PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Biochemical Approaches to the Study of Cytosolic Calcium Regulation in Nerve Endings [and Discussion]

D. G. Nicholls, K. E. O. Akerman, J. R. Garrett and P. F. Baker

Phil. Trans. R. Soc. Lond. B 1981 **296**, 115-122
doi: 10.1098/rstb.1981.0176

Phil. Trans. Soc. Lond. B **296**, 115–122 (1981) [115]
Printed in Great Britain

Biochemical approaches to the study of cytosolic calcium regulation in nerve endings

By D. G. Nicholls and K. E. O. Åkerman
Neurochemistry Laboratory, Department of Psychiatry, University of Dundee, Dundee DD1 4HN, U.K.

The nerve ending cytosol is bounded by the plasma membrane, the mitochondrial inner membrane and the endoplasmic reticulum membrane, transport across each of which is capable, in theory, of regulating the cytosolic free Ca^{2+} concentration. By parallel monitoring of mitochondrial and plasma membrane potentials, ATP levels, Na^+ gradients and intrasynaptosomal Ca^{2+} distribution in preparations of isolated synaptosomes, we conclude the following: (*a*) mitochondria *in situ* represent a major Ca^{2+} pool, regulating the upper steady-state limit of the cytosolic free Ca^{2+} concentration by sequestering Ca^{2+} reversibly; (*b*) this limit is responsive to the cytosolic Na^+ concentration, but is below the concentration required for significant exocytosis; (*c*) plasma membrane Ca^{2+} transport can be resolved into a constant slow influx, a voltage-dependent and verapamil-sensitive influx and an ATP-dependent efflux, while Ca^{2+} efflux driven by the sodium electrochemical potential cannot be detected; (*d*) Ca^{2+} regulation by intrasynaptosomal endoplasmic reticulum appears to be of minor significance in the present preparation.

Introduction

In neuronal tissue the plasma membrane, mitochondrial inner membrane and endoplasmic reticulum have each been implicated in the transport of Ca^{2+}, and hence in the regulation of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}_c]$). Since each of these membranes may possess more than one Ca^{2+} transport mechanism, the potential complexity of neuronal $[Ca^{2+}_c]$ regulation is considerable (figure 1). The two preparations that have been investigated most intensively in this context are the squid giant axon (for reviews see Baker (1978) and Requena & Mullins (1979)) and the mammalian synaptosome (Blaustein *et al.* 1978*a*, *b*). By virtue of its small size, the synaptosome has an approximately 1000-fold greater surface:volume ratio than the giant axon. While this allows relatively large ion fluxes to be measured, distinctive experimental techniques are required. In this paper we discuss the usefulness and limitations of methodologies derived from mitochondrial and hepatocyte studies when applied to the guinea-pig cerebral cortical synaptosome (Scott & Nicholls 1980; Scott *et al.* 1980; Åkerman & Nicholls 1981*a*, *b*, *c*).

Ca^{2+} transport by isolated cerebral cortical mitochondria

An understanding of the bioenergetic and Ca^{2+} transporting properties of isolated brain mitochondria is an aid to the interpretation of the complexities of the intact synaptosome. Mitochondria from non-synaptosomal fractions of the brain have properties similar to those prepared by synaptosomal fractionation (Nicholls 1978*a*). In accordance with the chemiosmotic theory (for review see Mitchell 1976), respiring brain mitochondria maintain a membrane potential ($\Delta\Psi_m$) in the region of 150 mV (Nicholls 1978; Nicholls & Scott 1980). This potential

drives the uptake of Ca^{2+} by a uniport mechanism (for review see Nicholls & Crompton 1980) whose activity is highly dependent upon the external free Ca^{2+} concentration (Nicholls & Scott 1980). An independent efflux pathway exists which probably operates by Na^+–Ca^{2+} exchange, and whose activity is essentially independent of the total matrix Ca^{2+} content. The distinctive kinetics of uptake and efflux allow the mitochondria to buffer selectively the free Ca^{2+} concentration in the medium. The precise Ca^{2+} concentration at which a kinetic balance between uptake and efflux is obtained can be varied over the range 0.3–3 μM by altering the kinetics of the two pathways (Nicholls & Scott 1980).

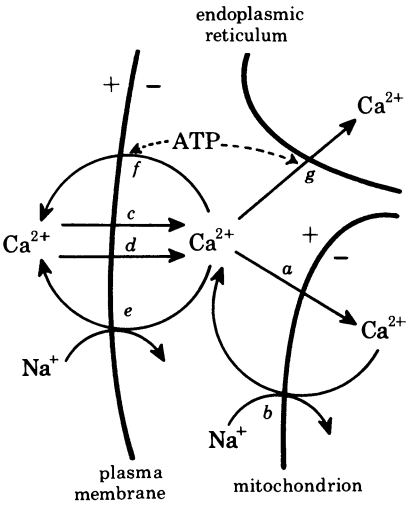

FIGURE 1. Possible pathways of Ca^{2+} transport in synaptosomes. Mitochondrial pathways: (*a*) uniporter; (*b*) Na^+–Ca^{2+} exchange. Plasma membrane pathways: (*c*) constant influx; (*d*) voltage-dependent influx; (*e*) Na^+–Ca^{2+} exchange; (*f*) Ca^{2+}-ATPase. Endoplasmic reticulum pathway; (*g*) Ca^{2+}-ATPase.

Although mitochondrial buffering is bidirectional – an increase in external Ca^{2+} concentration leads to a net uptake into the matrix, while a decrease results in a net loss – the maximal rates are very different. Net Ca^{2+} uptake when the external Ca^{2+} concentration is increased to 10 μM can be as rapid as 400 nmol Ca^{2+} min^{-1} mg^{-1} mitochondrial protein (Nicholls 1978), while maximal efflux does not exceed 10 nmol min^{-1} (Nicholls & Scott 1980). This suggests that the mitochondria *in situ* would be most effective in taking up Ca^{2+} after a temporary elevation in $[Ca^{2+}_c]$. Indeed, a crude calculation shows that, if the activity coefficient of cytosolic Ca^{2+} is 0.05, as it appears to be in the squid giant axon (Brinley *et al.* 1977), an instantaneous entry across the plasma membrane of 0.6 nmol Ca^{2+} mg^{-1} synaptosomal protein would raise $[Ca^{2+}_c]$ to a uniform 10 μM; after this the *in situ* mitochondria, amount to 10% of synaptosomal protein, would be capable of taking up this excess Ca^{2+} with a half time of about 2 s.

THE BIOENERGETICS OF INTACT SYNAPTOSOMES

The multiple pathways of Ca^{2+} transport in intact synaptosomes are influenced variously by ATP, by internal Na^+, by plasma membrane potential ($\Delta\Psi_p$) and by mitochondrial membrane potential ($\Delta\Psi_m$) (see figure 1). It is therefore important to monitor each of these parameters. As an example of the information that such determinations can yield, figure 2 compares the effects of rotenone (which inhibits respiration), rotenone and oligomycin together (which

additionally inhibits the mitochondrial ATP synthetase), and ouabain and veratridine together (which respectively inhibit the $(Na^+ + K^+)$ATPase and activate the plasma membrane Na^+ channels). Inhibition of respiration alone only depletes ATP partially since glycolysis is active. There is therefore only a slight decrease in $\Delta\Psi_m$ since glycolytic ATP can cause the mitochondrial ATP synthetase to reverse, pump out protons and maintain a significant potential. With rotenone and oligomycin together, this is no longer possible and $\Delta\Psi_m$ collapses completely. In neither case is $\Delta\Psi_p$ affected, since glycolytic ATP is adequate for the needs of the plasma membrane $(Na^+ + K^+)$ATPase (Scott & Nicholls 1980). Specific collapse of $\Delta\Psi_p$ can be induced by veratridine and ouabain together. There is little effect on $\Delta\Psi_m$ or ATP concentration, but a considerable increase in internal Na^+ concentration (Åkerman & Nicholls 1981*c*).

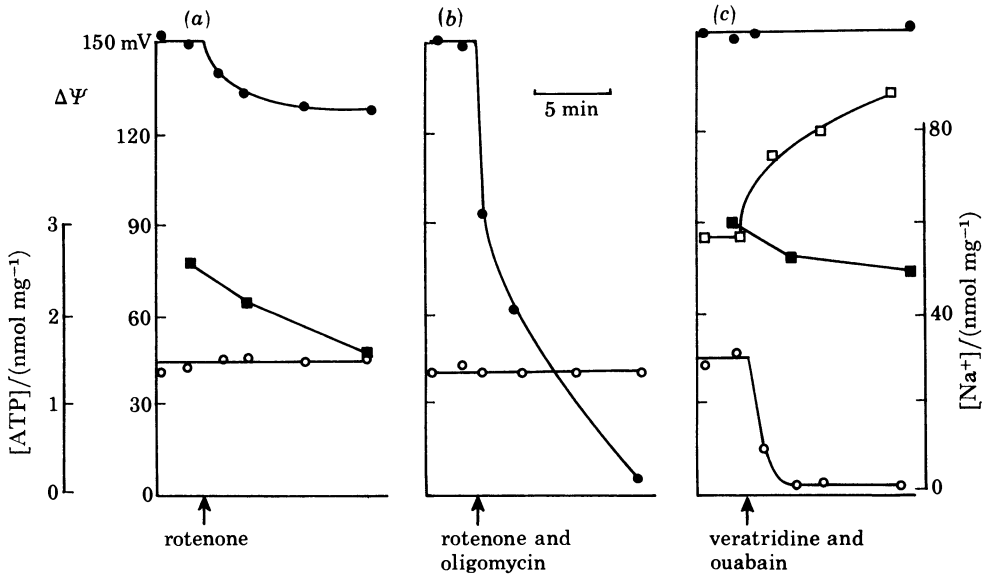

FIGURE 2. Plasma membrane potential ($\Delta\Psi_p$), *in situ* mitochondrial membrane potential($\Delta\Psi_m$), ATP content and Na^+ content of synaptosomes during specific depolarization of plasma or mitochondrial membrane potentials. $\Delta\Psi_p$ and $\Delta\Psi_m$ were estimated simultaneously from the relative accumulation of $^{86}Rb^+$ and [^{3}H]triphenylmethylphosphonium cation. Na^+ was assayed by using $^{22}Na^+$ after displacement of superficial Na^+ with Li^+. For details see Scott & Nicholls (1980) and Åkerman & Nicholls (1981*c*). ●, $\Delta\Psi_m$; ○, $\Delta\Psi_p$; ■, [ATP]; □, [Na^+].

INTRASYNAPTOSOMAL Ca^{2+} COMPARTMENTS

We have investigated three pools of synaptosomal Ca^{2+}: superficial Ca^{2+} bound to the outer surface of the plasma membrane, Ca^{2+} within the matrices of the *in situ* mitochondria, and Ca^{2+} present in non-mitochondrial compartments within the synaptosome (Scott *et al.* 1980; Åkerman & Nicholls 1981*b*). Superficial Ca^{2+} amounts to about 10 nmol mg^{-1} synaptosomal protein and may be removed specifically by chelation with EGTA (Blaustein 1975) followed by immediate centrifugation through silicone oil to forestall efflux from the cytosol as an artefact (Scott *et al.* 1980).

The most direct method to apportion the transported Ca^{2+} between mitochondrial and non-mitochondrial compartments is adapted from hepatocyte studies (Zuurendonk & Tager 1974) and relies on the selective disruption of the plasma membrane by digitonin followed by rapid centrifugation through silicone oil (Scott *et al.* 1980). The uptake of Ca^{2+} into the

mitochondria as an artefact after disruption is prevented by the presence of ruthenium red and EGTA, and the entire process of disruption and separation is complete within 30 s. When both plasma and mitochondrial membranes are polarized, the pellet Ca^{2+} at steady state averaged 2.5 nmol mg^{-1} protein, while the digitonin-releasable pool amounted to 3.3 nmol Ca^{2+} mg^{-1} protein (Scott *et al.* 1980). The pellet Ca^{2+} could be unequivocally identified with the mitochondrial pool as the result of control experiments in which rotenone and oligomycin together (which abolish $\Delta\Psi_m$ and decrease ATP; see figure 2) decreased the pellet Ca^{2+} content by 84% whereas oligomycin alone (which decreases ATP without affecting $\Delta\Psi_m$) caused no decrease in pellet Ca^{2+} content after disruption. This eliminated the possibility that the decrease in ATP was preventing Ca^{2+} uptake into digitonin-resistant non-mitochondrial organelles.

Specific depolarization of the plasma membrane by either veratridine plus ouabain or by high $[K^+]$ increases synaptosomal Ca^{2+} content (Blaustein 1975). In the present preparation total synaptosomal Ca^{2+} content doubled relative to the control after 20 min in high $[K^+]$ medium; 85% of this increased Ca^{2+} was found by subsequent digitonin fractionation to have been further transported into the *in situ* mitochondria (Åkerman & Nicholls 1981*b*). Mitochondrial uptake was less significant after depolarization induced by veratridine and ouabain together, probably because the consequent increase in cytosolic $[Na^+]$ (figure 2) increased the activity of the mitochondrial efflux pathway.

The other methods that we have employed to quantitate the mitochondrial Ca^{2+} pool are less direct, relying upon the assumption that the non-mitochondrial pool will revert to the same size if mitochondrial Ca^{2+} accumulation is abolished (Åkerman & Nicholls 1981*b*). Abolition of $\Delta\Psi_m$ before Ca^{2+} addition by the combination of rotenone and oligomycin decreased the steady-state synaptosomal Ca^{2+} content by 40% in both control and veratridine–ouabain-depolarized synaptosomes, and by 60% for $[K^+]$ depolarization; these decreases can be equated with the loss of the mitochondrial pool. In a related experiment (figure 3), $\Delta\Psi_m$ was abolished after Ca^{2+} accumulation had attained a steady state, by the addition of oligomycin with a proton translocator FCCP. The net efflux of Ca^{2+} from the synaptosomes again correlated with the expected magnitude of the matrix compartment.

The conclusion from these studies is that mitochondria represent a major pool of synaptosomal Ca^{2+}, and that the pool size is responsive to metabolic alterations *in situ*.

Ca^{2+} TRANSPORT ACROSS THE PLASMA MEMBRANE

When synaptosomes are exposed to 1.3 mM Ca^{2+} after preincubation in a Ca^{2+}-free medium there is a net Ca^{2+} uptake at about 0.2 nmol min^{-1} mg^{-1} under polarized conditions, which approaches a steady state after 30 min (figure 4). This net influx is unaffected by verapamil, an inhibitor of the voltage-sensitive Ca^{2+} channel, or by omission of Na^+ from the incubation medium. Superimposed upon this influx is a voltage-dependent pathway that results in the rapid uptake of an additional 4 nmol Ca^{2+} mg^{-1} when the plasma membrane is depolarized by either high $[K^+]$ or veratridine and ouabain together. Although verapamil does not prevent depolarization of the plasma membrane by these agents it completely inhibits the incremental uptake of Ca^{2+} (figure 4). Since veratridine activates Na^+ channels while ouabain inhibits Na^+ extrusion, this combination collapses the sodium electrochemical gradient across the plasma membrane, both by lowering $\Delta\Psi_p$ and by increasing internal $[Na^+]$ (figure 2). This action has no effect on plasma membrane Ca^{2+} transport in the presence of verapamil, showing that no

detectable Ca^{2+} efflux in exchange for Na^+ was occurring before addition of the inhibitors, as this would reverse when the sodium electrochemical potential was collapsed (Åkerman & Nicholls 1981*c*).

The mitochondrial membrane potential remains high after plasma membrane depolarization in the presence of Ca^{2+} (figure 2). Under steady-state conditions a high $\Delta\Psi_m$ implies a low $[Ca_c^{2+}]$. It follows that some mechanism other than the inoperative Na^+–Ca^{2+} exchange must exist in order to extrude Ca^{2+} from the synaptosome. One possibility is a Ca^{2+}-ATPase at the plasma membrane, for which evidence has been obtained in synaptosomal plasma membrane

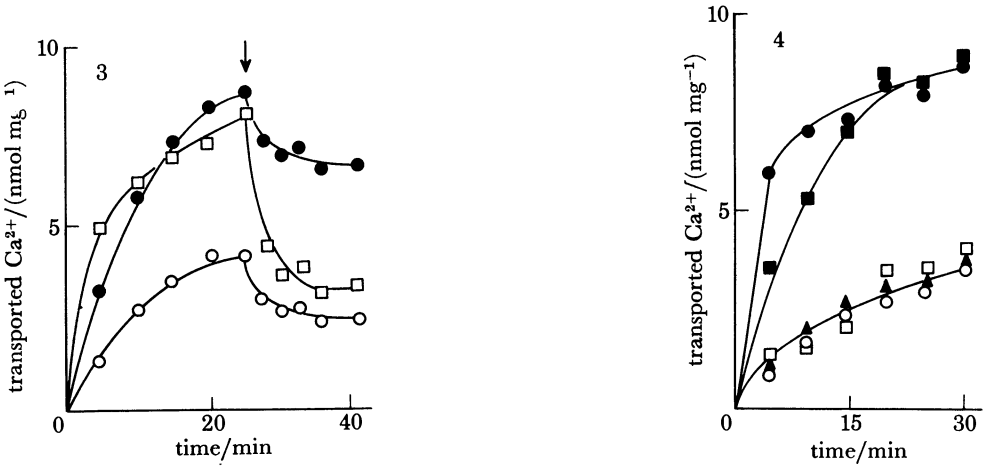

FIGURE 3. Ca^{2+} release from synaptosomes induced by abolition of $\Delta\Psi_m$. Synaptosomes were incubated with $^{45}Ca^{2+}$. ○, Plasma membrane polarized; □, plasma membrane depolarized by 48 mM K^+; ●, plasma membrane depolarized by veratridine and ouabain together. At the arrow the proton translocator FCCP was added together with oligomycin. For details see Åkerman & Nicholls (1981*c*).

FIGURE 4. The effect of verapamil on depolarization-induced Ca^{2+} uptake. Synaptosomes were incubated with $^{45}Ca^{2+}$. ▲, Plasma membrane polarized; ○, ●, plasma membrane depolarized by 40 mM K^+; □, ■, plasma membrane depolarized by veratridine plus ouabain. Filled symbols, verapamil absent; open symbols, verapamil present. For details see Åkerman & Nicholls (1981*c*).

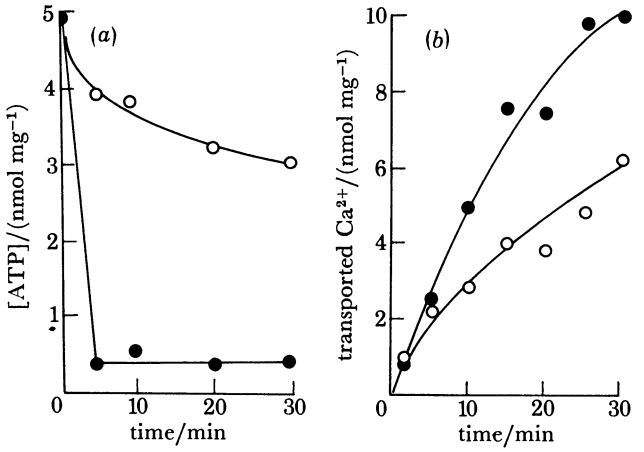

FIGURE 5. The effect of ATP depletion on net Ca^{2+} uptake. Synaptosomes were incubated with $^{45}Ca^{2+}$ in the presence of ouabain and FCCP. ○, Oligomycin additionally present; ●, iodoacetate additionally present.

preparations (Sobue *et al.* 1979). To obtain functional evidence for ATP-dependent Ca^{2+} extrusion, we have depleted synaptosomal ATP concentrations to less than 10% of controls by the combination of iodoacetate to inhibit glycolysis and FCCP to collapse $\Delta\Psi_m$, the latter allowing the mitochondrial ATP synthetase to reverse and hydrolyse residual ATP (figure 5). Relative to controls in which ATP concentrations were largely maintained, there was a significant increase in the rate and extent of synaptosomal Ca^{2+} accumulation, which suggests the inhibition, through lack of ATP, of a Ca^{2+} extrusion mechanism. The presence of ouabain eliminated any effect due to the $(Na^+ + K^+)$ATPase. It is significant that ATP depletion increases uptake, since the opposite would be predicted if the Ca^{2+}-ATPase proposed to accumulate cytosolic Ca^{2+} into endoplasmic reticulum (Blaustein *et al.* 1978*a*, *b*) were of major importance.

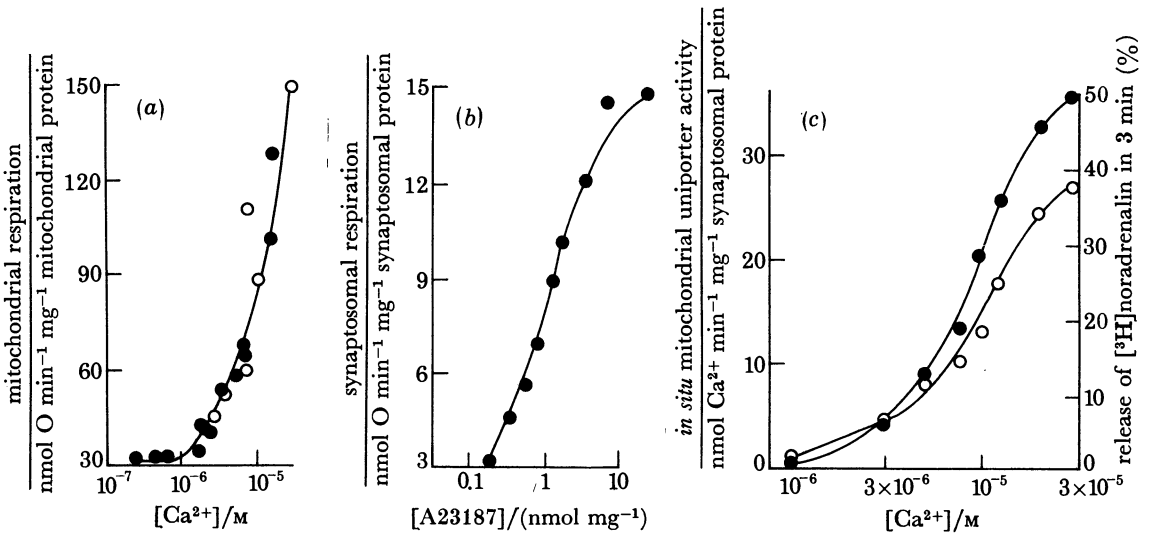

FIGURE 6. (*a*) Respiration of isolated brain mitochondria in the presence of ionophore A23187 as a function of $[Ca^{2+}]$. A23187 was present at 0.3 μM (●) or 6 μM (○). Ca^{2+} concentrations were established with nitrilotriacetate or *N*-hydroxyethylenediamine triacetate as Ca^{2+} chelators. (*b*) Respiration of intact synaptosomes as a function of A23187 concentration. Synaptosomes were incubated in the presence of varying concentrations of A23187. (*c*) *In situ* mitochondrial Ca^{2+} uniporter activity and release of [^{3}H]noradrenalin as a function of $[Ca^{2+}_c]$. Synaptosomes prelabelled with [^{3}H]noradrenalin were incubated with various concentrations of A23187. Respiratory stimulation and noradrenalin release were determined. The calibration of (*a*) was used to convert respiratory stimulation to $[Ca^{2+}_c]$. Mitochondrial uniporter activity was calculated on the basis that an increment of 1 nmol O min^{-1} mg^{-1} corresponded to an increase of 3 nmol Ca^{2+} min^{-1} mg^{-1} in the rate of Ca^{2+} cycling across the inner membrane of the *in situ* mitochondria. ●, Uniporter activity; ○, noradrenalin release.

Ca^{2+} TRANSPORT IN RELATION TO EXOCYTOSIS

Although *in situ* mitochondria clearly play a major role in the regulation of Ca^{2+} concentration in isolated synaptosomes, the possibility exists that this may merely represent a pathological response to a $[Ca^{2+}_c]$ abnormally elevated as a resulted of damage to the plasma membrane during isolation. If it is accepted that the Ca^{2+}-dependent exocytosis of neurotransmitters from synaptosomes has physiological relevance, then it is of interest to see whether *in situ* mitochondria are significant in Ca^{2+} buffering over the range required for exocytosis.

Ionophore A23187, a Ca^{2+}–$2H^+$ exchanger, provides an approach to this problem, while at the same time allowing an estimate to be made of the $[Ca^{2+}_c]$ necessary to induce exocytosis.

A23187 acts at two loci in synaptosomes, the plasma membrane, where it induces a concentration-dependent influx (Åkerman & Nicholls 1981*a*), and the mitochondrial inner membrane, where it induces an artificial Ca^{2+} efflux pathway from the matrix. This latter, in concert with the native mitochondrial Ca^{2+} uniporter induces a dissipative Ca^{2+} cycling, which lowers $\Delta\Psi_m$ and increases respiration. The rate of cycling, and hence the extent of respiration, depends only on $[Ca_c^{2+}]$. If a range of A23187 concentrations are presented to synaptosomes that have previously been allowed to take up [^{3}H]noradrenalin, the ionophore will induce a concentration-dependent increase in $[Ca_c^{2+}]$ due to plasma membrane Ca^{2+} influx; secondly, this increase in $[Ca_c^{2+}]$ will induce exocytosis of noradrenalin; thirdly, the increase in $[Ca_c^{2+}]$ can be monitored as an increase in respiration. To convert the respiratory increase into a Ca^{2+} concentration, a calibration is performed with isolated brain mitochondria. Figure 6 shows the result of such an experiment. In figure 6*a* the calibration with isolated mitochondria is shown; note that the stimulation is independent of ionophore concentration. In figure 6*b* the respiratory stimulation induced in intact synaptosomes by different concentrations of A23187 is shown; in figure 6*c* these data, together with the extent of noradrenalin release induced by these ionophore concentrations, are replotted as a function of $[Ca_c^{2+}]$ by using the calibration from figure 6*a*.

The results show the following: first, the mitochondrial uniporter is active over the range 1–10 μM Ca^{2+}; at 10 μM Ca^{2+} it can take up the cation at 20 nmol min^{-1} mg^{-1} synaptosomal protein; secondly, release of [^{3}H]noradrenalin occurs over the same range of $[Ca_c^{2+}]$. It is significant that this range of Ca^{2+} concentration is the same as that observed by Baker & Knight (1978) for exocytosis from adrenal medullary cells.

Conclusions

Our studies indicate that plasma membrane Ca^{2+} transport is the resultant of a Ca^{2+}-ATPase acting in opposition to a constant leak and a voltage-dependent channel, that mitochondria are the major intra-synaptosomal Ca^{2+} regulators, and, by elimination, that endoplasmic reticulum is of minor quantitative importance. We therefore reach a different conclusion from those workers who emphasize Na^+–Ca^{2+} exchange at the plasma membrane (Blaustein & Ector 1976) or a major role for endoplasmic reticulum (Blaustein *et al.* 1978*a*, *b*). While none of our work eliminates a role for endoplasmic reticulum, there are a number of problems. First, membranes with the appearance of endoplasmic reticulum are only occasionally visible in electron micrographs of nerve endings; secondly, microsomal preparations from synaptosomes have been insufficiently characterized to allow the possibility of contamination by plasma membrane to be eliminated; thirdly, the Ca^{2+} transport properties of these fractions, in terms both of kinetics and regulation, are not impressive in relation to those of isolated mitochondria.

The need for both plasma membrane and mitochondrial Ca^{2+} transport can be rationalized by the following cycle of events during exocytosis. Entry of Ca^{2+} through the voltage-sensitive channels raises $[Ca_c^{2+}]$ to 1–10 μM within 1 or 2 ms, initiating exocytosis. Subsequent uptake by mitochondria lowers $[Ca_c^{2+}]$ to 1 μM (below the threshold for transmission) on a timescale of a few seconds. Ca^{2+} efflux across the plasma membrane lowers $[Ca_c^{2+}]$ sufficiently for the mitochondria to release their Ca^{2+}. Finally a resting steady state is obtained, at which uptake and efflux across the plasma membrane balance, at a $[Ca_c^{2+}]$ of about 0.1 μM.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

K. E. O. Å. was supported by a fellowship from the European Molecular Biology Organization. D. G. N. is supported by grants from the Medical Research Council.

References (Nicholls & Åkerman)

Åkerman, K. E. O. & Nicholls, D. G. 1981*a* Calcium transport by intact synaptosomes: influence of ionophore A23187 on plasma membrane potential, plasma membrane calcium transport, mitochondrial membrane potential, respiration, cytosolic free calcium concentration and noradrenalin release. *Eur. J. Biochem.* **115**, 67–73.

Åkerman, K. E. O. & Nicholls, D. G. 1981*b* Intra-synaptosomal compartmentation of calcium during depolarization-induced calcium uptake across the plasma membrane. *Biochim. biophys. Acta* **645**, 41–48.

Åkerman, K. E. O. & Nicholls, D. G. 1981*c* Calcium transport by intact synaptosomes: the voltage-dependent calcium channel and a re-evaluation of the role of sodium/calcium exchange. *Eur. J. Biochem.* **117**, 491–497.

Baker, P. F. 1978 The regulation of intracellular calcium in giant axons of *Loligo* and *Myxicola*. *Ann. N.Y. Acad. Sci.* **307**, 250–268.

Baker, P. F. & Knight, D. E. 1978 Calcium-dependent exocytosis in adrenal medullary cells with leaky plasma membranes. *Nature, Lond.* **276**, 620–622.

Blaustein, M. P. 1975 Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. *J. Physiol, Lond.* **247**, 617–655.

Blaustein, M. P. & Ector, A. C. 1976 Carrier-mediated sodium-dependent and calcium-dependent efflux from pinched-off presynaptic nerve terminals (synaptosomes) *in vitro*. *Biochim. biophys. Acta* **419**, 295–308.

Blaustein, M. P., Ratzlaff, R. W., Kendrick, N. C. & Schweitzer, E. S. 1978*a* Calcium buffering in presynaptic nerve terminals: evidence for involvement of a non-mitochondrial ATP-dependent sequestration mechanism. *J. gen. Physiol.* **72**, 15–41.

Blaustein, M. P., Ratzlaff, R. W. & Schweitzer, E. S. 1978*b* Calcium buffering in presynaptic nerve terminals: kinetic properties of the non-mitochondrial calcium sequestration mechanism. *J. gen. Physiol.* **72**, 43–64.

Brinley, F. J., Tiffert, T., Scarpa, A. & Mullins, L. J. 1977 Intra-cellular calcium buffering capacity in isolated squid axons. *J. gen. Physiol.* **70**, 355–384.

Mitchell, P. 1976 Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: power transmission by proticity. *Biochem. Soc. Trans.* **4**, 399–430.

Nicholls, D. G. 1978 Calcium transport and proton electrochemical gradient in mitochondria from cerebral cortex and rat heart. *Biochem. J.* **170**, 511–522.

Nicholls, D. G. & Crompton, M. 1980 Mitochondrial calcium transport. *FEBS Lett.* **111**, 261–268.

Nicholls, D. G. & Scott, I. D. 1980 The regulation of brain mitochondrial calcium transport: the role of ATP in the discrimination between kinetic and membrane potential dependent calcium efflux mechanisms. *Biochem. J.* **186**, 833–839.

Requena, J. & Mullins, L. J. 1979 Calcium movements in nerve fibres. *Q. Rev. Biophys.* **12**, 371–460.

Scott, I. D. & Nicholls, D. G. 1980 Energy transduction in intact synaptosomes: influence of plasma membrane depolarization on the respiration and membrane potential of internal mitochondria determined *in situ*. *Biochem. J.* **186**, 21–33.

Scott, I. D., Åkerman, K. E. O. & Nicholls, D. G. 1980 Calcium transport by intact synaptosomes: intra-synaptosomal compartmentation and the role of the mitochondrial membrane potential. *Biochem. J.* **192**, 873–880.

Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. & Kakiuchi, S. 1979 Occurrence of a calcium- and modulator protein-activatable ATPase in the synaptic plasma membranes of brain. *FEBS Lett.* **99**, 199–202.

Zuurendonk, P. F. & Tager, J. M. 1974 Rapid separation of particulate components and soluble cytoplasm of isolated rat liver cells. *Biochim. biophys. Acta* **333**, 393–399.

Discussion

J. R. Garrett (*King's College Hospital Dental School, London, U.K.*). Does botulinum toxin have any effects on the authors' system?

D. G. Nicholls. We have not yet investigated the effects of neurotoxins on our synaptosomal preparation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES