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Forskolin activation of an identified peptide-sensitive motoneurone in *Aplysia*

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Activation of a physiological response by the adenylate cyclase activator, forskolin, has been suggested as a new criterion for testing the role of cyclic AMP. In *Aplysia*, motoneurone B16, which innervates muscle I5, is activated by the peptide egg-laying hormone (ELH). In high magnesium-low calcium medium, used to block synaptic activity, forskolin produced a similar response to ELH. Forskolin, at a concentration of 100 μM, consistently activated the ELH-sensitive neurone; vehicle produced no response while 30 μM forskolin usually produced lower levels of activity than 100 μM. The data are consistent with cyclic AMP mediation of the ELH response.

Introduction Forskolin is a diterpene which reversibly activates adenylate cyclase in intact tissues, including the brain (reviewed in Seamon & Daly, 1981). Forskolin activates adenylate cyclases normally stimulated by different agonists, and its effects are not perturbed by specific receptor antagonists (Seamon & Daly, 1981). The effect of forskolin on adenylate cyclase appears to be specific since it has been reported not to affect the activity of other enzymes that have been tested, including phosphodiesterase (Linder, Dohadwalla & Bhattacharya, 1978), sodium-potassium ATPase (Lindner *et al.* 1978), cyclic adenosine 3′, 5′-monophosphate (cyclic AMP)-dependent protein kinase (Metzger & Lindner, 1981), and guanylate cyclase (Seamon & Daly, 1981). Because forskolin activates adenylate cyclase from a wide variety of sources independently of specific agonist receptors, it has been suggested that an additional criterion for assessing the possible role of cyclic AMP in a physiological response is that forskolin should be able to elicit the same response.

In *Aplysia*, the neuropeptide egg laying hormone (ELH) activates motoneurone B16, which innervates buccal muscle I5 (Ram, 1982a,b; 1983a). Since peptide actions are often mediated by cyclic AMP, experiments were initiated to test the involvement of cyclic AMP in this electrophysiological response. Phosphodiesterase inhibitors, isobutylmethylxanthine and theophylline, and a cyclic AMP analogue, 8-bromo cyclic AMP, all mimic the physiological response to ELH (Ram, 1983b). I now show that forskolin activates neurone B16, producing a response similar to that of ELH.

Methods *Aplysia* buccal ganglia with attached I5 muscles were mounted in a two-chamber recording dish with the buccal ganglia in one chamber and the nerves going through two slots between the chambers to the muscles in the second chamber (Padgaonkar & Ram, 1983). The connecting slots were blocked with Vaseline, and each chamber was separately perfused with media at 0.3 ml min^{-1}. Experiments were done at 12°C. Perfusion media included (a) buffered sea water (BSW): (Instant Ocean), sterilized by cold filtration through 0.2 μm Metricel membrane filters, with the addition of 0.6 g l^{-1} THAM (tris hydroxymethyl aminomethane)-HCl (pH 8.0), 1.8 g l^{-1} dextrose, and 100 mg l^{-1} streptomycin and (b) high magnesium-low calcium artifical sea water (High Mg-Low Ca-ASW): 150 m M $MgCl_2$, 290 mM NaCl, 10 mMKCl, 28 mM Na_2SO_4, 1 mM $CaCl_2$, 0.6 g l^{-1} THAM-HCl (pH 8.0), 1.8 g l^{-1} dextrose, and 100 mg l^{-1} streptomycin. Stock solutions of forskolin of 10 mM and 100 mM, in 95% ethanol, were added to BSW or High Mg-Low Ca-ASW and applied by perfusion through the buccal ganglia chamber. ELH was prepared by gel filtration on Sephadex G-50 in 0.5 M formic acid of centrifuged homogenates of *Aplysia* bag cell neurones, as described previously (Padgaonkar & Ram, 1983). Freeze-dried ELH-containing fractions were redissolved in BSW or High Mg-Low Ca-ASW for application by perfusion through the buccal ganglia chamber. The muscle chamber was perfused with BSW throughout all experiments.

Muscle potentials (e.j.ps; no action potentials occur in the muscle; Cohen, Weiss & Kupfermann, 1978) were recorded bilaterally extracellularly. Of the two major inputs to muscle I5, B16 was identified by the characteristically smaller degree of facilitation at 1 Hz of its e.j.p. onto I5, in comparison with B15, the other major input to I5 (Ram, 1982; 1983a). B15 is not activated by ELH.

Results In BSW, forskolin activated bursting output from the buccal ganglia (data not shown), an effect similar to the response in BSW produced by theophylline and isobutylmethylxanthine (Ram, 1983b). This response is believed to result from

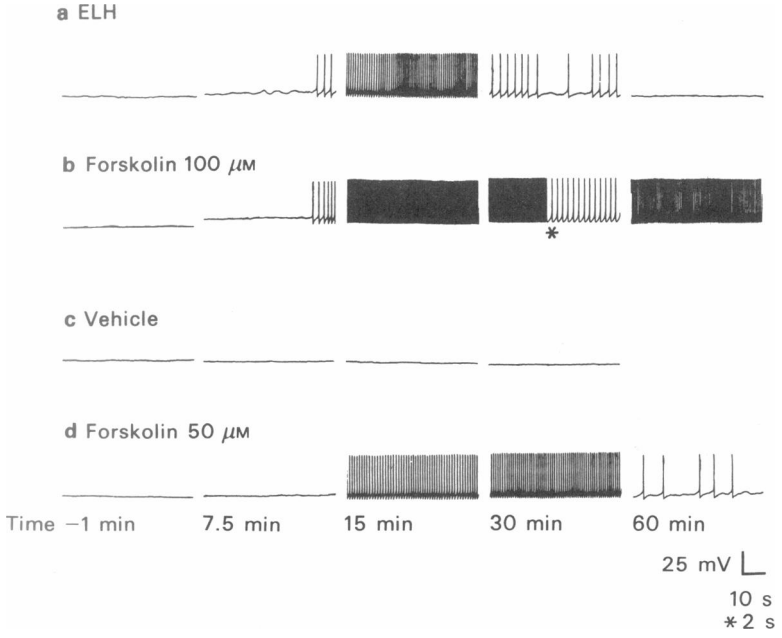

Figure 1 Intracellular recording from buccal neurone B16 in High Mg-Low Ca-ASW. (a) Response to egg-laying hormone (ELH), fractionated on Sephadex G-50 as described in Methods. (b) Response to 100 μM forskolin, prepared by adding 6 μl of 100 mM forskolin stock solution to 6 ml High Mg-Low Ca-ASW. (c) Response to the vehicle, 6 μl 95% ethanol added to 6 ml High Mg-Low Ca-ASW. (d) Response to 50 μM forskolin, prepared by adding 3 μl 100 mM forskolin stock and 3 μl 95% ethanol to 6 ml High Mg-Low Ca-ASW. In all cases the solution being tested was begun at time = 0 min. ELH was applied for 10 min, all others for 15 min.

synaptic interactions of many cells in the ganglion, possibly including B16, in which activity and/or transmitter release or efficacy may be cyclic nucleotide-modulated.

Synaptic interactions in the buccal ganglia were blocked by superfusion of buccal ganglia with High Mg-Low Ca-ASW. This medium did not block activation of B16 by ELH (Figure 1a), but spontaneous e.p.s.ps and e.p.s.ps into B16 caused by stimulation of buccal nerve B5 were completely blocked (data not shown). Addition of forskolin (30–100 μM) to this medium caused activation of B16, similar to the effect of ELH (Figure 1b, 1d).

Forskolin was applied to four preparations in which activity was recorded extracellularly from a total of eight I5 muscles. For all muscles 100 μM forskolin caused activation of the ELH-activated input. The latency to the first e.j.p. varied from 8.3 min to 27 min (median = 16 min), and the activity often persisted for more than 1 h (range 27 min to 175 min; median = 66 min) after perfusion with High Mg-Low Ca-ASW without foskolin was resumed. The maximal e.j.p. frequency observed with 100 μM forskolin was 1.9 ± 1.0 e.j.ps/s (mean ± s.d., $n = 8$). Of four muscles also tested with 30 μM forskolin, three muscles exhibited no response or a comparatively low maximal activity (0.3 e.j.ps/s), while the other muscle gave 3 e.j.ps/s. Application of as much as 100 μl 95% ethanol per 10 ml High Mg-Low Ca-ASW (the same amount of ethanol as when 100 μM forskolin perfusate was made with the 10 mM forskolin stock solution) never activated I5.

The response to forskolin was recorded intracellularly in three cells. In Figure 1b, in response to 100 μM forskolin B16 depolarized slowly, reached the spike threshold in less than 10 min, and achieved a maximal activity of 2.3 spikes s^{-1}. No depolarization occurred in response to a control application of the vehicle (Figure 1c), whereas application of 50 μM forskolin gave a maximal response of 0.6 spikes s^{-1} (Figure 1d). Application of 100 μM forskolin while recording from an ELH-insensitive I5 motoneurone gave no depolarization (data not shown).

Discussion Forskolin depolarized and activated cell B16. Forskolin could be producing this effect through an unknown cellular mechanism; however, previously demonstrated biochemical effects of forskolin suggest that the response may be mediated by

an increase in intracellular cyclic AMP. In dose-response studies of vertebrate brain slices, maximal accumulations of cyclic AMP occurred at concentrations of forskolin of 100 μM or more (Daly, Padgett & Seamon, 1982). Consistent with this result, B16 activity was usually greater in response to 100 μM forskolin than to lower concentrations. Half-maximal accumulations of cyclic AMP in vertebrate brain slices occurred at forskolin concentrations of 20–10 μM (Daly *et al.*, 1982), a concentration which usually produced no response or only a low level of activity, as recorded at muscle I5. In two intracellular recording experiments, application of 30 μM forskolin produced subthreshold depolarizations of 5 mV in B16 (data not shown). Additional experiments are needed to define the dose-response relationship between forskolin concentration and membrane potential of B16. Considering that the present experiments were done at 12°C, the response latencies compare favourably with the latency of 10 min at 37°C seen for maximal cyclic AMP accumulation in brain slice experiments (Daly *et al.*, 1982).

These data are consistent with the hypothesis that ELH-activation of motoneurone B16 is mediated by cyclic AMP. As predicted by this hypothesis, forskolin elicited the same response from B16 as did ELH. To my knowledge, this is the first reported use of forskolin to test mediation of a peptide response of a neurone by cyclic AMP.

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