $P < 0.001$) but, on stimulation with 60 μM veratridine for 4 min, O_2 consumption enhancement was anomalously low. This dependence on mode of stimulation suggests that ATP consumption in exocytosis itself is an inadequate explanation. Ouabain-sensitive oxygen consumption rose from undetectable levels to 18 ± 8% (±S.D., N = 4) of the basal respiration during prolonged 0.1 mM acetylcholine stimulation in the absence of Ca, indicating that Na,K-ATPase was not responsible for all of the oxygen consumption enhancement.

Enhanced oxygen consumption on stimulation of secretion has been reported in a number of secretory tissues within the past 20 years [1–7], and one of the earliest observations of this type was made by Banks [8] in perifused strips of ox adrenal medulla.

If this phenomenon arises from mitochondrial respiration, it is of considerable interest, as little is known about the mechanisms in secretory cells that consume ATP during or after secretion [9]. It has been shown that a combination of an inhibitor of glycolysis and an inhibitor of oxidative phosphorylation [10–13] can inhibit catecholamine secretion from perfused adrenal glands. Similarly, in the ingenious 'leaky chromaffin cell' experiments of Baker and Knight [9], it was demonstrated that catecholamine secretion could be stimulated by addition of Ca only if Mg-ATP was also present. These results suggest that ATP hydrolysis occurs in the process of secretion by exocytosis, but unfortunately the evidence is not conclusive, as loss of ATP from the cytoplasm may inhibit secretion indirectly by preventing some process, for example the phosphorylation of enzymes by protein kinases, on which initiation of secretion could depend [14].

Therefore, in this paper, Banks' original technique for measuring oxygen consumption was modified for use in perfused cortex-free ox adrenal medulla, in order to investigate the nature of the processes consuming ATP, which are stimulated during secretion.

* Correspondence to present address: Department of Human Metabolism and Clinical Biochemistry, University Medical School, Beech Hill Road, Sheffield, S10 2RX, U.K.

MATERIALS AND METHODS

Ox adrenal glands from the local slaughterhouse were obtained after approximately 30 min of warm ischaemia. At the slaughterhouse periadrenal fat was removed, and a series of lateral slits spaced 2–3 mm apart, perpendicular to the axis of the adrenal vein, were made through the adrenal cortex. Each gland was then flushed with 60 ml of cold perfusion medium (about 4°) in about 3 min. In the laboratory, the 2–3 mm strips of cortex were excised while the medulla (4–6 g) perfused under a pressure of 40 mm Hg at 12–13 ml/min. This occupied the first 30–45 min of the perfusion. The oxygen consumption and spontaneous catecholamine release were then allowed to stabilise for at least another 60 min at 37°, before commencing experiments.

During the perfusion, the medium was equilibrated with a water-saturated gas mixture of 95% O_2/5% CO_2 at 37°.

Oxygen consumption by the tissue was calculated from the decline in dissolved oxygen concentration in the perfusion medium measured before and after it had passed through the tissue. Oxygen leakage from perfusion lines was minimized by using glass, and Tygon tubing (V.A. Howe, London) of minimum wall thickness 1/16th inch. Residual leakage measured in the same apparatus in the absence of the tissue, was subtracted from all measurements. Dissolved oxygen was measured polarographically in the perfusion line using a Clarke oxygen-sensitive electrode [15].

Catecholamine was assayed as described previously [16].

The perfusion medium was a modified Krebs–

Henseleit comprising: NaCl 118 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.0 mM, $MgSO_4$ 1.2 mM, KCl 6.0 mM and D-glucose 10 mM. This was filtered through a 0.45 μm filter (Millipore HAWP 047 00) before use. For Ca-free perfusions, the 2.0 mM $CaCl_2$ in the perfusion medium was replaced by an equimolar amount of $MgCl_2$. Similarly, for K-free perfusions, the 6.0 mM KCl was replaced by NaCl.

Uncoupler 1799 was kindly donated by the Central Research Department of E.I. Du Pont de Nemours & Co., Wilmington, Delaware, U.S.A. A 1 mM stock was made in methanol. In the perfusion medium, the final concentration of methanol after infusion was, at most 0.2%.

All other drugs and biochemical agents were obtained from Sigma (London, U.K.) Chemical Co.

Statistical analysis. Degree of correlation has been expressed as the product moment correlation coefficient, r. Statistical significance was assessed from values of Student's t as tabulated in "Statistical Tables for Biological, Agricultural and Medical Research", Fisher, R.A. and Yates, F. (Oliver & Boyd).

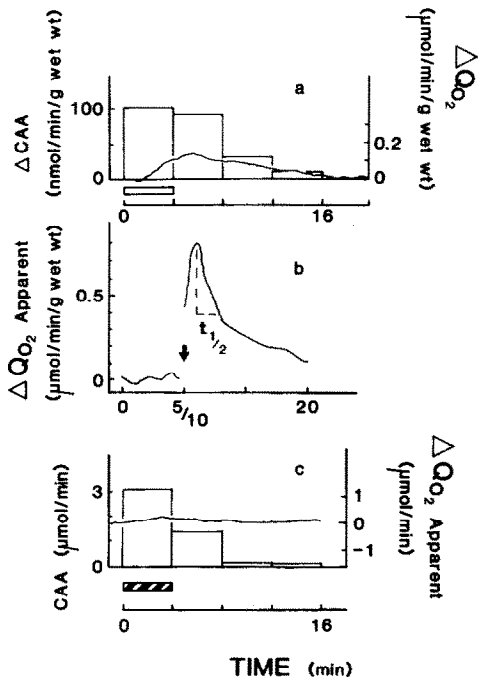

Fig. 1. (a) Comparison of time courses of net catecholamine secretion (ΔCAA) and oxygen consumption enhancement (ΔQO_2) in perfused cortex free ox adrenal medulla. Horizontal white bar denotes duration of 0.1 mM acetylcholine infusion. ΔCAA is plotted as a histogram and ΔQO_2 as a continuous trace. All perfusions were performed at 37°. Weight of tissue = 5.8 g. Abbreviations: CAA, total catecholamine (adrenaline plus noradrenaline); ΔCAA, net catecholamine secretion; QO_2, rate of oxygen (O_2) consumption by tissue; ΔQO_2, change in rate of oxygen (O_2) consumption. (b) Estimate of the time required for deoxygenated perfusion medium to wash out of a perfused ox adrenal medulla after 5 min of warm ischaemia (black arrow). $t_{\frac{1}{2}}$ denotes the half time for the declining phase. (c) Infusion of water soluble content of chromaffin granules in the absence of tissue. Chromaffin granule content was prepared as described in Ref. 17. Hatched horizontal bar denotes duration of infusion. Oxygen and catecholamine measurements are represented as in (a).

RESULTS

Oxygen consumption during stimulation

Figure 1(a) shows a comparison of the time courses of oxygen consumption and catecholamine secretion in a perfused ox adrenal medulla which was stimulated for 4 min. Catecholamine and partly deoxygenated perfusion medium continued to wash out of the apparatus for several minutes after stimulation had ceased. Figures 1(b) and (c) suggest that part, but not all, of the slow decline of both parameters can be explained by slow wash-out from the tissue and perfusion lines. Figure 1(c) also shows that the oxygen electrode response was not significantly affected by the secretion products.

In 12 such stimulations of previously unstimulated perfused medullae, the peak increase in oxygen consumption was 0.15 ± 0.07 (±S.D.) μmole O_2/min/g wet wt compared with a mean basal oxygen consumption of 0.49 ± 0.15 (±S.D.) μmol O_2/min/g wet wt in the same medullae ($P < 0.001$ by Student's t test).

Catecholamine/oxygen ratio

Integration of the areas under curves like that in Fig. 1(a) yields estimates of the total catecholamine secreted and total oxygen consumed after 4 min of stimulation. Basal secretion and consumption were subtracted in all cases and integrals were restricted to the first 16 min after the start of stimulation.

For the ratio of catecholamine secreted to oxygen consumed, a mean value of 1.4 ± 0.9 (±S.D.) moles of catecholamine per mole of O_2 was obtained from 12 preparations. The range was 0.5 to 3.1. In the same 12 preparations, the mean catecholamine

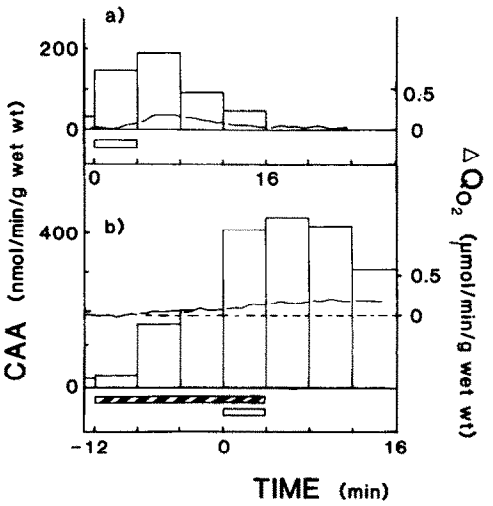

Fig. 2. Stimulation of perfused ox adrenal medulla with 0.1 mM acetylcholine in the presence of 0.1 mM ouabain: (a) control stimulation—no ouabain; (b) with ouabain. Hatched horizontal bar denotes duration of infusion. All other symbols are as in Fig. 1(a).

Table 1. Enhancement of O_2 consumption by 4 min 0.1 mM acetylcholine stimulations in the absence of calcium

Apparent increase	Injection artifact	Increase minus artifact	Basal O_2 consumption	Increase % of basal	Signal stability
0.052± 0.008	0.027± 0.005	0.026± 0.012	0.34± 0.08	8 ± 5%	±0.009

All values are μmole/min/g wet wt (±S.D., N = 5 from 3 different medullae).

secretion in the 16 min was 1.25 ± 0.33 μmole/g wet wt.

Secretion in the presence of ouabain

Pre-perfusion with 0.1 mM ouabain for 12 min, followed by stimulation with 0.1 mM acetylcholine, caused an enhancement of catecholamine release relative to the control (Fig. 2) [18]. Both catecholamine secretion and oxygen consumption were stimulated by ouabain alone, but the peak oxygen consumption observed on subsequent addition of acetylcholine did not differ significantly from the control with acetylcholine alone. Thus ouabain did not inhibit the peak oxygen response accompanying secretion, nor did it enhance it in proportion to the increase in catecholamine secretion.

Uncoupled oxygen consumption

Infusion of uncoupler 1799 (0.43 mg/l, approx. 1 μM) for 10–20 min in 4 medullae yielded a stable increase in oxygen consumption of 88 ± 20% (±S.D.) relative to the mean basal rate of 0.52 ± 0.22 μmole O_2/min/g wet wt. In a fifth preparation only 30% increase was observed with 1 μM 1799 but this rose to 66% when 2 μM uncoupler (0.86 mg/l) was infused. This may mean that values obtained with 1 μM 1799 underestimate the true uncoupled rate.

After the ouabain experiment discussed above (Fig. 2), infusion of 2 μM 1799 for 6 min in the same preparation increased oxygen consumption to 1.20 μmole O_2/min/g wet wt compared with a basal value of 0.62 and a maximum of 0.82 on stimulating with acetylcholine and ouabain.

Na,K-*ATPase and basal oxygen consumption*

Inhibition of Na,K-ATPase with 0.1 mM ouabain or extracellular potassium deprivation had no significant effect on basal oxygen consumption. A complication in such experiments is that both techniques are reported to stimulate secretion [18, 19] which may cause an increase in oxygen consumption that exactly balances the decrease arising from Na,K-ATPase inhibition. Secretion was therefore blocked by replacing the 2.0 mM calcium chloride in the perfusion medium with 2.0 mM magnesium chloride. However, negligible decline in oxygen consumption was detected even in calcium-free medium.

Na,K-*ATPase and stimulated oxygen consumption (in the absence of calcium)*

The contribution of Na,K-ATPase to the enhanced oxygen consumption elicited by acetylcholine was estimated in two ways:

(a) A very small increase in oxygen consumption was observed on stimulating ox adrenal medullae for 4 min in the absence of calcium. About half of this arose from infusion of deoxygenated acetylcholine (Injection artifact, Table 1) (0.027 μmole O_2/min/g wet wt) but the remainder, 0.026 ± 0.012 μmole O_2/min/g wet wt may have reflected enhanced Na,K-ATPase activity (Table 1).

(b) A more reliable estimate was obtained by prolonged stimulation of perfused medullae with acetylcholine in the absence of calcium. As in (a), an apparent increase in oxygen consumption was observed (Fig. 3) and when this had stabilised, 0.1 mM ouabain was infused until the oxygen consumption had fallen to a new stable value. The difference between these two values was taken to be the Na,K-ATPase contribution to the stimulated oxygen consumption (Table 2).

From these results it seems that, even on stimulation with acetylcholine, the Na,K-ATPase only accounts for about 10–20% of the total oxygen consumption. However, not all of this may contribute to the observed increase in overall oxygen consumption, as other components of the basal oxygen consumption may decline during ACh stimulation.

Correlation of catecholamine secretion with increased oxygen consumption

(a) *Prolonged stimulations.* A decline in secretion occurred throughout the stimulation (Fig. 4), but

Table 2. Inhibition of O_2 consumption by 0.1 mM ouabain during prolonged calcium-free 0.1 mM acetylcholine stimulation

Inhibition	Basal O_2 consumption	Inhibition % of basal	Mean signal stability
0.065± 0.022	0.41 ± 0.15	18 ± 8%	±0.011

All values are in μmole/min/g wet wt (±S.D., N = 4).

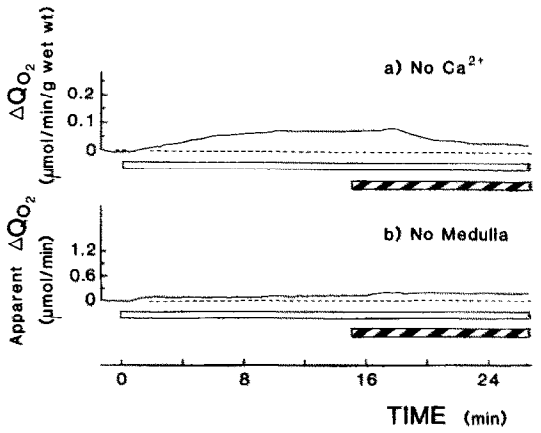

Fig. 3. Stimulation of oxygen consumption in perfused ox adrenal medulla by 0.1 mM acetylcholine in the absence of Ca. The white horizontal bars denote duration of acetylcholine infusion. The hatched bars denote duration of 0.1 mM ouabain infusion. The apparent increase in oxygen consumption in (b) arises because the ouabain solution contained a lower concentration of oxygen than the perfusion medium into which it was infused. The ordinates in (a) and (b) are on directly comparable scales.

decline in the enhancement of oxygen consumption lagged behind secretion in Fig. 4(a) and was not detectable in Fig. 4(b).

(b) *Intermittent stimulations.* As the amount of catecholamine secretion declined in successive 4 min

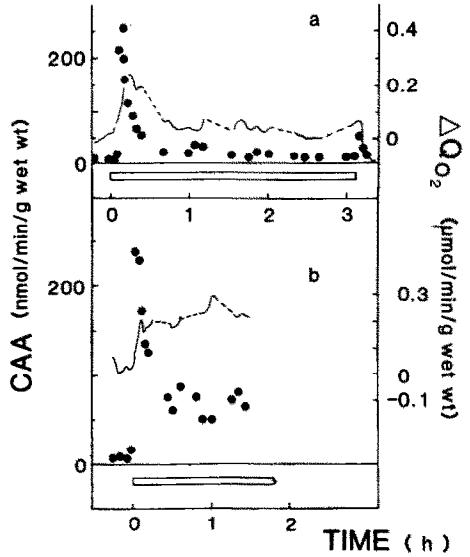

Fig. 4. Comparison of time courses of catecholamine secretion and oxygen consumption enhancement during continuous stimulation of 2 perfused ox adrenal medullae: (●) total catecholamine secreted. Oxygen consumption results are shown as continuous traces joined by interpolations (dashed lines) which represent intervals in which the oxygen concentration supplied to the tissue was measured. This involved interrupting the sampling of perfusion effluent from the tissue.

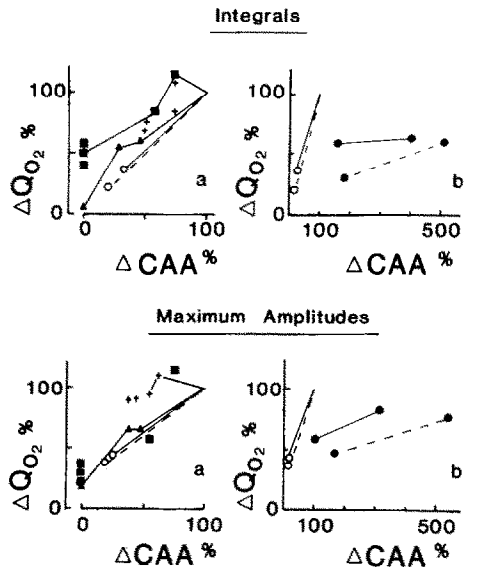

Fig. 5. The correlation between oxygen consumption enhancement and net catecholamine secretion during intermittent stimulations (4 min each). (a) 0.1 mM acetylcholine stimulations. Each series of different symbols represents a different medulla. Results (○) also appear in (b). (b) Acetylcholine and veratridine stimulations. Commencing with acetylcholine, the tissue was given 4 alternate 4 min stimulations with 0.1 mM acetylcholine (○), and 69 μM veratridine + 0.55% v/v dimethylsulphoxide (●). In order to reduce the size of the secretory response between the second (veratridine) and third (acetylcholine) stimulations, a 1 hr stimulation with 0.1 mM acetylcholine was inserted. The continuous and dashed lines denote 2 separate preparations. *Integral values* denote total catecholamine secreted and oxygen consumed in the 16 min after stimulation commenced, expressed as % of the value in the first 4 min acetylcholine stimulation. *Maximum amplitudes* are the corresponding peak values.

stimulations (Fig. 5) a similar but erratic decline was observed in the corresponding oxygen responses (for integrals $r = 0.82, P < 0.001$) [8]. However, more intense secretion obtained with veratridine failed to give a proportional increase in oxygen consumption.

DISCUSSION

Na,K-*ATPase*

If the increased oxygen consumption during secretion is mitochondrial respiration, this effect probably arises from stimulation of an ATPase [20] (although, in parotid, a similar effect was attributed to a direct action of calcium on mitochondria [6]). In many cells, a major contributor to the basal respiration is Na,K-ATPase in the plasma membrane [3].

Electrophysiological evidence suggests that sodium entry is the major contributor to the depolarization of the plasma membrane observed on cholinergic stimulation [21] and blocking of the sodium channels with tetrodotoxin is known to inhibit secretion [22]. This evidence suggests a rise

in the intracellular concentration of sodium on stimulation which, in its turn, should stimulate Na,K-ATPase.

However, experiments on the perfused medulla proved disappointing in this respect. In unstimulated medullae, respiration coupled to Na,K-ATPase was undetectable; and was only 10–20% of the total when the sodium channels were opened with acetylcholine in the absence of calcium (Tables 1 and 2). The acetylcholine experiment was performed in the absence of calcium, as ouabain did not cause any significant inhibition of oxygen consumption enhancement when the tissue was stimulated by acetylcholine in the presence of calcium (Fig. 2). It was concluded that any respiratory inhibition by ouabain in that experiment was being masked by a respiratory enhancement coupled in some way to secretion. This clearly indicates that the Na,K-ATPase is not the sole contributor to the enhancement.

The chromaffin granule Mg-*ATPase*

Pollard *et al.* [23] have proposed that the final step in exocytosis, the fission of the granule at its site of fusion with the plasma membrane, involves osmotic lysis driven by the granule's proton-translocating Mg-ATPase [24]. It has also been suggested recently that, if the granule membrane remains in the plasma membrane after fission and release of the granule content, ATP hydrolysis will continue at an increased rate as the Mg-ATPase no longer has to pump against a proton electro-chemical gradient.* An analogous mechanism has been observed in the regulation of urinary acidification in turtle bladder [25].

These hypotheses, and any other involving an energy requirement during exocytosis [26], predict a strong correlation between the number of exocytotic fusion events (and hence the amount of secretion) and the magnitude of the increase in oxygen consumption. In the prolonged stimulations depicted in Fig. 4, the oxygen response declined more slowly than the secretion. This would not be anticipated if ATP hydrolysis during exocytosis itself were a major contributor: but proton pumping after exocytosis may still be consistent with these results if the granule membrane remains in the plasma membrane for several minutes before retrieval into the cytoplasm.

Owing to these uncertainties, the correlation of secretion and respiration was also investigated by a series of intermittent stimulations on each ox medulla. A positive correlation was obtained (Fig. 5) but more intense secretion induced by veratridine (Fig. 5) did not conform to the same pattern as that induced by acetylcholine. This could simply arise because, when subjected to a high oxygen demand, the partly perfused ox medullae become anoxic so that oxygen consumption is no longer proportional to the rate of ATP hydrolysis. However, the higher rate of respiration obtained with uncoupler 1799 suggests that this is not the case.

Significance of the catecholamine/oxygen ratio

The number of moles of catecholamine secreted per mole of additional oxygen consumed was between 0.5 and 3.1 in a sample of 12 × 4 min acetylcholine stimulations. The highest ratio of 3.1 was derived from an experiment in which 1.46 μmole of catecholamine was secreted and an extra 0.47 μmole of oxygen was consumed per g wet wt in the 16 min after the start of stimulation. If the additional oxygen consumption arose from mitochondrial respiration and a P/O ratio of 3 is assumed [27], 2.8 μmole of ATP was hydrolysed (and resynthesised) while 1.46 μmole of catecholamine was secreted. A chromaffin granule contains approximately 3 million catecholamine molecules [28] so, each exocytotic event was accompanied, on average over the 16 min, by the hydrolysis of at least 6 million additional molecules of ATP.

At first sight, these values seem extremely high, suggesting that oxygenases [1] are also contributing to the oxygen consumption enhancement. However, it should be stressed that, if this is mitochondrial respiration, the large ATP hydrolysis merely accompanied and was not necessarily caused by exocytosis. Processes such as ion pumping, biosynthesis and changes in the cytoskeleton may also have contributed.

Proton pumping

The catecholamine/oxygen ratio may also be used to estimate an upper limit for the contribution of proton pumping to the observed increase in oxygen consumption.

Uncoupled isolated ox chromaffin granules hydrolyse ATP at the rate of 60 nmole/min/mg protein† and contain 3 μmole of catecholamine/mg protein [29]. In the experiment discussed above, the perfused medulla secreted 1.46 μmole catecholamine per g wet wt in 16 min. If the granules which fused with the plasma membrane in secreting this catecholamine remained there and hydrolysed ATP at the uncoupled rate, 2.9×10^{-8} mole of ATP would be hydrolysed per g wet wt per min. The average time of residence in the plasma membrane during the 16 min period is unknown, but it must have been less than 16 min; a value which is consistent with immunochemical evidence [30]. Thus, at most, 0.46 μmole of ATP would have been consumed per g wet wt, corresponding to an oxygen consumption of <0.08 μmole O_2/g wet wt. The observed additional oxygen consumption in this 16 min period was 0.46 μmol O_2/g wet wt.

From these estimates, the measured enhancement of oxygen consumption does seem large enough to accommodate the postulated bout of proton pumping during and after exocytosis. However, it also seems likely, in view of the disparity between calculated and observed values, that there are other contributors to the effect which have yet to be determined.

Acknowledgements—This work was supported by the Science Research Council. A.B. thanks the Medical Research Council and Exeter College, Oxford for financial support.

* Balaban, R. S. and Drake, R. A. L. Personal communication.

† Wakefield, L. M. Personal communication.

REFERENCES

1. M. H. Fukami, H. Holmsen and J. Bauer, *Biochim. biophys. Acta* **428**, 253 (1976).

2. W. R. Henderson and M. Kaliner, *J. Clin. Invest.* **61**, 187 (1978).
3. J. M. Ritchie and R. W. Straub, *J. Physiol. (Lond.)* **304**, 109 (1980).
4. H. J. M. Beijer, A. H. J. Maas and G. A. Charbon, *Blood Vess.* **17**, 144 (1980).
5. T. Kombayashi, S. Sakamoto and M. Tsuboi, *Jap. J. Pharmacol.* **29**, 707 (1979).
6. J. W. Putney, Jr., *Life Sci.* **22**, 1731 (1978).
7. V. Herzog, H. Sies and F. Miller, *J. Cell. Biol.* **70**, 692 (1976).
8. P. Banks, *Biochem. J.* **97**, 555 (1965).
9. P. F. Baker and D. E. Knight, *Phil. Trans. R. Soc. Lond. (B)* **296**, 83 (1981).
10. N. Kirshner and W. J. Smith, *Science* **154**, 422 (1966).
11. N. Kirshner and W. J. Smith, *Life Sci.* **8**, 799 (1969).
12. R. P. Rubin, *J. Physiol. (Lond.)* **202**, 197 (1969).
13. R. P. Rubin, *J. Physiol. (Lond.)* **206**, 181 (1970).
14. F. Konings and W. de Potter, *Biochem. biophys. Res. Commun.* **110**, 55 (1983).
15. B. J. Sproule, W. F. Miller, I. E. Cushing and C. B. Chapman, *J. appl. Physiol.* **11**, 365 (1957).
16. A. Bevington, R. W. Briggs, G. K. Radda and K. R. Thulborn, *Neuroscience* **11**, 281 (1984).
17. D. L. Njus, P. A. Sehr, G. K. Radda, G. A. Ritchie and P. J. Seeley, *Biochemistry* **17**, 4337 (1978).
18. P. Banks, *J. Physiol. (Lond.)* **193**, 631 (1967).
19. A. R. Wakade, *J. Physiol. (Lond.)* **313**, 481 (1981).
20. B. Chance and G. R. Williams, *J. biol. Chem.* **217**, 383 (1955).
21. B. L. Brandt, S. Hagiwara, Y. Kidokoro and S. Miyazaki, *J. Physiol. (Lond.)* **263**, 417 (1976).
22. D. L. Kilpatrick, R. Slepetis and N. Kirshner, *J. Neurochem.* **36**, 1245 (1981).
23. H. B. Pollard, C. J. Pazoles, C. E. Creutz and O. Zinder, *Int. Rev. Cytol.* **58**, 159 (1979).
24. R. W. Holz, R. A. Senter and R. R. Sharp, *J. biol. Chem.* **258**, 7506 (1983).
25. S. Gluck, C. Cannon and Q. Alawqati, *Proc. natn. Acad. Sci. U.S.A.* **79**, 4327 (1982).
26. J. M. Trifaró, *Neuroscience* **3**, 1 (1978).
27. S. Ochoa, *J. biol. Chem.* **151**, 493 (1943).
28. H. Winkler and E. Westhead, *Neuroscience* **5**, 1803 (1980).
29. G. Taugner, *Biochem. J.* **130**, 969 (1972).
30. G. Lingg, R. Fischer-Colbrie, W. Schmidt and H. Winkler *Nature* **301**, 610 (1983).