A Study of the Kinetics of the Muscarinic Effect on Phosphatidylinositol and Phosphatidic Acid Metabolism in Rat Brain Synaptosomes

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(Received 8 June 1977)

The uptake of [^{32}P]phosphate into phosphatidylinositol and phosphatidate was measured in synaptosomes incubated in Krebs–Ringer bicarbonate buffer, pH 7.4. The apparent dissociation constants for acetylcholine and carbamoylcholine were estimated from the increase in ^{32}P uptake caused by these agents. These apparent constants were similar for both phosphatidylinositol and phosphatidate and were $2.7 \pm 0.5 \mu\text{M}$ for acetylcholine and $12 \pm 2 \mu\text{M}$ for carbamoylcholine when the Ca^{2+} concentration was 0.75 mM. Under the same conditions the inhibition of the carbamoylcholine-induced increase in ^{32}P uptake, caused by atropine, is consistent with atropine being a competitive inhibitor, with an apparent inhibition constant of $0.35 \pm 0.05 \text{ nM}$. The apparent constants were dependent on the Ca^{2+} concentration, and were greater in 2.54 mM- Ca^{2+} . The former values for the kinetic constants are similar to the muscarinic-receptor dissociation constant, which indicates that the binding of the agonist to the receptor may be rate-limiting in this series of reactions when the Ca^{2+} concentration is 0.75 mM.

An increase in the turnover of phosphatidylinositol and phosphatidate has been observed in a number of tissues after stimulation by several neurotransmitters and hormones (Michell, 1975). This phenomenon has been associated with muscarinic receptors in several tissues, including the brain (Pumphrey, 1969), synaptosomes (Schacht & Agranoff, 1972), ganglia (Lapetina *et al.*, 1976), smooth muscle (Jafferji & Michell, 1976), parotid gland (Jones & Michell, 1974) and pancreas (Hokin-Neaverson, 1974). Consequently, it has been suggested that this phospholipid effect may have an important function in the response to the muscarinic receptor (Michell, 1975).

However, the relationship of the binding of the agonist to the receptor, and the subsequent breakdown and resynthesis of these phospholipids, is not yet fully understood. The step in the turnover cycle that is thought to be stimulated in most tissues is the hydrolysis of phosphatidylinositol (Jones & Michell, 1974; Hokin-Neaverson, 1974; Michell, 1975). In synaptosomes, however, increases in ^{32}P uptake into both phosphatidylinositol and phosphatidate have been observed (Redman & Hokin, 1964; Schacht & Agranoff, 1972) but stimulated chemical breakdown, or loss of label from pre-labelled synaptosomes, has only been found for phosphatidate (Yagihara *et al.*, 1973; Lapetina & Michell, 1974; Schacht & Agranoff, 1974). Therefore there may be a mechanism of stimulation of ^{32}P uptake into phosphatidylinositol and phosphatidate in synaptosomes that differs from

that in other tissues, although this seems surprising (Michell, 1975).

In the experiments described below we have estimated the apparent dissociation constants of acetylcholine, carbamoylcholine and atropine by measuring the effect of these agents on ^{32}P uptake into both phosphatidylinositol and phosphatidate. The aim of this estimation was to ascertain whether agonist binding to the receptor is the rate-limiting step in this series of reactions, as it appears to be for carbamoylcholine in smooth muscle (Jafferji & Michell, 1976) and in other physiological responses such as stimulated K^+ release in smooth muscle (Burgen & Spero, 1968). A preliminary report of some of these findings has been made (Miller, 1975).

Materials and Methods

Preparation of synaptosomes

The method was based on that of De Robertis *et al.* (1962) and of Gray & Whittaker (1962). Male Sprague–Dawley rats (200–300 g) were killed by decapitation. Their brains were removed, chilled in ice-cold 0.32 M-sucrose and the cerebellum and some white matter were discarded. All subsequent operations were carried out at 4°C. The forebrain was homogenized in 0.32 M-sucrose with a glass/Teflon homogenizer with a radial clearance of 250 μm , and the homogenate was centrifuged at 1000g for 10 min, in a Sorvall SS-34 rotor in a RC-2B centrifuge. In

some experiments, the sediment was resuspended by homogenization and the centrifugation repeated. The combined supernatants were centrifuged at 14500g for 20 min, and the pellet was resuspended and centrifuged again. The pellet (crude mitochondrial fraction) was resuspended in a small volume of 0.32M-sucrose, layered on a sucrose density gradient of 1.4, 1.2, 1.0 and 0.8M-sucrose and centrifuged for 2 h at 50000g in an SW-27 rotor in a Beckman L2-65B ultracentrifuge. Synaptosomes are found at the 1.4/1.2M- and 1.2/1.0M-sucrose interfaces (De Robertis *et al.*, 1962). The lighter fraction was used only, unless otherwise noted. It was diluted with chilled water (slowly) to a sucrose concentration of 0.45M (Gray & Whittaker, 1962), and centrifuged for 20 min at 20000g. The pellet was suspended in Krebs-Ringer bicarbonate buffer, pH 7.4 (Dawson, 1969), modified to contain 0.75 mM-Ca²⁺, 10 mM-glucose and 10 mM-sodium succinate, unless otherwise noted. This preparation may contain some contaminating mitochondria and microsomal fraction. However, it has been shown that under similar conditions microsomal fractions are unable to take up ³²P into phospholipids, and that the acetylcholine-induced increase does not occur in mitochondria (Yagihara *et al.*, 1973).

Uptake of ³²P into synaptosomes

The synaptosome suspension was injected into vials (to a final concentration of 0.5–0.9 mg of protein/ml) that contained the same buffer as described above, 30–50 μ Ci/ml of [³²P]phosphate (New England Nuclear Corp., Boston, MA, U.S.A.), cholinergic drugs (Sigma Chemical Co., St. Louis, MO, U.S.A.) and a gas phase of O₂/CO₂ (19:1), and that were equilibrated at 30°C. Eserine salicylate was included in all experiments with acetylcholine. The reaction was carried out for 1 h at 30°C, unless otherwise noted, and was stopped by the addition of an excess of chloroform and methanol, the first step in lipid extraction.

Extraction and purification of phospholipids

The phospholipids were extracted either by the method of Folch *et al.* (1957) or by modification of the method of Bligh & Dyer (Kates, 1972). The excess of water-soluble ³²P was washed out with 1 mM-potassium phosphate in 0.9% NaCl. The lipid extracts were dried with a stream of N₂, and were redissolved in 50 μ l of chloroform/methanol/water (75:25:2, by vol.) containing 50 mg of butylated hydroxytoluene/litre to prevent oxidation of the lipids (Neudoerffer & Lea, 1966).

The phospholipids were separated on thin-layer plates prepared with Camag D-0 silica gel in 1 mM-Na₂CO₃, and developed in chloroform/methanol/acetic acid/water (50:25:7:3, by vol.) (Skipski *et al.*, 1963). Occasionally the separation of phosphatidyl-

serine and phosphatidylinositol was inadequate, but as control experiments showed that the uptake of ³²P into phosphatidylserine was approximately 13% of that into phosphatidylinositol (unstimulated) and was not affected by cholinergic drugs, on those few occasions the increased uptake was assumed to be into phosphatidylinositol only. It is noteworthy that separation of phosphatidylserine and phosphatidylinositol was not achieved by using Merck silica gel H. The lipids were stained with iodine and the phospholipid bands identified by comparison with standards (General Biochemical Inc., Chagrin Falls, OH, U.S.A.). The lipid bands were scraped into vials, suspended in 1 ml of methanol and 10 ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO)/litre and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (Interex Corp., Natick, MA, U.S.A.)/litre (Roscoe *et al.*, 1970) and the ³²P uptake was measured with a Packard Tri-Carb liquid-scintillation spectrometer.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard.

Acetylcholinesterase assay

The assay was a modification of the method of Garry & Routh (1965). The assay mixture contained 4 ml of 0.25 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), 10–20 μ l of synaptosome suspension and 0.5 ml of 9 mM-acetylthiocholine iodide (Sigma). The mixture was incubated at 37°C for 5–10 mins, and the assay stopped by the addition of 1 ml of 0.2% cetyltrimethylammonium bromide (Sigma). The A₄₁₅ was then measured.

Results

Comparison of the effect of acetylcholine on phospholipids in light and dense fractions of synaptosomes

The effect of acetylcholine on the two fractions of synaptosomes was measured, as it has been reported that cholinergic synaptosomes are concentrated in the lighter fraction (De Robertis *et al.*, 1962). There was no significant difference in the unstimulated incorporation of ³²P into the phospholipids in the two fractions. However, the increase in ³²P uptake caused by acetylcholine was much greater in the lighter fraction, Band C (Table 1). A similar finding has been reported previously (Schacht & Agranoff, 1972). In all subsequent experiments the lighter fraction was used.

Linearity of ³²P uptake

The uptake of ³²P into both phosphatidylinositol and phosphatidate was linear with time during a

Table 1. Comparison of the effect of acetylcholine on the light and dense fractions of synaptosomes

Band-C synaptosomes were collected from the interface of 1.0 and 1.2M-sucrose, and Band D from 1.2 and 1.4M-sucrose, after sucrose-density-gradient centrifugation. They were incubated for 1 h at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 11 mM-glucose and 50 μ Ci of [32 P]phosphate/ml. The results are the mean values of two experiments. The mean acetylcholinesterase activity of Band C was 8.7 units (μ mol of acetylthiocholine hydrolysed/h per mg of protein) and the protein yield was 7.12 mg/g of brain (wet wt.). The mean acetylcholinesterase activity of Band D was 2.92 units and the protein yield was 2.30 mg/g of brain.

	Phosphatidylinositol		Phosphatidate	
	(c.p.m./mg of protein)	(% increase by acetylcholine)	(c.p.m./mg of protein)	(% increase by acetylcholine)
Band C				
Control	606		1220	
Acetylcholine (30 μ M)+eserine salicylate (0.1 mM)	1188	96	2041	67
Band D				
Control	535		948	
Acetylcholine (30 μ M)+eserine salicylate (0.1 mM)	601	12	1134	19

Fig. 1. Time course of 32 P uptake into phosphatidylinositol and phosphatidate

Synaptosomes were incubated (a) in the absence and (b) in the presence of 30 μ M-acetylcholine and 0.1 mM-eserine salicylate. Δ , Phosphatidate; $\bullet$, phosphatidylinositol.

period of at least 90 min (Fig. 1). Incubations in all subsequent experiments were for 1 h.

Effects of Ca^{2+} on 32 P incorporation into phosphatidylinositol and phosphatidate

A comparison was made between the uptake of 32 P into phosphatidylinositol and phosphatidate in the

absence of Ca^{2+} , and that in 0.75 mM- and 2.45 mM- Ca^{2+} . These two concentrations were chosen as the former appears to be the free extracellular concentration in brain (Lolley, 1963) and the latter is the concentration in Krebs-Ringer bicarbonate medium. Although the latter is probably higher than the physiological concentration, it has been used frequently in muscarinic-receptor studies. The effects of Ca^{2+} on the uptake of 32 P into phosphatidate were small, but significant, and it appeared that 32 P uptake into phosphatidylinositol may follow a similar trend (Table 2). The effect of increasing the Ca^{2+} concentration was an inhibition of 32 P uptake in the absence of acetylcholine, and an increase in 32 P uptake in the presence of acetylcholine. The net effect was a greater stimulation of 32 P uptake by acetylcholine on increasing the Ca^{2+} concentration from zero to 2.54 mM. The concentration of Ca^{2+} affects the sensitivity of the synaptosomes to acetylcholine (Fig. 2) and atropine (Fig. 5), as discussed below.

Measurement of the apparent dissociation constant of acetylcholine and carbamoylcholine

The apparent dissociation constants were estimated by measuring the increase in 32 P uptake caused by various concentrations of agonist. Each measurement was made with a single preparation of synaptosomes. The results of experiments with acetylcholine and carbamoylcholine are plotted in Figs. 2 and 3 respectively. The apparent dissociation constants were similar for phosphatidylinositol and phosphatidate, and those measured in 0.75 mM- Ca^{2+} are similar to the equilibrium dissociation constant for the muscarinic receptor itself, which has been measured by several different methods (Furchgott & Bursztyn, 1967; Burgen & Spero, 1970; Burgen *et al.*, 1974; Yamamura & Snyder, 1974), and suggests that

Table 2. *Effect of Ca^{2+} on the uptake of ^{32}P into phosphatidylinositol and phosphatidate*

Results are expressed as c.p.m./mg of protein, $\pm$ s.d. ($n=3$). The statistical significance (P) of the effect of Ca^{2+} (compared with ^{32}P uptake in the absence of Ca^{2+}) was tested by Student's t test: n.s., not significant ($P > 0.05$).

	Control	P for Ca^{2+} inhibition	30 μM -Acetylcholine and 0.1 mM- eserine	P for Ca^{2+} stimulation
Phosphatidylinositol				
No Ca^{2+}	1932 $\pm$ 220		1985 $\pm$ 89	
0.75 mM- Ca^{2+}	1860 $\pm$ 120	n.s.	2245 $\pm$ 150	n.s.
2.54 mM- Ca^{2+}	1725 $\pm$ 250	n.s.	2238 $\pm$ 193	n.s.
Phosphatidate				
No Ca^{2+}	4364 $\pm$ 127		5946 $\pm$ 202	
0.75 mM- Ca^{2+}	4098 $\pm$ 143	n.s.	6464 $\pm$ 195	<0.02
2.54 mM- Ca^{2+}	3575 $\pm$ 356	<0.02	6906 $\pm$ 241	<0.02

Fig. 2. *Estimation of the apparent dissociation constant K_{app} for acetylcholine*

All samples in these experiments contained 0.1 mM-eserine salicylate. Δ , Phosphatidate; $\bullet$, phosphatidylinositol; c.p.m._c, c.p.m./mg of protein in the absence of acetylcholine; c.p.m._s, c.p.m./mg of protein in the presence of acetylcholine. The slopes were calculated by regression analysis. From two experiments with 2.54 mM- Ca^{2+} (a), the average K_{app} was 11 μM for phosphatidylinositol and 17 μM for phosphatidate. These differences were not statistically significant and the mean of the pooled estimates is $14 \pm 2 \mu\text{M}$ (S.E.M.). The average K_{app} from two experiments at 0.75 mM- Ca^{2+} (b) was 3.5 μM for phosphatidylinositol and 2.0 μM for phosphatidate. The mean of the pooled estimates was $2.7 \pm 0.5 \mu\text{M}$ (S.E.M.).

Fig. 3. *Estimation of the K_{app} for carbamoylcholine* Δ , Phosphatidate; $\bullet$, phosphatidylinositol; c.p.m._c, c.p.m./mg of protein in the absence of carbamoylcholine; c.p.m._s, c.p.m./mg of protein in the presence of carbamoylcholine. The slopes were calculated by regression analysis. The average K_{app} (from two experiments) was 9.2 μM for phosphatidylinositol and 13.5 μM for phosphatidate. The mean of the pooled estimates was $12 \pm 2 \mu\text{M}$ (S.E.M.).

under these conditions agonist binding to the receptor may be the rate-limiting step.

Measurement of the apparent inhibition constant of atropine

To determine the inhibition constant of atropine and characterize the type of inhibition, three preparations of synaptosomes were incubated, each with a single concentration of carbamoylcholine and various concentrations of atropine, and the results are plotted in Fig. 4. The scatter in the analysis is inevitably considerable, as the error in the method is relatively high and the increase in ^{32}P uptake in the presence of atropine is small. Nevertheless, the slopes vary

Fig. 4. Estimation of the apparent inhibition constant of atropine

Quadruplicate control samples were incubated in the presence (c.p.m.s) and absence (c.p.m.e) of carbamylcholine. Duplicate test samples contained carbamoylcholine and various concentrations of atropine (c.p.m.i). The inhibition of the increase in ^{32}P uptake (c.p.m.i - c.p.m.e) was averaged for each atropine concentration, and the ratio of the non-inhibited to the inhibited increase in ^{32}P uptake was calculated. The slopes were calculated by regression analysis. ●, 10 μM -Carbamoylcholine. The slope was 1.33 ± 0.72 for phosphatidylinositol and 0.74 ± 0.36 for phosphatidate. These differences are not statistically significant, and the data were combined. The slope of the combined data (plotted) was 0.96 ± 0.36 . Δ, 20 μM -Carbamoylcholine. The slope was 0.46 ± 0.28 for phosphatidylinositol, 0.85 ± 0.12 for phosphatidate and 0.65 ± 0.23 for the combined data. ■, 50 μM -Carbamoylcholine. The slope was 0.43 ± 0.12 for phosphatidylinositol, 0.59 ± 0.07 for phosphatidate, and 0.51 ± 0.07 for the combined data. The slopes were significantly different at 10 μM - and 50 μM -carbamoylcholine ($P < 0.001$), indicating competitive inhibition, and the inhibition constant was calculated at each carbamoylcholine concentration. The mean value was $0.35 \pm 0.05 \text{ nM}$.

significantly, and are consistent with competitive inhibition. The apparent inhibition constant was $0.35 \pm 0.05 \text{ nM}$ (S.E.M.) (Fig. 4), which is similar to the dissociation constant for the muscarinic receptor (Paton, 1961; Burgen & Spero, 1970; Farrow & O'Brien, 1973; Burgen *et al.*, 1974; Yamamura & Snyder, 1974). However, the inhibition constant measured in buffer containing 2.54 mM- Ca^{2+} is approx. 5 nM (Fig. 5).

Discussion

An increase of the turnover of phosphatidylinositol and phosphatidate appears to be a universal response to the stimulation of muscarinic receptors, and it has been suggested that this turnover may play a central

Fig. 5. Apparent inhibition constant of atropine in the presence of 2.54 mM- Ca^{2+}

The experimental conditions were similar to those in Fig. 4, except that 30 μM -acetylcholine and 0.1 mM- eserine salicylate were present in place of carbamoylcholine, the Ca^{2+} concentration was 2.54 mM and all tests were carried out in triplicate. The slope was calculated by regression analysis and was 0.057 ± 0.004 for phosphatidylinositol, 0.10 ± 0.02 for phosphatidate and 0.063 ± 0.003 for the combined data. The apparent inhibition constant was calculated to be approx. 5 nM, assuming competitive inhibition.

role in the response to the muscarinic receptor (Michell, 1975; Michell *et al.*, 1976). If this is so, and the binding of the agonist to the receptor is the rate-limiting step, the apparent dissociation constants derived from the increase in ^{32}P uptake into these lipids should be similar to the muscarinic-receptor dissociation constants. Recent results indicate that this may be so in smooth muscle and pancreas (Michell *et al.*, 1976). In the more detailed studies of the present paper, estimates have been made for the apparent dissociation constants of acetylcholine, carbamoylcholine and the muscarinic inhibitor atropine, and the experiments were carried out with synaptosomes, which may have a different mechanism of stimulation of phospholipid turnover (Yagihara *et al.*, 1973; Lapetina & Michell, 1974; Schacht & Agranoff, 1974).

The muscarinic receptor has both a high- and a low-affinity binding site for agonists (Birdsall & Hulme, 1976). From the data presented in the present paper, it is not possible to detect whether or not the binding of agonist to the high-affinity binding site results in an increased uptake of ^{32}P into phosphatidate and phosphatidylinositol, but the apparent dissociation constants measured are comparable, under certain conditions, with those of the low-affinity site, as are the estimates of the muscarinic-receptor dissociation constants, which do not separate the high- and low-affinity sites (Paton, 1961;

Furchgott & Bursztyn, 1967; Burgen & Spero, 1970; Farrow & O'Brien, 1973; Burgen *et al.*, 1974; Yamamura & Snyder, 1974). Similarly, the apparent dissociation constants for carbamoylcholine and acetylcholine, estimated from the increase in ^{32}P uptake into phosphatidylinositol in smooth muscle (Jafferji & Michell, 1976; Michell *et al.*, 1976) and pancreas (Michell *et al.*, 1976) respectively, are comparable with that of the muscarinic receptor. This seems to provide confirmation of the suggestion that the phospholipid turnover has an important role in the response to the muscarinic receptor in synaptosomes as well as in other tissues.

However, the apparent dissociation constants were larger when measured in buffer containing 2.54 mM- Ca^{2+} than in 0.75 mM- Ca^{2+} . This is noteworthy, since Ca^{2+} is not required for an increase in ^{32}P uptake to occur into these lipids (Table 2; see also Trifaro, 1969; Oron *et al.*, 1975). However, the concentration of Ca^{2+} has a small but statistically significant effect on the uptake of ^{32}P into phosphatide (Table 2), and a similar effect has been observed previously in pancreas (Hokin, 1966) and, in the absence of acetylcholine, in brain cortex (Brossard & Quastel, 1963). No such effects were observed in adrenal medulla (Trifaro, 1969) or parotid gland (Oron *et al.*, 1975). It is noteworthy that differences in apparent dissociation constants were obtained when the Ca^{2+} concentration was varied, as there do not appear to be any variations in equilibrium dissociation constants for the muscarinic receptor measured at different Ca^{2+} concentrations (Farrow & O'Brien, 1973; Burgen *et al.*, 1974; Yamamura & Snyder, 1974). This conclusion is reached by comparing data from different studies, and few measurements have been made at low Ca^{2+} . In support of this it has been reported that Ca^{2+} (between 1.25 and 2.5 mM) has no effect on the carbamoylcholine-induced increase in Rb^{+} efflux from smooth muscle (Burgen & Spero, 1970). However, varying the Ca^{2+} concentration has marked effects on the physiological responses of smooth muscle and brain to agonists (Burgen & Spero, 1970; Richards & Sercombe, 1970), although the inhibition by atropine is not affected (Burgen & Spero, 1970). Also, it has been observed that varying the Ca^{2+} concentration affects the atropine-binding capacity of muscarinic receptors isolated from rat brain, and the maximum binding was found at 0.5 mM- Ca^{2+} (Alberts & Bartfai, 1976).

Since the apparent dissociation constants are comparable with those of the muscarinic receptor when they are measured at physiological concentrations of Ca^{2+} , it may be postulated that the turnover of these lipids is important to the response of the muscarinic receptor. However, the muscarinic-receptor dissociation constants do not appear to be sensitive to Ca^{2+} , nor does the stimulated uptake of

^{32}P into phosphatidylinositol in some other tissues (Trifaro, 1969; Oron *et al.*, 1975). If this sensitivity to Ca^{2+} is found to occur in brain tissues, and not in some other tissues, this would support the evidence that the mechanism of phospholipid turnover in synaptosomes differs from that in other tissues.

This work was supported by Research Grant NIGMS-15904 from the National Institute of General Medical Sciences.

References

- Alberts, P. & Bartfai, T. (1976) *J. Biol. Chem.* **251**, 1543–1547
- Birdsall, N. J. M. & Hulme, E. C. (1976) *J. Neurochem.* **27**, 7–16
- Brossard, M. & Quastel, J. H. (1963) *Can. J. Biochem. Physiol.* **41**, 1243–1256
- Burgen, A. S. V. & Spero, L. (1968) *Br. J. Pharmacol.* **34**, 99–115
- Burgen, A. S. V. & Spero, L. (1970) *Br. J. Pharmacol.* **40**, 492–500
- Burgen, A. S. V., Hiley, C. R. & Young, J. M. (1974) *Br. J. Pharmacol.* **51**, 279–285
- Dawson, R. M. C. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), 2nd edn., pp. 476–508, Oxford University Press, New York and Oxford
- De Robertis, E., Pellegrino de Iraldi, A., Rodriguez de Lores Arnaiz, G. & Salganicoff, L. (1962) *J. Neurochem.* **9**, 23–35
- Farrow, J. T. & O'Brien, R. D. (1973) *Mol. Pharmacol.* **9**, 33–40
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Furchgott, R. F. & Bursztyn, P. (1967) *Ann. N. Y. Acad. Sci.* **144**, 882–899
- Garry, P. J. & Routh, J. I. (1965) *Clin. Chem. (Winston-Salem, N.C.)* **11**, 91–96
- Gray, E. G. & Whittaker, V. P. (1962) *J. Anat.* **96**, 79–88
- Hokin, L. E. (1966) *Biochim. Biophys. Acta* **115**, 219–221
- Hokin-Neaverson, M. (1974) *Biochem. Biophys. Res. Commun.* **58**, 763–768
- Jafferji, S. S. & Michell, R. H. (1976) *Biochem. J.* **154**, 653–657
- Jones, L. M. & Michell, R. H. (1974) *Biochem. J.* **142**, 583–590
- Kates, M. (1972) in *Techniques of Lipidology: Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E. eds.), pp. 347–353, North-Holland/American Elsevier, New York
- Lapetina, E. G. & Michell, R. H. (1974) *J. Neurochem.* **23**, 283–287
- Lapetina, E. G., Brown, W. E. & Michell, R. H. (1976) *J. Neurochem.* **26**, 649–651
- Lolley, R. N. (1963) *J. Neurochem.* **10**, 665–676
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, J. (1951) *J. Biol. Chem.* **193**, 265–275
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Michell, R. H., Jafferji, S. S. & Jones, L. M. (1976) *FEBS Lett.* **69**, 1–5

- Miller, J. C. (1975) in *Progress in Anesthesiology*, vol. 1, *Molecular Mechanisms of Anesthesia* (B. R. Fink, ed.), pp. 439–447, Raven Press, New York
- Neudoerffer, T. S. & Lea, C. H. (1966) *J. Chromatogr.* **21**, 138–140
- Oron, Y., Lowe, M. & Selinger, Z. (1975) *Mol. Pharmacol.* **11**, 79–86
- Paton, W. D. M. (1961) *Proc. R. Soc. London Ser. B* **154**, 21–69
- Pumphrey, A. M. (1969) *Biochem. J.* **112**, 61–70
- Redman, C. M. & Hokin, L. E. (1964) *J. Neurochem.* **11**, 155–163
- Richards, C. D. & Sercombe, R. (1970) *J. Physiol. (London)* **211**, 571–584
- Roscoe, H. G., Goldstein, R., Ricciardi, B. A. & Fahrenbach, M. J. (1970) *Arch. Biochem. Biophys.* **138**, 329–337
- Schacht, J. & Agranoff, B. W. (1972) *J. Biol. Chem.* **247**, 771–777
- Schacht, J. & Agranoff, B. W. (1974) *J. Biol. Chem.* **249**, 1551–1557
- Skipski, V. P., Peterson, R. F., Sanders, J. & Barclay, M. (1963) *J. Lipid Res.* **4**, 227–228
- Trifaro, J. M. (1969) *Mol. Pharmacol.* **5**, 424–427
- Yagihara, Y., Bleasdale, J. E. & Hawthorne, J. N. (1973) *J. Neurochem.* **21**, 173–190
- Yamamura, H. I. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1725–1729