cells. (Fab against aggregation-competent cells did, however, block the adhesion of these cells.)

Recently, Geltosky *et al.*[10] described a glycoprotein of molecular weight 150,000, present on the surface of vegetative *D. discoideum* cells, whose concentration increased during the first 10 h of development. Fab directed against this protein blocked the ability of aggregation-competent cells to reaggregate. However, the synthesis of such a glycoprotein was not detected in labelling experiments[5]. (A glycoprotein of molecular weight 155,000 was synthesized later, between early aggregation and culmination[5].) One explanation for this discrepancy would be that the glycoprotein described by Geltosky *et al.*[10] is stored in the cytoplasm of vegetative cells and transported to the cell surface during early development. It may bind to one of the glycoproteins of molecular weights 82,000 or 95,000 on adjacent cells. In our plasma membrane preparations no proteins with molecular weights of approximately 150,000 were markedly antigenic. Hence, our Fab contained few, if any, molecules directed against the glycoprotein of Geltosky *et al.*[10]. When the region of the gel corresponding to a molecular weight of 150,000 was preincubated with anti-slug Fab, the remaining Fab still inhibited aggregation.

Our results suggest that the antigen of molecular weight 95,000 is involved in cell adhesion, although we cannot be absolutely sure that it is not another protein running at around that region on gels that is responsible. However, we consider this unlikely as butanol extracts contained no other detectable proteins which ran so near to this antigen as to be included in the gel slice. The possibility that binding of Fab to the antigen of molecular weight 95,000 indirectly inhibits reaggregation by selective steric impairment of another set of molecules cannot be excluded.

The plasma membrane glycoproteins involved in cell adhesion during the early developmental stages of both *D. discoideum* (molecular weight 82,000)[2] and *Polysphondylium pallidum*[11] (molecular weight 71,000)[9] are no longer synthesized when aggregation is complete and are subsequently lost from the cells[4,5,8,9]. This may be related to the sorting out of pre-spore and pre-stalk cells that occurs during the post-aggregation phase[12–16]. The glycoprotein of molecular weight 95,000 of *D. discoideum*, for example, may be restricted to one cell type or occur at different concentrations on the two types. We are using immunofluorescence to check these possiblities. The glycoprotein of molecular weight 150,000 apparently does not disappear from the plasma membrane during differentiation[10]. However, it may be part of a second type of adhesion which is not involved in sorting out. Alternatively, a change in only one component of an adhesion system may be sufficient to induce sorting out.

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Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation

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Phosphorylation of membrane components is thought to be an important process in membrane function[1]. Phosphorylated proteins[2] and a special class of phospholipids, the (poly)phosphoinositides (poly PI)[3], are implicated in the regulation of membrane permeability and synaptic transmission in neurones. For many years, protein phosphorylation and poly PI metabolism have been studied in parallel without knowledge of their possible interaction. We report here that the ACTH-sensitive protein kinase/B-50 protein complex which we recently isolated in soluble form from rat brain synaptosomal plasma membranes[4,5] has lipid phosphorylating activity. Exogenously added phosphatidylinositol 4-phosphate (DPI) is phosphorylated to phosphatidylinositol 4,5-diphosphate (TPI), and this DPI-kinase activity is dependent on the state of phosphorylation of the protein kinase/B-50 protein complex. The results imply that phosphorylation of protein may affect the metabolism of (poly)PI in brain cell membranes.

Effects of peptide hormones on phosphatidylinositol (PI) metabolism in cell membranes of peripheral tissues have been obtained[6–8]. Similarly, corticotropin (ACTH) is known to affect (poly)PI metabolism in membrane fractions from rat brain[9]. An enhanced metabolism of TPI was also found in response to cholinergic and noradrenergic receptor stimulation[10] and after the influx of Ca^{2+} into the cell[11]. With respect to the phosphorylation of proteins, we found that ACTH exerts a dose- and structure-dependent inhibitory effect on the *in vitro* phosphorylation of specific proteins in synaptosomal plasma membranes[4]. The ACTH-sensitive protein kinase (molecular weight 71,000; isoelectric point 5.5) and one of its substrate proteins (B-50; molecular weight 48,000; isoelectric point 4.5) were isolated and the phosphorylation of the B-50 protein was found to be Ca^{2+}-dependent[5].

The protein kinase/B-50 complex used in the present study was obtained from a Triton–KCl solubilized synaptosomal plasma membrane fraction and subjected to further purification by DEAE-cellulose chromatography and ammonium sulphate precipitation (ASP) as described before (see legend to Fig. 1). The ASP fraction has been characterized by SDS–polyacrylamide slab gel electrophoresis: the position of B-50 and its protein kinase are indicated in Fig. 1*a*. Upon incubation of this ASP fraction with [γ-^{32}P]ATP only the B-50 protein became labelled (Fig. 1*b*). Interestingly, addition of DPI to the ASP fraction in the phosphorylation medium produced [^{32}P]TPI as shown by autoradiography after thin layer chromatography of the lipid extract (Fig. 1*c*).

When the eluate of the DEAE–cellulose column was assayed for B-50 protein and DPI-kinase activity, it was found that the two activities cochromatographed, suggesting a possible relationship (J.J. *et al.*, in preparation). Confirming previous results[6], incubation of the ASP fraction in the presence of $ACTH_{1-24}$ resulted in a dose-dependent inhibition of B-50 phosphorylation (Table 1). Concomitantly, the phosphorylation

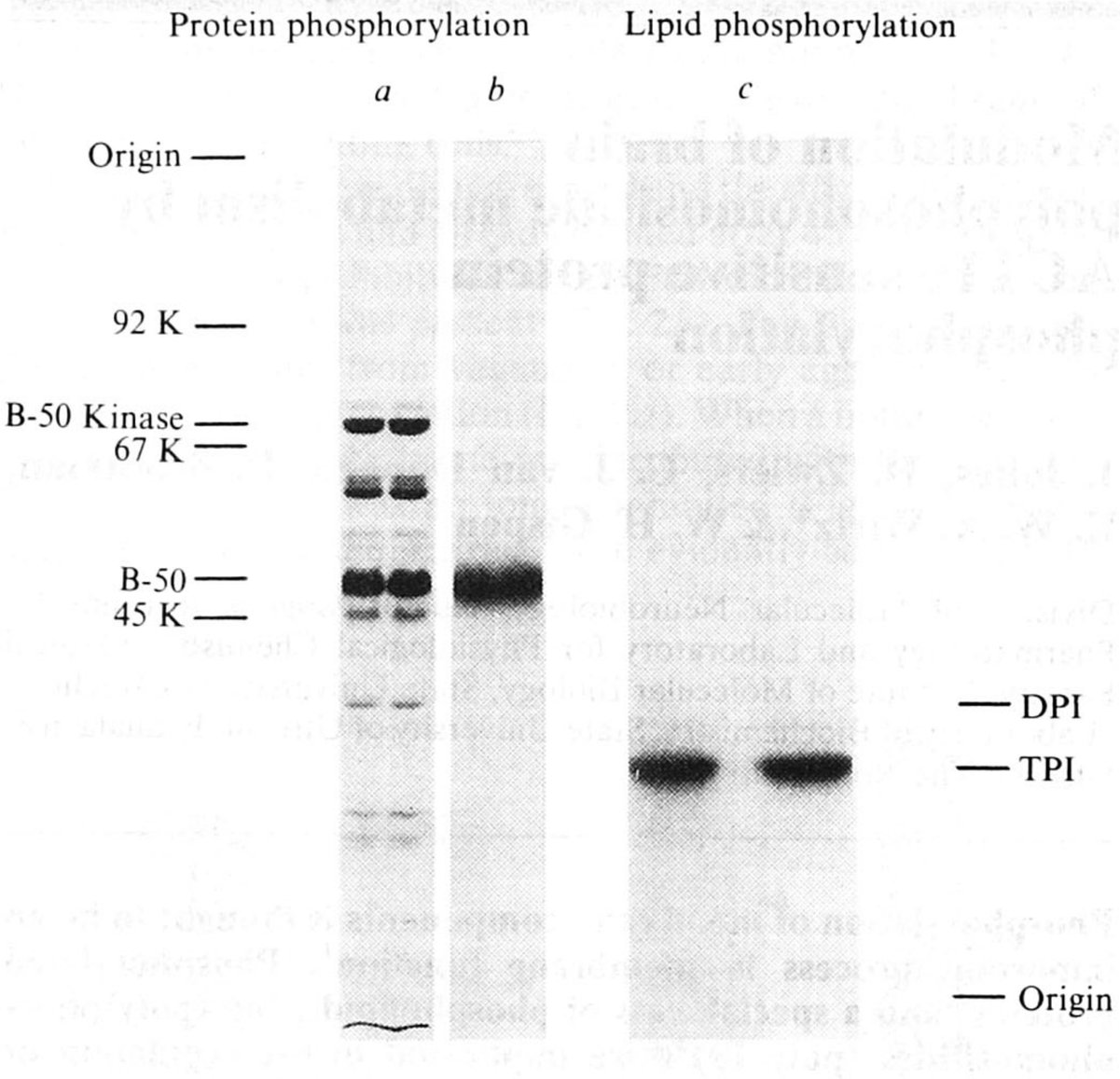

Fig. 1 *a*, Gel staining pattern showing the position of the protein bands, and *b*, autoradiogram showing the endogenous phosphorylation profile of the $ASP_{55-80\%}$ fraction. Each track contains 6 μg of total ASP protein. The position of molecular weight marker proteins, and of B-50 and B-50 kinase is indicated. *c*, Autoradiogram after extraction and thin layer chromatography of ASP fraction incubated with exogenous DPI. The position of DPI and TPI is indicated. Each lane shows the results of duplicate determinations. The ACTH-sensitive protein kinase and its protein substrate, the B-50 protein, were isolated as described by Zwiers *et al.*[5,6]. Briefly, a crude synaptosomal plasma membrane fraction was prepared from rat brain. The membrane-bound B-50 protein kinase activity was solubilized with 0.5% Triton X-100 with 75 mM KCl in 6 mM Tris, 0.1 mM dithiothreitol (*p*H 8.1) and the solubilized proteins separated by DEAE-cellulose chromatography. The proteins were eluted with a salt gradient in 10 mM Tris, 1 mM $CaCl_2$, 0.1 mM dithiothreitol, *p*H 7.4 (buffer A). The eluate fractions containing the peak of B-50 protein and B-50 phosphorylating activity were pooled and subjected to 55% ammonium sulphate saturation. After separation of the precipitated proteins by centrifugation the supernatant was saturated to 80% with ammonium sulphate and the precipitate collected by centrifugation. This $ASP_{55-80\%}$ fraction was dissolved in 400 μl buffer A and dialysed overnight against 1 l of buffer A. The phosphorylation assay was performed in the following conditions: 50 mM Na acetate, 10 mM Mg acetate, 1 mM Ca acetate (*p*H 6.5), 7.5 μM ATP, 2 μCi [γ-^{32}P]ATP (~3,000 Ci mmol^{-1}, Amersham), 7.5 μl ASP fraction, 40 μM DPI (disodium salt, Sigma) in a final volume of 25 μl. The enzyme fraction was routinely preincubated for 5 min before the incubation was started by the addition of DPI and ATP. Buffer with or without $ACTH_{1-24}$ was added 15 s before the ATP and incubation continued for 15 min unless indicated otherwise. Protein phosphorylation and lipid phosphorylation assays were always run in parallel. The protein phosphorylation reaction was terminated by the addition of a denaturating solution and the proteins were separated by SDS–polyacrylamide gel electrophoresis. After staining with Fast Green FCF and autoradiography the labelled protein bands were cut from the gel and measured for radioactivity by liquid scintillation counting[4,5]. The lipid phosphorylation reaction was terminated by the addition of ice-cold chloroform/methanol/13 N HCl solution (200 : 100 : 0.75 v/v). Carrier poly PI were added and an acid extraction was carried out. The lipid extract was separated by one-dimensional high performance thin layer chromatography (HPTLC) on precoated HPTLC plates (Merck) impregnated with 1% K oxalate. After lipid staining and autoradiography the labelled lipids were scraped from the plate and counted for radioactivity in a liquid scintillation counter[8,12]. All experiments were performed at least three times.

of exogenously added DPI to TPI was stimulated (+35% and +149% at 10 μM and 100 μM ACTH respectively). Because similar ACTH effects have been obtained with brain synaptosomal membrane fractions[4,12], it seems very probable that the enzymes responsive to the peptide in synaptosomal plasma membranes are present in the ASP fraction. The correlation between inhibited B-50 phosphorylation and stimulated TPI formation suggests that the state of B-50 phosphorylation may affect the DPI kinase activity (see ref. 13 for a recent review on the regulation of enzymatic activity by phosphorylation). To test this hypothesis the ASP fraction was preincubated with [γ-^{32}P]ATP for various periods of time, and DPI kinase activity was measured after this B-50 prephosphorylation period. As Fig. 2*a* shows, the extent of B-50 protein phosphorylation increased with time up to 40 min but DPI kinase activity diminished with increasing B-50 phosphorylation, providing additional evidence for a direct relationship between B-50 protein and DPI phosphorylation. It appears that the formation of TPI is especially affected by the final 30% of the protein phosphorylation; more insight into the exact molecular mechanisms involved is needed to appreciate the significance of this finding more fully. To eliminate the possibility that the decreased formation of TPI is due to enzyme breakdown during the phosphorylation period,

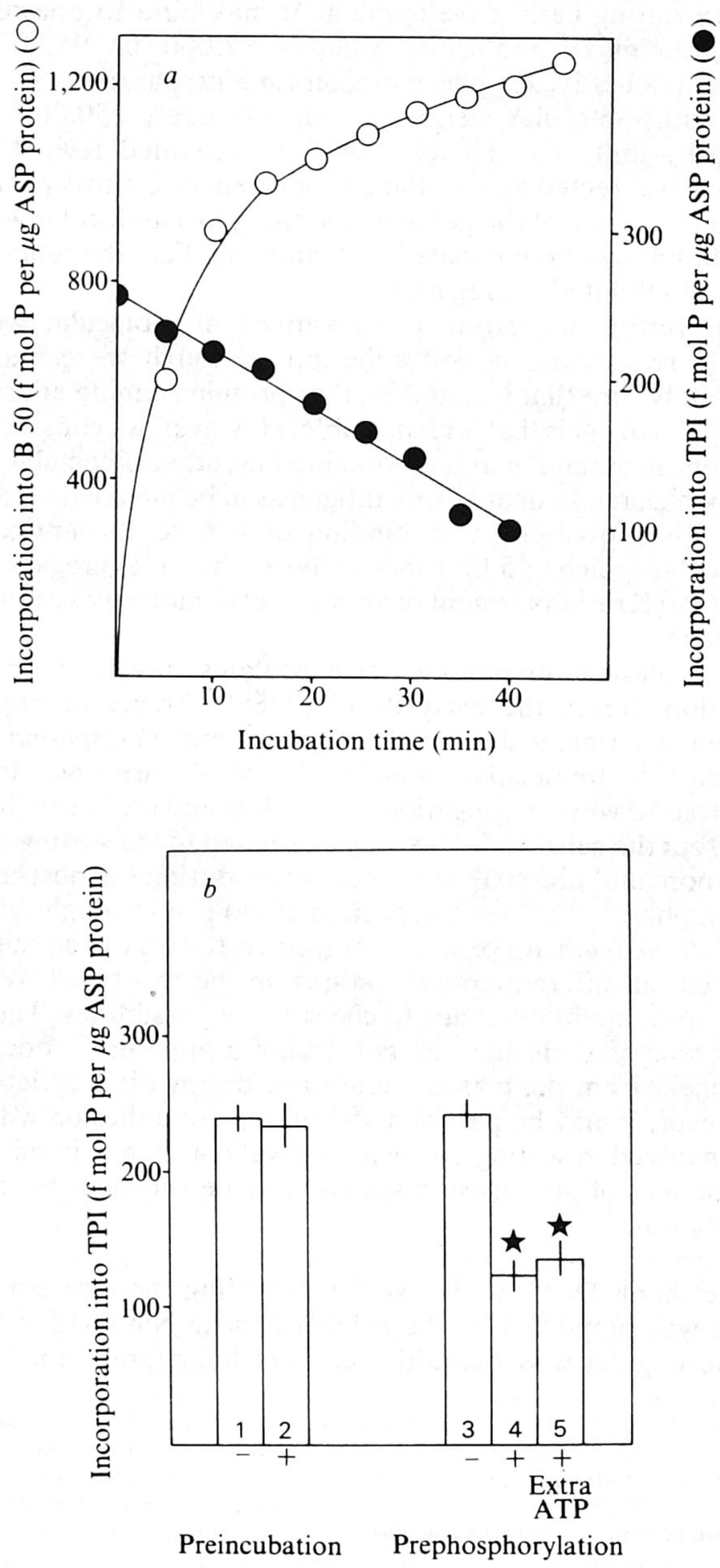

Fig. 2 *a*, The effect of B-50 prephosphorylation on TPI formation. Incubations were performed as described in the legend to Fig. 1, with the following modifications: ASP fractions were incubated in the absence of DPI for 5–45 min (○). Exogenous DPI was added to the ASP fraction after the various prephosphorylation time periods (0–40 min) and incubation continued for another 5 min. The amount of TPI formation in these 5 min periods (0–5, 5–10 min etc.) was measured (●). *b*, The effect of B-50 prephosphorylation on TPI formation: influence of preincubation and ATP. ASP fractions were incubated for 15 min in the presence of ATP and DPI as indicated in the legend to Fig. 1 (group 1 and 3). Group 2 was preincubated for 30 min before addition of DPI and ATP. Group 4 was preincubated for 30 min in the presence of ATP before DPI was added and the incubation continued for another 15 min. Group 5 was treated like group 4 but extra ATP containing 2 μCi [γ-^{32}P]ATP was added together with the DPI to give a final concentration of 15 μM ATP.

TPI formation was measured with and without 30 min preincubation in the absence of ATP. As Fig. 2*b* shows, preincubation had no effect. Furthermore, the decreased TPI formation seemed not to be related to exhaustion of ATP during the prephosphorylation, as the amount of labelled lipid was significantly depressed even after the addition of extra ATP (Fig. 2b). These results are in line with those obtained with ACTH (Table 1 and refs 4, 5, 12): there seems to be an inverse relationship between B-50 phosphorylation and TPI formation. Therefore, and in view of the studies on enzyme phosphorylation[13], we propose that the effect of ACTH on TPI production is secondary to the effect on protein phosphorylation: the peptide inhibits the B-50 kinase[4], and the B-50 substrate protein in its non-phosphorylated form, stimulates TPI formation. It awaits further experimentation to determine whether the B-50 protein is a regulatory factor of the DPI-kinase or the kinase itself.

Table 1 The effect of $ACTH_{1-24}$ on B-50 phosphorylation and TPI formation

Treatment	Incorporation (f mol P per μg ASP protein) B-50	Increase (%)	TPI	Increase (%)
No addition	994.9±8.0		180.0±9.1	
$ACTH_{1-24}$ (10 μM)	517.3±21.2	−48†	242.2±16.2	+35*
$ACTH_{1-24}$ (100 μM)	308.4±25.4	−69†	448.4±30.0	+149†

Incubations were performed as described in the legend to Fig. 1. Values represent mean±s.e.m. ($n = 5$). Differences were tested with Student's t-test.
* $P < 0.01$.
† $P < 0.001$.

Interestingly, Takai *et al.*[14,15] recently demonstrated that the activity of a Ca^{2+}-dependent protein kinase is linked to PI and its breakdown product 1,2-diacylglycerol, suggesting that a functional relation exists between protein phosphorylation and phosphatidylinositol metabolism. DPI and especially TPI are very potent chelators of Ca^{2+} and Mg^{2+} (refs 3, 16); they interact strongly with proteins and, due to their very negative headgroup which contains three (DPI) or five (TPI) negative charges, these lipids may carry the negative potential of the membrane[17,18]. Thus a change in the relative amounts of the phosphoinositides PI/DPI/TPI in the membrane may affect the conformation of membrane proteins and change the amount of Ca^{2+} and Mg^{2+} bound to the membrane. Both protein phosphorylation[1,2] and (poly)PI metabolism[3] are implicated in the regulation of membrane permeability and synaptic transmission in neurones. The results obtained in the present study suggest that the phosphorylation of a quantitatively minor membrane protein (B-50 protein), may bring about profound changes in membrane characteristics by changing the content of poly PIs. The demonstration that ACTH influences this process may suggest new avenues for investigating the biochemical mechanisms underlying agonist–effector cell membrane interactions.

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Chemically induced myotonia in amphibia

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Frogs and toads treated with high doses of anthracene-9-carboxylic acid (A-9-C) develop prolonged muscular contractions and 'divebomber' electromyograms characteristic of myotonia. Hitherto, myotonia has been considered peculiar to homeotherms where it is associated with several hereditable diseases and can be induced by specific treatments, most of which seem to act by decreasing membrane chloride conductance[1]. Our work indicates that myotonia can be induced in amphibia by similar means. We offer possible reasons why others have missed seeing myotonia in amphibia.

Toads, *Bufo marinus*, weighing about 120 g and frogs, *Littoria aurea*, of 30–60 g were kept at room temperatures of 20–25 °C and injected subcutaneously with A-9-C (167 mg per kg as a 10 mg ml^{-1} solution neutralized with NaOH). Myotonic behaviour could then be observed in the toads within half an hour. In the frogs it took a number of hours to develop. If jumping was provoked it resulted in one or a few normal jumps followed by jumps with severe extensor spasm on landing. This often caused the animal to freeze in a bizarre attitude for some seconds (Fig. 1), before slowly relaxing and then resuming a normal squatting position. Continued provocation to jump eventually resulted in a return to normal. After rest for half an hour, however, the whole cycle could be repeated. This kind of response could be obtained for 2 or 3 days following the injection of A-9-C after which jumping behaviour gradually returned to normal over the next few days.

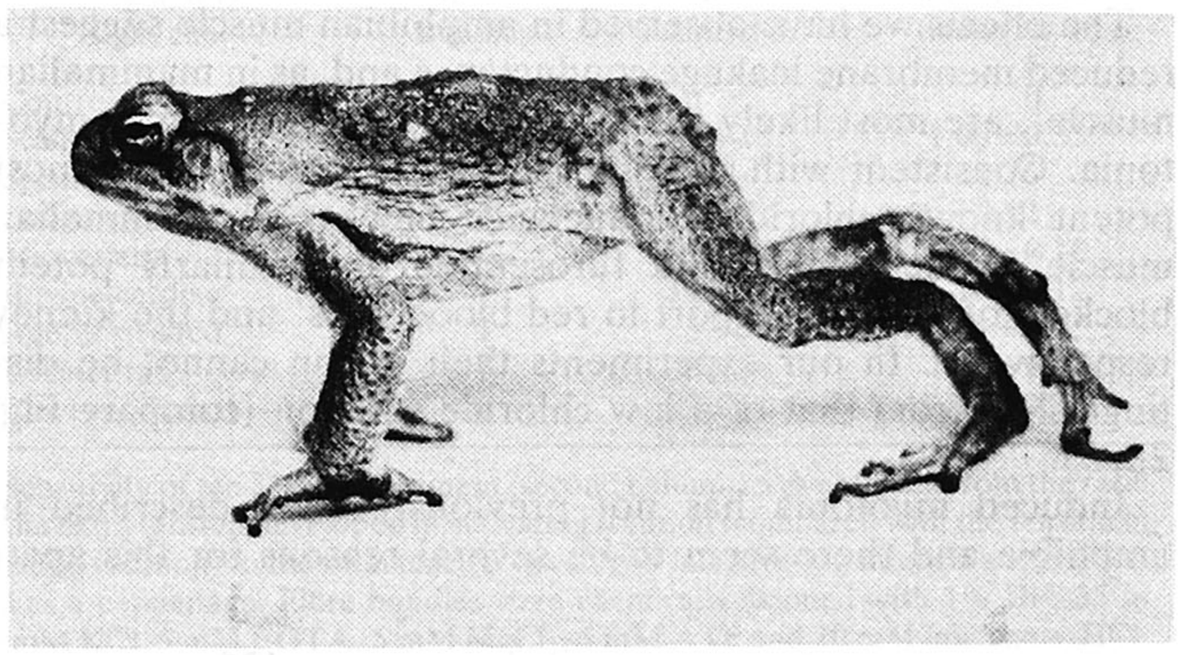

Fig. 1 An A-9-C treated toad, still immobile and stiff-legged, ~3s after landing from a jump. We have occasionally seen these animals overbalance on landing to become supported on their forelegs and snout with their hind legs extended above them. By contrast, untreated toads jump, land and resume their normal squatting position within half a second.

While animals were displaying myotonic behaviour, audio electromyograms could be recorded from leg muscles with frequent 'divebomber' bursts of activity in response to slight movements of the concentric recording needle. After exercise 'warm up', divebomber sounds were virtually eliminated.

Sartorius muscles, *in vitro*, could also be made to produce myotonic contractions (Fig. 2*a*) in response to A-9-C at concentrations comparable to those expected to pertain in the *in vivo* experiments. Similar results could be obtained using 4,