Calcium-dependent polyphosphoinositide hydrolysis is associated with exocytosis in vitro

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Micromolar calcium ions stimulate both exocytosis and polyphosphoinositide hydrolysis in sea urchin egg plasma membrane in vitro. Strontium and barium ions also stimulate both processes equally. Magnesium ions reduce the calcium sensitivity of both. Neomycin, a drug which prevents phosphoinositide hydrolysis, inhibits exocytosis in vitro. We suggest that hydrolysis of plasma membrane phosphoinositides may be an essential step in the fusion of the secretory granule and plasma membranes.

Calcium *Exocytosis* *Phosphoinositide* *Membrane fusion* *Phospholipase C* *Egg*

1. INTRODUCTION

The hydrolysis of plasma membrane polyphosphoinositides occurs in a wide range of eukaryotic cells during secretion [1]. The hydrolysis products act as second messengers [2]. In some cases it appears that hydrolysis of phosphatidylinositol 4,5-phosphate (PtdIns4,5P_2) and phosphatidylinositol 4-phosphate (PtdIns4P) is part of the plasma membrane transduction process and precedes the increase in cytoplasmic calcium which stimulates secretion [3]; indeed, there is evidence that polyphosphoinositide hydrolysis is a necessary precursor of the rise in cytosolic calcium [4–7]. But in other secretory cells, an increased turnover of plasma membrane PtdIns4,5P_2 and PtdIns4P seems to be a consequence rather than a cause of an increase in cytoplasmic calcium [8–12]. We show here that hydrolysis of PtdIns4,5P_2 and PtdIns4P can be stimulated in isolated plasma membrane preparations from sea urchin eggs by addition of physiological calcium concentrations. Moreover, the calcium-dependent hydrolysis of polyphosphoinositides is correlated with the secretory exocytosis which occurs in this preparation on addition of calcium [13–17].

2. MATERIALS AND METHODS

Eggs of *Lytechinus pictus* (Pacific Biomarine, Venice, CA) were labelled in artificial seawater [14] for 2–3 h in the presence of [^{32}P]orthophosphate (200 μCi/ml; 50% (v/v) egg suspension). The jelly coat was removed subsequently by passage through Nitex mesh. The egg cortex was isolated by sticking eggs to cover slides treated with polylysine (0.1 mg/ml) and then shearing away the bulk of the egg with a jet of medium (220 mM potassium glutatamate, 500 mM glycine, 10 mM NaCl, 5 mM $MgCl_2$, 2.5 mM adenosine 5′-triphosphate, 10 mM EGTA, pH 6.7) [14]. In most experiments neomycin (10 mM) was included in the shearing solution and the cortical preparation subsequently washed with neomycin-free medium 30 s after shearing. The washing medium contained varying concentrations of calcium buffered with EGTA [14]. EGTA concentration was 1 mM. The calcium-EGTA ratios and measured free calcium concentrations of the experimental solutions were: 0.340, 0.6 μM; 0.607, 1.9 μM; 0.756, 4.0 μM; 0.838, 5.9 μM; 0.942, 17 μM. After 2 min the aqueous phase was removed and the lipids extracted for chromatography [18]. Chromatograms

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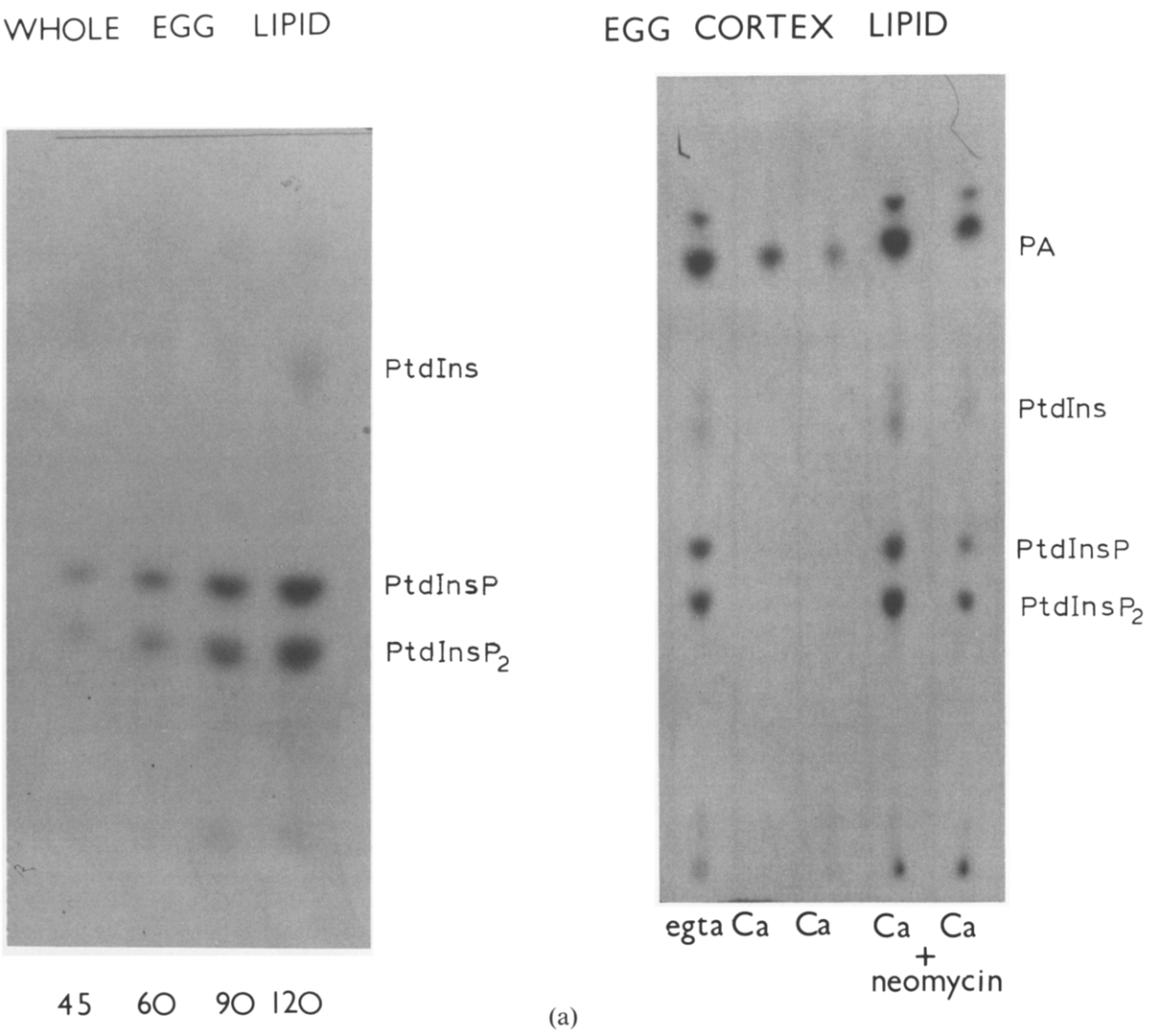

(a)

were developed on oxalate-treated silica gel 60 thin-layer chromatography plates (Merck, Darmstadt) in chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8) [19]. The loss of radioactivity from the spots identified by autoradiography was measured by recovering lipid from the substrate and determining ^{32}P by liquid scintillation counting [20].

The enzymatic activity of the cortical granule glucanohydrolase was measured as glucose production from the hydrolysis of laminarin by the supernatant of a cortical preparation prepared by homogenisation [14].

Exocytosis was also measured as the decrease in intensity of scattered light from a dark field image of the isolated arrays of cortical granules and plasma membrane attached to coverslips with polylysine [21]. Light was measured using a photomultiplier at low gain.

3. RESULTS

Sea urchin eggs incubated with ^{32}P in seawater incorporate label into PtdIns4,5P_2 and PtdIns4P (fig.1a). Plasma membrane fragments of these eggs contain ^{32}P-labelled PtdIns4,5P_2 and PtdIns 4P. Label is lost from the plasma membrane fragments when calcium (10 μM) is added to

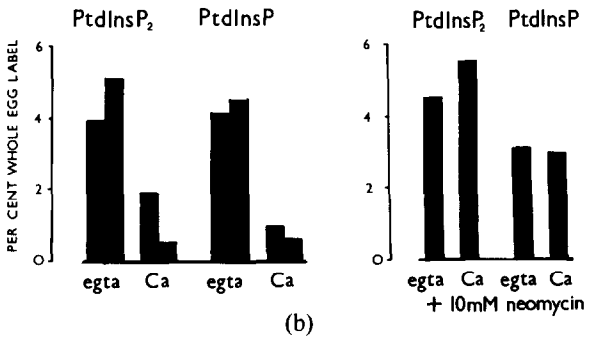

Fig.1. Isolation of egg plasma membrane polyphosphoinositides and their calcium-stimulated hydrolysis. (a) Autoradiochromatograms of ^{32}P-labelled lipid extracts. The chromatogram on the left shows the appearance of label with time in lipid extracts from whole eggs incubated with carrier-free ^{32}P-labelled phosphate (100 μCi/ml). The radioactivity is incorporated predominantly into lipid which runs as PtdIns4,5P_2 and PtdIns4P [20]. The chromatogram on the right shows the pattern of label in lipids extracted from the isolated egg cortex (which consists of secretory granules and plasma membrane [14]). The pattern of labelling is altered by treatment of the cortex with 10 μM calcium before lipid extraction. Radiolabel is lost from the PtdIns4,5P_2 and PtdIns4P spots. The effects of calcium addition on phosphatidic acid (PA) labelling are variable. In the experiment illustrated PA decreases. In other experiments PA labelling was unchanged or increased slightly. Pre-treating the cortex with neomycin (10 mM; Sigma) inhibits the calcium-dependent loss of label. (b) Quantitation of the amounts of ^{32}P from PtdIns4,5P_2 and PtdIns4P in the egg cortex. A representative experiment is shown. (i) Treatment with 10 μM calcium causes a loss of four-fifths of the radioactivity in PtdIns4P and PtdIns4,5P_2. (ii) The loss of radiolabel is prevented by pre-treatment with 10 mM neomycin. The radioactivity incorporated into PtdIns4,5P_2 and PtdIns4P measured in this way is expressed in these and other figures as a percentage of the radioactivity in PtdIns4,5P_2 and PtdIns4P in 2 μl of packed eggs. We have determined by labelling egg surface proteins with fluorescein isothiocyanate that the amount of egg cortex we use for each experimental point corresponds to the cortex of 0.14 μl of packed eggs; 16°C.

the cytoplasmic face. The calcium-dependent loss of ^{32}P label from the plasma membrane polyphosphoinositide is prevented by neomycin, a drug known to prevent hydrolysis of PtdIns4,5P_2 and PtdIns4P (fig.1a,b). Loss of ^{32}P label from the polyphosphoinositide does not in itself imply hydrolysis at the glycerol phosphate ester linkage: it is well-established that phosphomonoesterase activity can result in dephosphorylation of the hexose ring [22]. However, eggs labelled with [^{3}H]inositol, permeabilised and treated with micromolar calcium release a compound which behaves identically to inositol trisphosphate (InsP_3) on anion exchange chromatography (not shown). We therefore conclude that physiological calcium concentrations stimulate a phospholipase C activity which hydrolysis PtdIns4,5P_2 in the plasma membrane fragments to InsP_3 and diacylglycerol.

The phospholipase C activity is half-maximally stimulated at around 5 μM free calcium in the presence of 2.5 mM free Mg^{2+} (fig.2). We determined the calcium requirement of secretory exocytosis in these membrane fragments under identical conditions by measuring the decrease in light scattered by the cortical granules as they underwent exocytosis. Calcium stimulated a half-maximal decrease in light scattering at 3 μM, the concentration at which it stimulates half-maximal release of a glucanase, an enzyme which is released by the cortical granules when they undergo exocytosis. Fig.2 shows that polyphosphoinositide hydrolysis and exocytosis occur in vitro over the same range of calcium concentrations.

The calcium-stimulated exocytosis of sea urchin egg cortical granules is inhibited in vitro by magnesium. Increasing the magnesium ion concentration increases the free calcium concentration required for half-maximal stimulation of exocytosis [14]. Fig.3a shows that increasing the magnesium ion concentration also increases the calcium concentration required for half-maximal stimulation of PtdIns4,5P_2 hydrolysis. Again, the concentrations of calcium required to stimulate the two processes are quantitatively very similar (half-maximum concentration for PtdIns4,5P_2 hydrolysis, 27 μM; for exocytosis, 32 μM). In contrast to magnesium ions, strontium and barium ions stimulate exocytosis in vitro. The correlation between the strontium ion concentrations required to stimulate exocytosis and those required to stimulate PtdIns 4,5P_2 hydrolysis is shown in fig.3b. Similarly, barium stimulates both PtdIns4,5P_2 hydrolysis and exocytosis at concentrations above 10 mM under these conditions. Hydrolysis of PtdIns4P

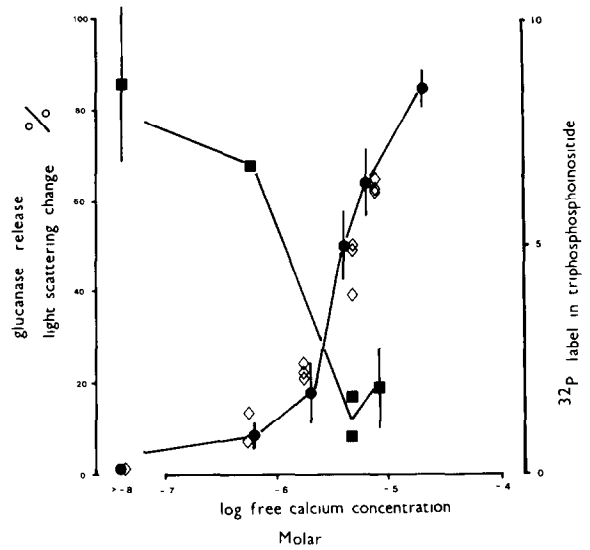

Fig.2. The calcium dependence of TPI hydrolysis (■) and cortical granule exocytosis (●, ◇) in isolated egg cortex. PtdIns4,5P$_2$ associated with the cortex in low calcium medium (free calcium $< 10^{-8}$ M) is 8.6 ± 2.09 (mean ± SE, $n = 9$); PtdIns4,5P$_2$ associated with the cortex after treatment with 10 μM calcium is 1.9 ± 0.81 (mean ± SE, $n = 8$). Cortical granule exocytosis was measured under identical conditions in separate experiments by measuring the decrease in light scattering from a dark field image of the isolated cortices (◇). The maximal decrease in light scattering was measured at the end of each experiment by adding 10 mM calcium-containing medium to the preparation at the end of each experiment. The calcium sensitivity of the decrease in light scattering agrees well with the calcium sensitivity of the release of an enzyme (a β-1,3-glucanohydrolase) from the granule interior (●). Bars are SE, $n = 8$, 16°C.

was similarly affected by divalent cations (not shown).

The similarity of the divalent cation requirements of polyphosphoinositide hydrolysis and exocytosis suggests the possibility of a causal connection. The data of fig.4 support the idea of a causal link between these two calcium-dependent phenomena. Neomycin, a drug which inhibits polyphosphoinositide hydrolysis [23–25], inhibits cortical granule exocytosis in vitro over the same range of concentrations. Neomycin has been shown to inhibit phosphoinositide hydrolysis both in intact synaptosomes [25] and in homogenates [23,24]. As a polar molecule it would not be expected to enter cells readily and its external action has been attributed to inhibition of a synaptosomal calcium

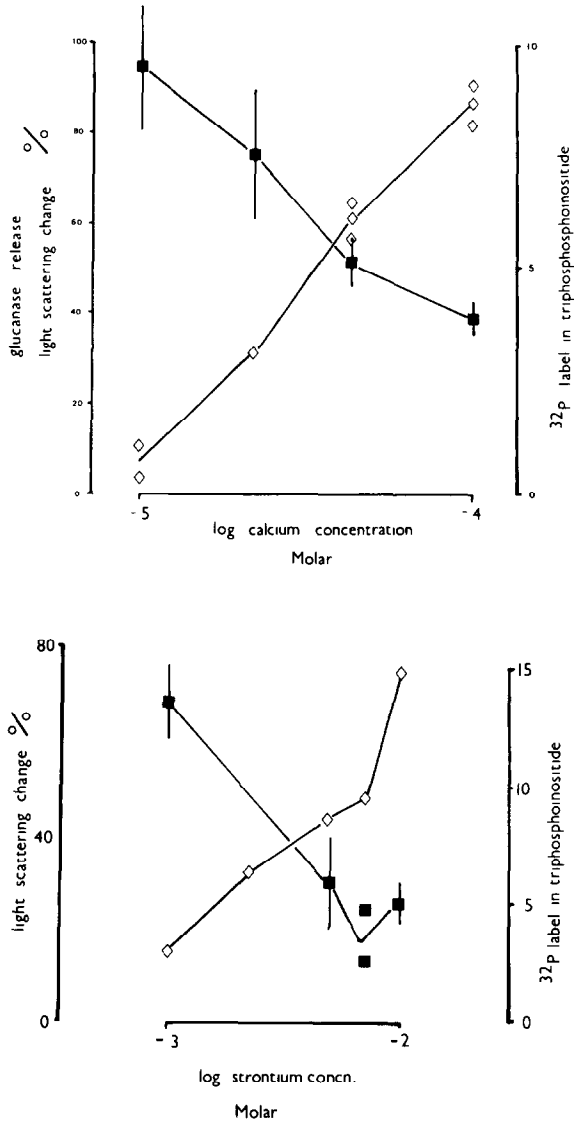

Fig.3. (a) The calcium dependence of PtdIns4,5P$_2$ hydrolysis (■) and exocytosis (◇) in the presence of 50 mM magnesium ions. (b) PtdInsP$_2$ hydrolysis and exocytosis induced by strontium in the presence of 50 mM magnesium. The bars represent the SE; $n = 6,4,6$ in ascending order of concentration. Under these conditions barium ions induced both exocytosis and PtdIns4,5P$_2$ hydrolysis at concentrations between 10 and 100 mM. 10 mM barium-containing solutions cause no significant decrease in light scattering and PtdIns4,5P$_2$ associated with the egg cortex is 14.1%; 100 mM barium-containing solutions cause a 70% reduction of light scattering by the egg cortex and PtdIns4,5P$_2$ associated with the egg cortex under these conditions is 3.4%, 16°C.

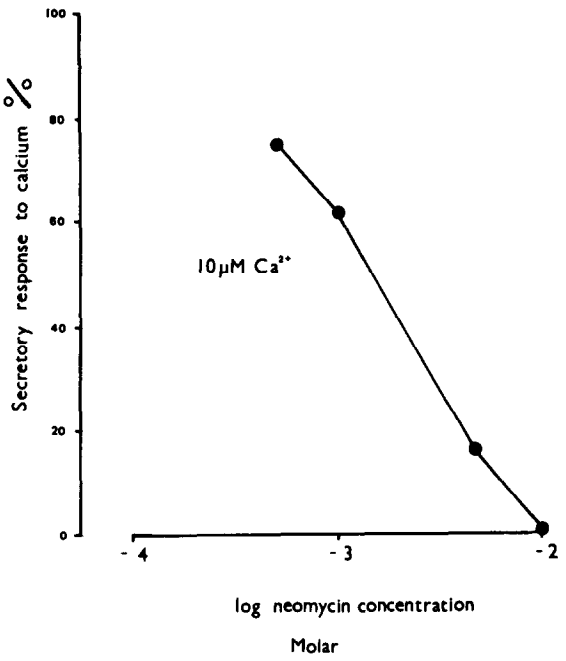

Fig.4. Inhibition of calcium-dependent exocytosis by neomycin. Neomycin was present for 5 min prior to the addition of calcium and included in the calcium-containing solutions. The presence of neomycin did not significantly affect the free calcium concentration of the solution measured with a calcium-selective electrode. The extent of exocytosis in vitro was measured as a decrease in light scattering, 16°C.

channel [25]. Neomycin is effective in inhibiting exocytosis only when applied to the cytoplasmic face of the egg plasma membrane. Gentamycin, a related polycation antibiotic, binds specifically to PtdIns (4,5)P_2: the intrinsic association constant is two orders of magnitude greater than for binding to phosphatidylserine or PtdIns ([26] and S. Maclaughlin, personal communication). Phosphoinositide hydrolysis is also inhibited by polylysine [23], though not at the concentrations we use to attach eggs to coverslides when preparing cortices. We have chosen a concentration (0.01 mg/ml) which promotes good adhesion but causes no inhibition. Inhibition does occur at higher concentrations.

4. DISCUSSION

Our findings raise three main points of interest. First is the evidence for the existence of a plasma membrane-associated phospholipase C activity in sea urchin eggs which is responsive to calcium in the micromolar range, concentrations which are attained at fertilisation. Elsewhere, phospholipase C activities have been described whose action is dependent on calcium but insensitive to the variations in calcium concentration which are thought to occur within the cell [22,27–29].

The second point concerns the mechanism of egg activation [30]. There are two primary triggers to development in sea urchin eggs, a transient increase in cytoplasmic calcium and a sustained increase in intracellular pH [31]. The increase in cytoplasmic pH is due to the stimulation of a sodium-hydrogen exchanger in the egg plasma membrane at fertilisation [32]. The exchanger is stimulated by the transient increase in free calcium concentration. It is also stimulated by the tumour promoter, phorbol myristate acetate (PMA) [33]. PMA and diacylglycerol act at similar sites [34], suggesting that calcium stimulates the sodium-hydrogen exchange indirectly by causing PtdIns 4,5P_2 hydrolysis to produce diacylglycerol. The other hydrolysis product, InsP_3, causes calcium release from the egg's intracellular calcium store [35], suggesting that a regenerative mechanism involving calcium release and polyphosphoinositide hydrolysis may occur at fertilisation.

The third point raised by our data concerns the mechanism of secretory granule plasma membrane fusion. We have shown that preventing polyphosphoinositide hydrolysis prevents exocytosis. One plausible explanation of this observation is that inhibiting polyphosphoinositide hydrolysis prevents the formation of diacylglycerol, a potent fusogen [36,37] which may promote fusion of the secretory granule and plasma membrane [38,39]. A second and equally important factor in promoting fusion may be the loss of the multiply charged inositol phosphates from the surface of the lipid bilayer. Rand and Parsegian have shown that the main energy barrier to the close apposition of lipid bilayers is the energy required to remove the water molecules from polar lipid head groups [40–42]. Another possibility, that phosphoinositide hydrolysis stimulates exocytosis by activating protein kinase C [34], seems unlikely because neither TPA nor oleylacetylglycerol stimulate exocytosis nor affect its sensitivity to calcium when applied directly

to the cytoplasmic face of the isolated egg cortex. Inositol lipid breakdown is correlated with exocytotic secretion in a variety of secretory tissues [2]. It has been demonstrated to be a consequence of an increase in cytoplasmic calcium in neutrophils [9,10], endocrine pancreas [11], brain synaptosomes [8] and in adrenal medullary cells [43], and occurs when myoblasts fuse [44]. Our present results suggest that the hydrolysis of polyphosphoinositides may be a crucial step in the process of calcium-dependent membrane fusion.

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REFERENCES

[1] Michell, R.H. (1979) Trends Biochem. Sci. 8, 263–265.
[2] Berridge, M.J. (1984) Biochem. J. 220, 345–360.
[3] Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) Phil. Trans. R. Soc. Ser. B 296, 123–137.
[4] Fain, J.N. and Berridge, M.J. (1979) Biochem. J. 178, 45–58.
[5] Berridge, M.J. and Fain, J.N. (1979) Biochem. J. 178, 59–69.
[6] Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482.
[7] Berridge, M.J. (1983) Biochem. J. 212, 849–858.
[8] Griffin, H.D. and Hawthorne, J.N. (1978) Biochem. J. 176, 541–552.
[9] Cockcroft, S., Bennett, J.P. and Gomperts, B.D. (1980) Nature 288, 275–277.
[10] Cockcroft, S., Bennett, J.P. and Gomperts, B.D. (1981) Biochem. J. 200, 501–508.
[11] Laychok, S.G. (1983) Biochem. J. 216, 101–106.
[12] Axen, K.V., Schubart, U.K., Blake, A.D. and Fleischer, N. (1983) J. Clin. Invest. 72, 13–21.
[13] Baker, P.F. and Whitaker, M.J. (1978) Nature 276, 513–515.
[14] Whitaker, M.J. and Baker, P.F. (1983) Proc. R. Soc. Lond. Ser. B, 218, 397–413.
[15] Haggerty, J.G. and Jackson, R.C. (1983) J. Biol. Chem. 258, 1819–1825.
[16] Sasaki, H. and Epel, D. (1983) Dev. Biol. 98, 327–337.
[17] Moy, G.W., Kopf, G.S., Gache, C. and Vacquier, V.D. (1983) Dev. Biol. 100, 267–274.
[18] Seiffert, U.B. and Agranoff, B.W. (1964) Biochim. Biophys. Acta 98, 574–581.
[19] Jolles, J., Zwiers, H., Dekker, A., Wurtz, K.W.A. and Gispen, W.H. (1981) Biochem. J. 214, 77–82.
[20] Allan, D. and Cockroft, S. (1983) Biochem. J. 213, 555–557.
[21] Zimmerberg, J. (1984) Biophys. J. 45, 88A.
[22] Irvine, R.F., Letcher, A.J. and Dawson, R.M.C. (1984) Biochem. J. 218, 177–185.
[23] Schacht, J. (1976) J. Neurochem. 27, 1119–1124.
[24] Schibechi, A. and Schacht, J. (1977) Biochem. Pharmacol. 26, 1769–1774.
[25] Das, S. and Rand, R.P. (1984) Biochem. Biophys. Res. Commun., in press.
[26] Chung, L., Kaloyanides, G., McDaniel, R., Maclaughlin, A. and Maclaughlin, S. (1985) Biochemistry, in press.
[27] Irvine, R.F. (1982) Cell Calcium 3, 295–309.
[28] Downes, C.P. and Michell, R.H. (1982) Biochem. J. 202, 53–58.
[29] Cockcroft, S., Baldwin, J.M. and Allen, D. (1984) Biochem. J. 221, 477–482.
[30] Turner, P.R., Sheetz, M.P. and Jaffe, L.A. (1984) Nature (Lond.) 310, 414–415.
[31] Whitaker, M.J. and Steinhardt, R.A. (1982) Q. Rev. Biophys. 15, 593–664.
[32] Payan, P., Girard, J.-P. and Ciapa, B. (1983) Dev. Biol. 100, 29–38.
[33] Swann, K. and Whitaker, M.J. (1984) Nature, in press.
[34] Nishizuka, Y. (1983) Phil. Trans. R. Soc. Lond. Ser B, 302, 101–112.
[35] Whitaker, M.J. and Irvine, R.F. (1984) Nature 312, 636–639.
[36] Ohki, K., Sekiya, T., Yamauchi, T. and Nozawa, Y. (1982) Biochim. Biophys. Acta 693, 341–350.
[37] Das, S. and Rand, R.P. (1984) Biochem. Biophys. Res. Commun., in press.
[38] Allan, D. and Michell, R.H. (1975) Nature (Lond.) 258, 343–348.
[39] Hawthorne, J.N. and Pickard, M.R. (1979) J. Neurochem. 32, 5–14.
[40] Le Neveu, D.M., Rand, R.P. and Parsegian, V.A. (1976) Nature 259, 601–603.
[41] Parsegian, V.A. (1977) in: Soc. Neurosci. Symp.: Approaches to the Cell Biology of Neurons (Cowan, W.M. and Ferrarelli, J.A. eds) vol. 2, pp. 162–171.
[42] Rand, R.P. (1981) Ann. Rev. Biophys. Bioeng. 10, 277–314.
[43] Whitaker, M.J., in preparation.
[44] Wakelam, M.J.O. (1983) Biochem. J. 214, 77–82; (1981) Biochem. J. 194, 283–291.