Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 471–474, January 1992
Neurobiology

Termination of second messenger signaling in olfaction

(cAMP/inositol trisphosphate/rapid kinetics/kinases/inhibitors)

INGRID BOEKHOFF AND HEINZ BREER

University Stuttgart-Hohenheim, Institute of Zoophysiology, 7000 Stuttgart 70, Federal Republic of Germany

Communicated by M. Lindauer, October 3, 1991 (received for review August 1, 1991)

ABSTRACT **By using isolated rat olfactory cilia and a fast kinetics methodology, it has been demonstrated that odorant-induced second messenger signaling in the millisecond time range is terminated via phosphorylation reactions catalyzed by specific protein kinases. The cyclic adenosine nucleotide pathway is turned off by kinase A activity, whereas the inositol trisphosphate cascade is terminated by kinase C. The data support the concept that desensitization of odorant responses involves phosphorylation of key elements in the transduction cascade.**

Although the molecular elements and processes in olfactory receptor cells underlying the transduction of odor stimulation into electrical responses are still elusive, several lines of evidence suggest that the chemo–electrical signal transduction is mediated by second messenger cascades (1). The application of a rapid kinetics methodology has recently allowed us to monitor the odorant-induced formation of second messengers in rat olfactory cilia in the subsecond time range (2, 3); it has been found that odorants induce a large and rapid elevation of either cAMP or inositol trisphosphate (IP_3) concentrations followed by a rapid decay. This molecular response clearly precedes, and may thus be causally involved in, the induction of the generator current in olfactory receptor cells, which can be recorded only after a latency of a few hundred milliseconds (4). One of the characteristic features of the second messenger signal is its transient nature. The rapid increase and subsequent decrease in cAMP or IP_3 levels is supposed to be essential for receptor cells, like olfactory neurons, which can repeatedly be stimulated. However, it is presently unclear how the intracellular signaling is terminated. Because the time course of second messenger signaling is very similar over a wide concentration range of stimulatory odorants (2, 3) the "turning off" reaction cannot be attributed to odorant inactivation—e.g., via cytochrome P450 or gluconyltransferase reactions (5). By a number of criteria, the odorant-induced second messenger formation in olfactory cilia resembles the receptor-mediated cAMP response in hormone-sensitive cells. One remarkable feature of hormone-induced cAMP responses is that, even when the agonist is continuously present, intracellular cAMP levels generally plateau or even return to near basal level within a short period of time (6). This waning of the stimulated response in the presence of continuous agonist exposure has been termed desensitization (6). Desensitization has been demonstrated in many hormone and neurotransmitter receptor systems, and evidence is accumulating indicating that receptor phosphorylation may be involved in signal termination (6, 7).

In the present study we provide evidence indicating that the odorant-induced second messenger signaling in olfactory cilia is turned off in a negative feedback reaction via specific kinases.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Sprague–Dawley rats were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). Radioligand assay kits for cAMP as well as for IP_3 were supplied by Amersham. Citralva (3,7-dimethyl-2,6-octadienenitrile) was obtained from the International Flavors & Fragrances (Union Beach, NJ); lyral [4-(4-hydroxy-4-methylphenyl)-3-cyclohexene-1-carboxyaldehyde] was kindly provided by W. Steiner (Baierbrunn, F.R.G.). Phorbol dibutyrate and 3-isobutyl-1-methylxanthine (IBMX), as well as the protein kinase inhibitor (Walsh inhibitor) and sphingosine, were obtained from Sigma. Okadaic acid was obtained from Boehringer Mannheim. The purity of all chemicals used in this study was >99%.

Partially purified preparations of chemosensory cilia from rat olfactory epithelia were produced according to the procedure described by Anholt *et al.* (8) and Chen *et al.* (9). A rapid-quench device was used to determine the subsecond kinetics of the odorant-induced changes in second messenger concentrations. Rapid kinetics experiments were performed as described (2, 3). The concentration of cAMP was determined following the procedure of Steiner *et al.* (10). IP_3 was estimated according to the procedure of Palmer *et al.* (11). Protein was measured according to the method of Bradford (12).

RESULTS

Upon application of micromolar concentrations of citralva, a very rapid and transient increase of the cAMP level in rat olfactory cilia could be monitored using a stopped flow approach (Fig. 1), thus confirming recent results (3). A key feature of this primary molecular response is its transient nature; a rapid and transient response to a short stimulus is essential and typical for olfactory receptor cells. As in any biochemical pathway, the actual concentration of cAMP is determined by the balance between synthesis and degradation; thus, the rapid decay of the cAMP signal could either be due to a delayed stimulation of the permanently active phosphodiesterases (PDEs) or to a termination of the elevated synthesis rate for cAMP.

Phosphodiesterase inhibitors, like IBMX, caused an elevated cAMP level for a long period of time (Fig. 1), indicating that IBMX-sensitive, permanently active PDEs are involved in catabolizing cAMP in olfactory cilia. However, this does, of course, not answer the question of whether the rapid decay of the cAMP concentration is due to an increase in PDE activity. Measurements of PDE under steady-state conditions gave a specific activity of 190 nmol per min per mg of protein. No indications for odorant-induced changes in the activity of PDE were observed; this finding confirms recent observations by Dickinson *et al.* (13).

Alternatively, the transient nature of the signal may be due to a delayed turn off of the stimulated anabolic pathway. In

Abbreviations: IP_3, inositol trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase.

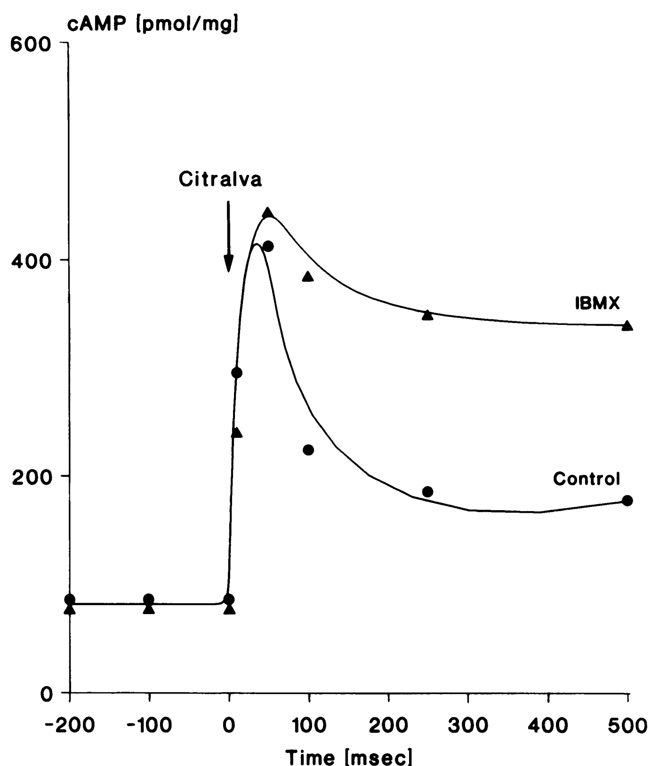

FIG. 1. Effect of IBMX on the time course of the cAMP signal in rat olfactory cilia stimulated by 1 μM citralva. Samples of isolated olfactory cilia were stimulated in a stopped flow device with either citralva (1 μM) or with citralva plus IBMX (1 mM). The reaction was quenched after appropriate incubation intervals, and the concentration of cAMP was determined. Note that the characteristic decay of the elevated cAMP level after 50 msec is prevented by the presence of IBMX. Data are the mean of three to five experiments with a variation of <10%.

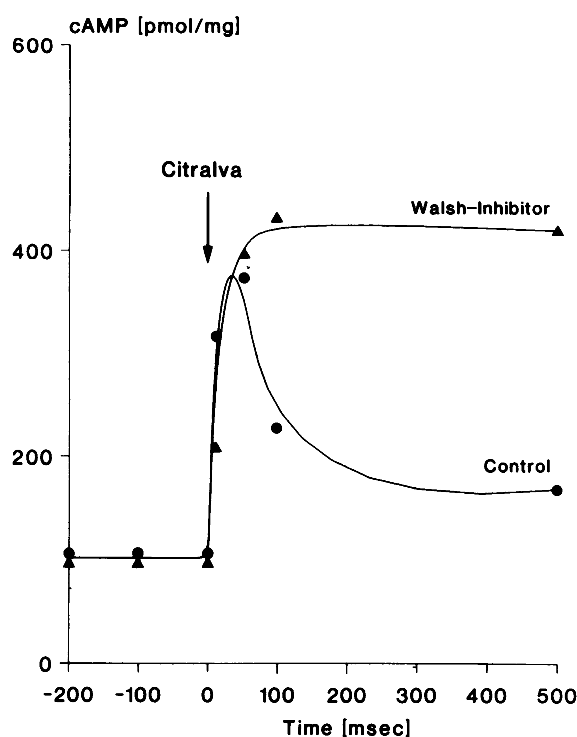

FIG. 2. Effect of the specific inhibitor for kinase A, the Walsh inhibitor, on an odorant-induced cAMP signal. Isolated olfactory cilia were preincubated with 3.8 μM protein kinase A (Walsh) inhibitor for 15 min at 4°C and subsequently stimulated with 1 μM citralva. Concentrations of cAMP were determined at appropriate time points after stimulation. Note that the onset kinetics of the cAMP accumulation in the presence of the Walsh inhibitor is unchanged; however, the cAMP level further increases and remains constant between 100 and 500 msec. Data represent the mean of three to four experiments with a variation of <10%.

the visual reaction cascade, as well as in hormone-induced reactions, signal termination is achieved by phosphorylation of the receptor protein; thus, the activation of further guanine nucleotide-binding regulatory proteins is prevented, and the anabolic reaction cascade is uncoupled (7, 14, 15). This possibility was analyzed for olfactory signaling by using specific protein kinase inhibitors.

As shown in Fig. 2, in the presence of a specific inhibitor for protein kinase A (16), the rising phase of the odor-induced cAMP signal was prolonged, reaching a plateau after about 100 msec; furthermore, the elevated cAMP level persisted over a long time period, up into the second range. This observation clearly indicates that a kinase reaction is an important step in reducing the odor-induced cAMP level. To explore the specificity of the inhibitor effect on the "offset" kinetics of the cAMP signal, dose–response experiments were performed to estimate the level of cAMP 500 msec after stimulus application. The results documented in Table 1 indicate that the persistent level of cAMP still detectable after 500 msec depends on the applied concentration of kinase inhibitor. An apparent saturation of this effect was obtained at an inhibitor concentration of 0.38 μM.

To analyze if the persistence of the cAMP signal may be due to a missing activation of a PDE in the presence of the kinase inhibitor, experiments were performed applying both Walsh inhibitor and IBMX. The results in Fig. 3 indicate that a combination of both inhibitors, each at a high concentration, gave an additive effect, suggesting that each compound affects a different target. Preventing an inactivation of the anabolic pathway and a simultaneous blockade of the catabolic reaction would account for the observed continuous increase in cAMP concentration. Thus, it has to be assumed that the odor-induced formation of cAMP is terminated via phosphorylation of a key element in the reaction cascade, most likely the odorant receptor.

This concept implies that the inactivated reaction cascade can be reactivated by dephosphorylating the modified elements; so an important functional role has to be expected for phosphatases. To approach this issue, the specific phosphatase inhibitor okadaic acid (17) was employed. As shown in Fig. 4, application of okadaic acid does not change the kinetics of the cAMP signal but rather reduces the intensity of the response. This observation indicates that inhibition of phosphatases results in a reduced reactivity of the signaling pathway due to elements that remain in the phosphorylated, inactive state. Furthermore, it is interesting to note that, in

Table 1. Effect of different concentrations of the kinase A (Walsh) inhibitor on the offset kinetics of odorant-induced cAMP signals

Walsh inhibitor, μM	cAMP, pmol/mg of protein	n
0	180 ± 80	6
0.0038	224 ± 22	3
0.038	347 ± 64	3
0.38	446 ± 78	3
3.8	480 ± 98	6
38	472 ± 15	3

Data represent the concentration of cAMP determined 500 msec after stimulating isolated olfactory cilia with 1 μM citralva. Cilia were preincubated with different concentrations of kinase A (Walsh) inhibitor for 15 min at 4°C. Data are the mean of n experiments ± SD.

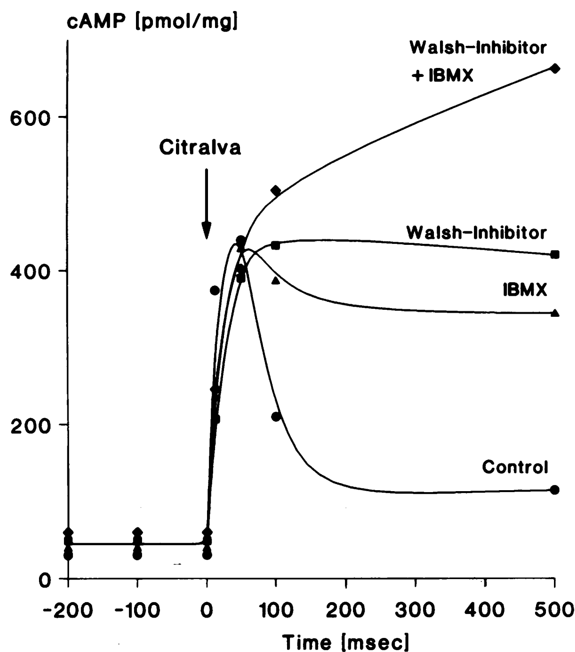

FIG. 3. Additive effect of IBMX and Walsh inhibitor on the accumulation of cAMP after stimulating rat olfactory cilia with citralva. Samples of isolated olfactory cilia were incubated with 3.8 μM protein kinase A (Walsh) inhibitor for 15 min at 4°C and subsequently stimulated with citralva (1 μM) plus IBMX (1 mM). Upon inhibition of PDEs and protein kinase A, the level of cAMP continued to increase even after 500 msec. Data are the mean of three to five experiments.

the presence of a phosphatase inhibitor, the cAMP level decays almost completely to the basal level within 50–500 msec. Under control conditions, the decaying cAMP signal levels off at an intermediate concentration. This observation suggests that in the presence of active phosphatases the pathway for generating cAMP can be activated again by odorants that are still present in the medium after a short period of time; thus the persistent intermediate cAMP level may reflect a new equilibrium of active and inactivated elements.

To determine if a similar termination mechanism controls the alternative olfactory second messenger pathway, odorant-induced generation of IP_3, the effect of protein kinase C inhibitors was analyzed. Fig. 5 demonstrated the time course of an IP_3 signal induced by lyral, confirming the rapid and transient IP_3 signal. In the presence of 100 μM sphingosine, which at this concentration is considered as a specific kinase C blocker (18), the decay of the IP_3 signal was almost completely prevented. Similar results were obtained using alternative kinase C inhibitors, like H7 or staurosporine. These results may be considered as an indication that the reaction cascade of the odorant-activated IP_3-generating pathway is blocked by the action of protein kinase C. This enzyme may be activated by diacylglycerol, which is generated simultaneously with IP_3. To prove whether or not stimulation of protein kinase C activity in olfactory cilia will affect the odorant-induced IP_3 responses, cilia preparations were challenged with phorbol esters, which are known to enhance kinase C activity. As shown in Fig. 6, pretreatment of olfactory cilia with phorbol dibutyrate significantly reduced the lyral-induced IP_3 signal, indicating that the efficiency of the odor-activated phospholipase C pathway is controlled by kinase C activity.

The effect of both kinases seems to be specific for each pathway, as kinase C inhibitors did not affect the kinetics of

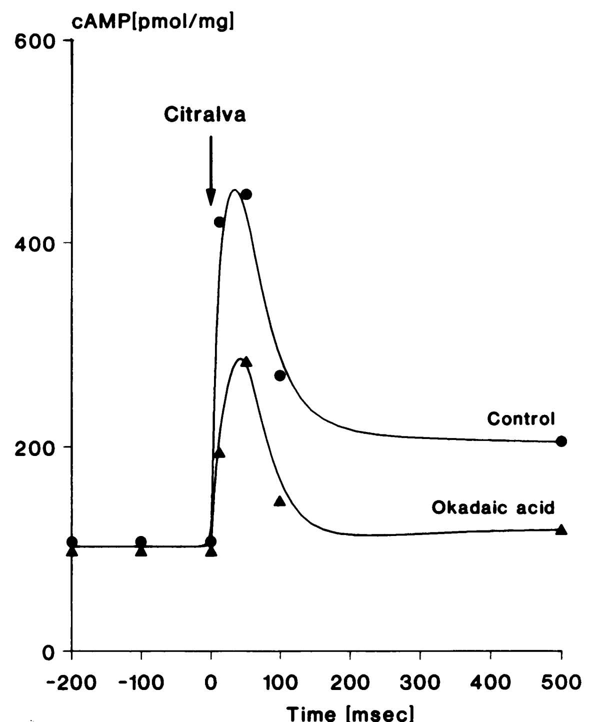

FIG. 4. Okadaic acid affects the citralva-induced cAMP signal in rat olfactory cilia. Cilia preparations were incubated for 15 min at 4°C with either buffer or 1 μM okadaic acid prior to stimulation with citralva. Inhibition of phosphatase activity significantly reduced the cAMP response; the kinetics was virtually unchanged but the cAMP level decayed to the basal level within 100 msec. Data represent the mean of three experiments performed in duplicate. Significance of the differences in cAMP concentration 50 msec after stimulus application was $P < 0.005$.

the cAMP signal, and the Walsh inhibitor was ineffective on the lyral-induced IP_3 response.

DISCUSSION

The rapid decay of odorant-activated second messenger signals in rat olfactory cilia is apparently due to a rapid desensitization of olfactory reaction cascades. The odor-induced increase of either cAMP or IP_3 concentrations peaks after ≈50 msec; thereafter the second messenger level rapidly decays, returning to the basal level within a few hundred milliseconds. The time course for termination of the molecular signal is reminiscent of the immediate psychophysical desensitization to odors (19, 20). A pronounced, immediate desensitization has recently been reported for odorant-stimulated adenylate cyclase activity in a primary culture of neonatal rat olfactory neurons (21).

In this study we have presented evidence indicating that the termination of olfactory signaling is brought about via a phosphorylation reaction mediated by specific protein kinases. It is interesting to note that an odorant-induced second messenger pathway is turned off only by a kinase that is activated by the messenger generated in this cascade: adenylyl cyclase activity and cAMP generation are affected by kinase A but not by kinase C; phospholipase C activity and IP_3/diacylglycerol formation are only affected by kinase C. These observations suggest that specific protein substrates are phosphorylated.

It is still elusive which elements of the reaction cascades are modified upon kinase reaction and thus uncouple the process of cAMP and IP_3 generation. However, desensitization phenomena in photoreceptors and hormone-sensitive cells have been attributed to phosphorylation of receptor

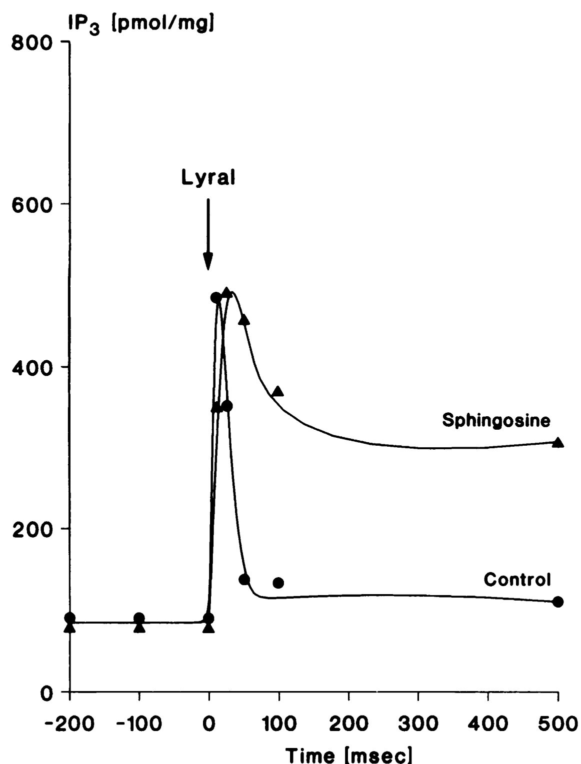

FIG. 5. Effect of kinase C inhibitors, such as sphingosine, on the IP_3 signal induced by lyral. Preparations of rat olfactory cilia were preincubated with sphingosine (100 μM) for 15 min at 4°C prior to stimulation with lyral (1 μM). At various intervals after stimulation, the concentration of IP_3 was determined according to Palmer *et al.* (11). Note that the offset kinetics of the IP_3 signal was significantly reduced; the IP_3 level stayed elevated even after 500 msec. Data represent the mean of three experiments, with a variation of <10%.

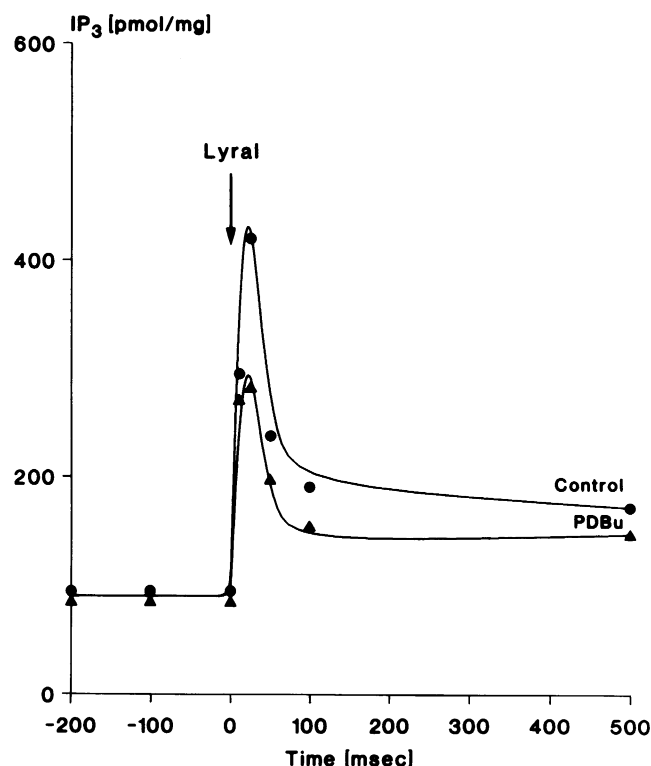

FIG. 6. Reduction of the lyral induced IP_3 signal upon activation of protein kinase C by phorbol ester. Rat cilia preparations were preincubated with phorbol dibutyrate (PDBu; 1 μM) for 15 min prior to stimulation. The IP_3 response of olfactory cilia was significantly reduced after stimulation of kinase C. The values are the means of three experiments performed in duplicate. Significance of differences in IP_3 concentrations 25 msec after stimulus application was $P < 0.005$.

proteins, notably rhodopsin, and β-adrenergic receptors (7, 15), respectively.

Unfortunately, until now odor receptor proteins have not been isolated; thus appropriate approaches like receptor phosphorylation *in vitro* have not been feasible yet. Phosphorylation experiments *in situ* may be hampered by the fact that active phosphatases are present in the preparation. Molecular cloning approaches have recently elucidated the primary structure of several putative odorant receptors, which are members of the seven-membrane-domain receptor superfamily (22). Upon inspection of the sequences, it turned out that all of them contain putative phosphorylation sites; therefore, these putative odorant receptors may represent substrates for protein kinases. Thus it is conceivable that desensitization upon olfactory signaling may be due to phosphorylation of odorant receptors.

This work was supported by the Deutsche Forschungsgemeinschaft Br 712/10-1.

1. Lancet, D. (1986) *Annu. Rev. Neurosci.* **9**, 329–355.
2. Breer, H., Boekhoff, I. & Tareilus, E. (1990) *Nature (London)* **344**, 65–68.
3. Boekhoff, I., Tareilus, E., Strotmann, J. & Breer, H. (1990) *EMBO J.* **9**, 2453–2458.
4. Firestein, S. & Werblin, F. (1989) *Science* **244**, 79–89.
5. Lazard, D., Zupko, K., Poria, Y., Nef, P., Lazarovits, J., Horn, S., Khen, M. & Lancet, D. (1991) *Nature (London)* **349**, 790–793.
6. Hausdorff, W. P., Caron, M. G. & Lefkowitz, R. J. (1990) *FASEB J.* **4**, 2881–2889.
7. Liebman, P. A. & Pugh, E. N., Jr. (1980) *Nature (London)* **287**, 734–736.
8. Anholt, R. R. H., Aebi, U. & Snyder, S. H. (1986) *J. Neurosci.* **6**, 1962–1969.
9. Chen, Z., Pace, U., Heldman, J., Shapira, A. & Lancet, D. (1986) *J. Neurosci.* **6**, 2146–2154.
10. Steiner, A. L., Pagliara, A. S., Chase, L. R. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1114–1120.
11. Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1989) *Cell. Signaling* **1**, 147–156.
12. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
13. Dickinson, K., Shirley, S. G. & Dodd, G. H. (1989) *Chem. Senses* **14**, 205.
14. Sibley, D. R., Benovic, J. L., Caron, M. G. & Lefkowitz, J. (1987) *Cell* **48**, 913–922.
15. Benovic, J. L. & Lefkowitz, R. J. (1987) *Enzymes* **18**, 319–333.
16. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1977–1985.
17. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102.
18. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., & Bell, M. (1986) *J. Biol. Chem.* **261**, 12604–12609.
19. Getchell, T. V. & Shepherd, G. M. (1978) *J. Physiol. (London)* **282**, 541–560.
20. Kurahashi, T. & Shibuya, T. (1990) *Brain Res.* **515**, 261–268.
21. Ronnett, G. V., Parfitt, D. J., Hester, L. D. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2366–2369.
22. Buck, L. & Axel, R. (1991) *Cell* **65**, 175–187.