*Proc. Natl. Acad. Sci. USA*
Vol. 73, No. 7, pp. 2520–2523, July 1976
Physiology

# Tetrodotoxin-resistant dendritic spikes in avian Purkinje cells

**(calcium action potentials/cerebellum)**

RODOLFO LLINÁS AND RAINER HESS*

Division of Neurobiology, Department of Physiology and Biophysics, University of Iowa, Oakdale, Iowa 52319

*Communicated by Francis O. Schmitt, April 12, 1976*

ABSTRACT **Electrophysiological evidence for the existence of dendritic spikes in the Purkinje cells of pigeon cerebellar cortex is presented. Intradendritic records indicate that the electroresponsive properties of the dendrites linger after voltage-dependent sodium and potassium conductances are reduced by superfusion of the cortex with Ringer's solution containing 20 μg/ml tetrodotoxin and 5 mM 3-aminopyridine.**

**Dendritic spikes could be evoked, in the complete absence of activity from all cerebellar afferents and from the soma and axon of the Purkinje cells, by direct electrical activation of the surface of the cerebellar cortex or by intracellular injection of current via the recording electrode. The dendritic electroresponsiveness was blocked by superfusion with 20 mM $Mn^{2+}$ or $Co^{2+}$. It is thus concluded that dendrites of Purkinje cells in birds are capable of generating calcium-dependent spikes. The possible role of such a calcium current in neuronal function is discussed.**

The presence of action potentials in the dendrites of Purkinje cells was originally demonstrated in alligators by field potential analysis (1, 2) and by means of direct intradendritic recording at the level of the molecular layer (3).

Comparative studies of cerebellar electrophysiology indicated that surface stimulation of the cerebellar cortex evokes a sharp, superficial, positive-negative wave produced by direct stimulation of the parallel fibers, followed by a secondary negativity (cf. ref. 4). In most vertebrates this latter negativity, which is produced by the activation of the parallel fiber–Purkinje cell junction, reverses to a positive wave at 100 μm depth and remains positive throughout the depth of the molecular layer. The above comparative study further determined that in pigeons, as in alligators (2), the superficial negativity does not reverse in depth. This particular distribution of the late negative wave has been found to be correlated with the active current sinks which characterize dendritic electroresponsiveness in the cerebellar cortex (2, 3).

The present study provides experimental evidence for the presence of dendritic spikes in avian Purkinje cells and considers the possible role of calcium as an active component in this form of activity.

## MATERIALS AND METHODS

Experiments were performed in domestic pigeons (*Columba livia*), 300–400 g in weight, anesthetized with pentobarbital (Nembutal®, 35 mg/kg), immobilized with gallamine triethiodide (Flaxedil®, 5 mg/kg), and artificially ventilated by perfusion with a 95% oxygen and 5% carbon dioxide mixture which escaped through the abdominal air sacs. The cerebellar cortex was exposed by carefully removing the occipital bone with a dental drill. The border of the severed cranial skin was sutured around a circular metal holder in order to establish a continuous perfusion pool over the cerebellar cortex. Intrasomatic and dendritic recordings were obtained with 2 M potassium citrate-filled microelectrodes with a dc resistance of 10–15 MΩ selected for linear properties with test sinusoidal currents from 100 to 1000 Hz. The surface of the cerebellar cortex was electrically activated with a bipolar local stimulating electrode (Loc) and the underlying cerebellar white matter, with a bipolar concentric electrode (Wm), located at a depth near the cerebellar nuclei.

Abbreviations: Loc, local stimulating electrode; Wm, white matter stimulating electrode; TTX, tetrodotoxin; 3-AmP, 3-aminopyridine; cGMP, guanosine 3′:5′-cyclic monophosphate.

* On leave from the Max-Planck-Institut für Biophysikalische Chemie, Göttingen, German Federal Republic.

## RESULTS

Local surface stimulation of the cerebellar cortex evoked the set of extracellular field potentials illustrated in Fig. 1A. Analysis of the laminar field potential confirmed the presence of large negative potentials attributable to inward ionic transmembrane current in the molecular layer between 100 and 300 μm from the surface. Characteristically, this negative field demonstrated a shift in latency with depth, as indicated by the second broken line in the figure. This delay has been interpreted as the conduction time of dendritic current sinks from their site of origin to the somatic level (2).

Following Loc stimulation it is possible to obtain, at 400 μm depth, intrasomatically recorded spikes from Purkinje cells which are identified by their antidromic invasion (3, 5). These intracellular records are characterized by a resting potential of −60 to −65 mV and by the generation of increasing numbers of action potentials as the Loc stimulus intensity is increased (Fig. 1B). Note that, as in alligators, local stimulation can produce burst responses which are graded, as opposed to the all-or-nothing climbing fiber activation (6). This type of graded burst response is characteristic of neurons having dendritic electroresponsiveness (7); the prolonged nature of the potential indicates summation of long-lasting dendritic spikes (3).

Typical intradendritic recordings (3) (i.e., long-lasting action potentials with multinotched rising phase) were routinely obtained in the molecular layer at 150–300 μm from the cerebellar surface. The resting potential of the dendrites was similar to that of the soma; the spikes evoked by Loc stimulation had, on the average, a duration of approximately 10 msec and a complex waveform (Fig. 1C). As reported for the alligator, hyperpolarizing current pulses injected through the recording pipette uncovered several distinct all-or-nothing components, indicating that these action potentials had more than one site of spike initiation (Fig. 1C). As in other Purkinje cells (3), the dendritic spikes thus appear to be conducted to the soma in a noncontinuous manner and produce, at that level, the prolonged after-depolarization seen in Fig. 1B. In conclusion, the striking similarity of the present potentials with those reported in alligators (3) strongly suggests that the Purkinje cells in pigeons have dendritic electroresponsiveness.

Following this initial investigation, a set of experiments was developed to study the possible ionic mechanisms responsible

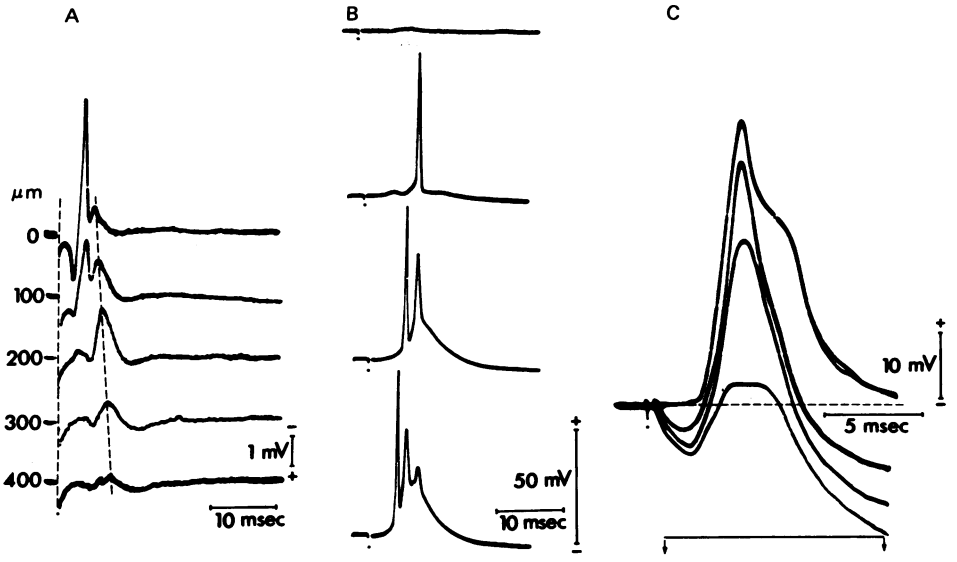


FIG. 1. Electrophysiological evidence for dendritic spikes in pigeon Purkinje cells. (A) Laminar field potentials evoked by local stimulation of the cerebellar cortex. Recordings were taken under a beam of activated parallel fibers at the depth indicated to the left in micrometers. Note the marked late negative field indicated by the second broken line and its increased latency with depth. (B) Intrasomatic penetration of a Purkinje cell. Spikes were evoked with Loc stimuli of increasing strength. (C) Intradendritic recordings show the amplitude and duration of dendritic spikes and their multiple all-or-none components as revealed by intracellular injection of hyperpolarizing currents. In C, the line marked by arrows indicates duration of the hyperpolarizing current pulse. Time and voltage calibration as indicated.

for these spikes. Thus, the potential-dependent sodium and potassium membrane conductances were blocked with tetrodotoxin (TTX) and 3-aminopyridine (3-AmP), respectively. TTX is known to block such sodium permeability in many types of excitable cells (8, 9) with few exceptions (10–12); however, this drug spares the "late" calcium conductance change (13, 14). The potassium currents were reduced with 3-AmP, which decreases voltage-dependent potassium conductance change in invertebrate nerve (15, 16) and synapse (17) without blocking the voltage-dependent late calcium current (17). After the superfusion of 5 mM 3-AmP for 10 min, Wm and Loc stimulation generated field potentials with an unusually late negative component (note arrows in Fig. 2B and E), probably due to prolonged synaptic action of parallel fibers on Purkinje cells and/or of the electroresponsive properties of the Purkinje cells themselves. This slow response has been seen also in the cat cerebellum, where it is apparently accompanied by a large transient increase in extracellular potassium (C. Nicholson, G.

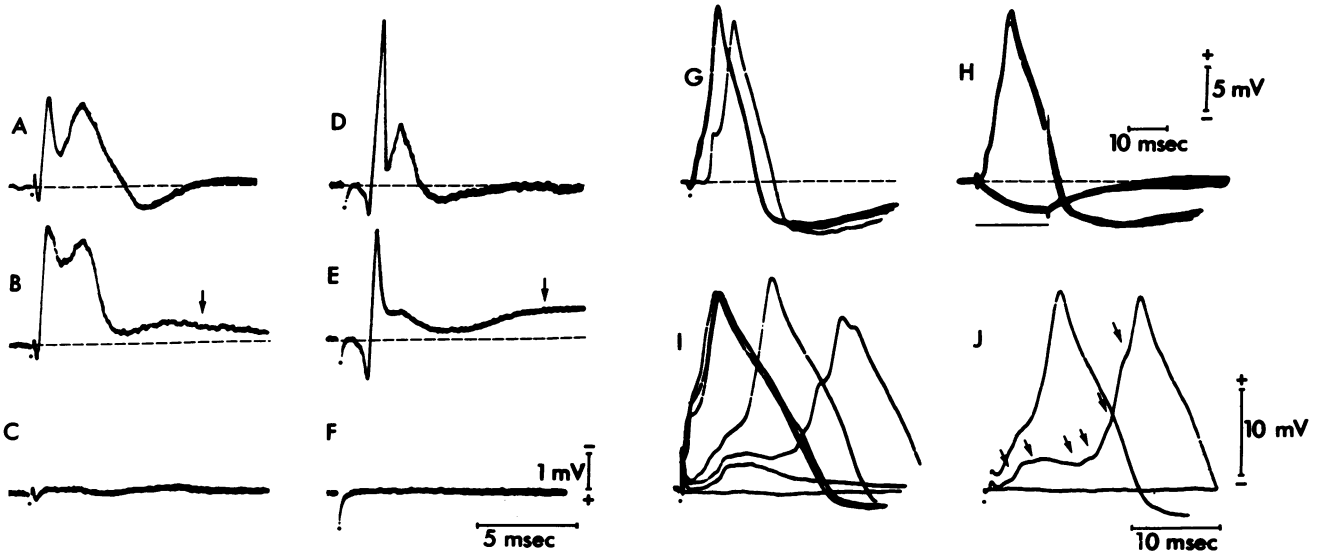


FIG. 2. Dendritic spike generation after administration of TTX and 3-AmP. (A and D) Field potentials generated by Wm and Loc stimulation, respectively. (B and E) Changes of these waveforms following administration of 3-AmP. (C and F) Complete blockage of these responses after 15 min of 20 μg/ml of TTX perfusion. (G–J) Intradendritic action potentials recorded 250 μm from cerebellar surface after 30 min of TTX and 3-AmP superperfusion. In G, Loc stimulation produces either a direct activation of dendritic spike or activation after a delay. In H, a similar action potential is activated in the same dendrite by intracellular outward current injection through the recording microelectrode; hyperpolarization with inward current pulse is also shown. (I and J) Records similar to those in G at a higher sweep speed. Note that as the Loc stimulus is decreased, the response moves from a direct activation (four superimposed records) to those showing progressive increase in latency. In J, several all-or-none components generating this spike are indicated by arrows. Note the prolonged nature of the action potential and the rather slow rate of rise. In H, the current injection is indicated by bar at the bottom of the record. Time and voltage calibration for A–F are indicated in F.

ten Bruggencate, and R. Senekowitsch, personal communication). This drug was followed by 20 μg/ml of TTX continuously superfused over the cortex of the cerebellum for up to 2 hr. After about 15 min of this superfusion, all electrical activity produced by Wm and parallel fiber stimulation was completely abolished (Fig. 2C and F). (In fact, a similar finding was obtained with a TTX superfusion of 2 μg/ml.) The blockage of the field potentials evoked by Loc and Wm stimuli was used as a probe to test the completeness of the TTX poisoning. A depth study of the antidromic field potential revealed the TTX blockage of cerebellar activity to be complete down to 2000 μm from the surface. No active field potential response of any type could be evoked by the Wm stimulus down to that depth.

Under these conditions, microelectrode penetrations into cellular elements of the molecular layer of the cerebellar cortex revealed the persistence of prolonged, low-voltage, action potentials which could be generated either by strong Loc stimulation or by passing current through the recording electrode. The action potentials were recorded most frequently between 150 and 300 μm from the surface of the cortex. They consisted of several components, as illustrated in Fig. 2G–J. As in the case of dendritic action potentials seen in pigeons under normal circumstances, several sites of spike generation were probably involved, given the multiple all-or-nothing components present in the recording.

Their break points were, however, more numerous and their amplitude smaller. The overall duration of the spikes was, in fact, as long as 40 msec. No difference in the resting dendritic membrane potential was apparent. On several occasions, Loc stimulation near the site of impalement produced, besides direct activation of these elements, a more delayed spike response (Fig. 2G, I, and J). As this happened in the absence of any parallel fiber action potentials, it is most probably due to direct depolarization of parallel fiber terminals. Such depolarization should be capable of releasing synaptic transmitter from these fibers, and thus of depolarizing Purkinje cells in a manner similar to that observed in squid giant synapse under similar conditions (17, 18).

The dendritic electroresponsiveness encountered after the TTX and 3-AmP superfusion was abolished by substances known to block the late calcium channel such as $Mn^{2+}$ or $Co^{2+}$ (13, 14, 19). These ions were applied to the cerebellar cortex in concentrations of 10 and 20 mM in Ringer's solution, and the excitability of Purkinje cells was tested after periods of 0.5–2 hr of continuous exposure. At these concentrations, the cerebellar cortex showed no action potentials or other electrical activity, either spontaneously or following strong Loc stimulation.

In order to ensure that neither $Co^{2+}$ or $Mn^{2+}$ had a marked effect on the TTX-sensitive excitability of either Purkinje cells or parallel fibers, a separate set of experiments was designed. The effects of these ions on the field potentials produced by the parallel fiber volley following Loc stimulation and on the field potential produced by the antidromic invasion of the cerebellar cortex was tested during superfusion with $Co^{2+}$ and $Mn^{2+}$ as utilized above. Under these circumstances, the positive-negative field potential produced by the parallel fiber activation was slightly reduced, while that corresponding to the activation of the parallel fiber–Purkinje cell synapse was totally abolished. This is to be expected, since the above ions blocked synaptic transmission through the blockage of the calcium current. Similarly, the antidromic field potential produced by Wm stimulation consisted of a large negative field at the level of the Purkinje cell layer and below, with none of the complex potentials produced by the synaptic activation of the granular layer. Again, as with the parallel fiber volley, there was a slight decrease in the field potentials and a reduction of the invasion of the field into the molecular layer.

These experiments indicate, therefore, that the concentrations of $Co^{2+}$ and $Mn^{2+}$ which block the TTX-insensitive dendritic potentials do not interfere significantly with excitability of the soma or axon of the Purkinje cell nor with that of the parallel fibers. We conclude, therefore, that the dendritic spiking observed after TTX and 3-AmP superfusion is produced by inward calcium current similar to that reported in the squid nerve by Baker *et al.* (19) and named by them the "late calcium current."

## DISCUSSION

This paper presents electrophysiological evidence that the dendrites of Purkinje cells in pigeons have electroresponsive properties similar to those of alligators. The evidence obtained by field potential analysis and intracellular recording at the molecular layer strongly suggests that the dendrites of these Purkinje cells are capable of generating spikes. Our suggestion that these dendritic spikes are generated by calcium currents is based on two sets of evidence. First, they persist after superfusion with TTX at a concentration one order of magnitude higher than needed for complete blockage of parallel fiber activation and of antidromic Purkinje cell invasion. (Since the antidromic fields were blocked to a depth of 2000 μm, this indicates that TTX diffuses through the molecular layer and reaches the Purkinje cell and granular layer, continuing into the white matter of the cerebellar cortex.) Second, $Co^{2+}$ and $Mn^{2+}$—which are known to block potential-dependent calcium conductances (13, 14, 19)—produce total abolition of these TTX-resistant spikes but do not themselves block either parallel fiber action potentials or axonic or somatic activation (i.e., they do not appear to interfere largely with the TTX-sensitive-electroresponsiveness).

In conclusion, therefore, the Purkinje cell dendrites appear to have calcium electroresponsiveness, very much in keeping with similar findings in invertebrate neurons and in the frog spinal ganglion and motoneuron, which have been reported to generate calcium-dependent action potentials (20–23; cf. ref. 24). As in the case of the presynaptic terminal in the squid, it is probable that the calcium electroresponsiveness may not normally be large enough to produce full-size action potentials due to the shunting effect produced by potassium conductance change (25). Indeed, the addition of 3-AmP, which can act in the squid synapse very much like tetraethylammonium (17), does tend to make the presence of these TTX-resistant spikes more conspicuous.

The presence of calcium electroresponsive properties in dendrites of Purkinje cells in birds suggests that the dendritic spikes present in many different types of central neurons (26, 27) may be partly carried by this ion. In fact, indirect evidence for this possibility is provided by field potential analysis of the cat cerebral cortex (28).

The functional significance of a calcium current in dendrites is difficult to assess at this moment. It may be speculated, however, that calcium current may be an important component in the dendro-dendritic interaction (cf. ref. 29), where calcium entry must be essential for the release of transmitter from the "reciprocal" synapses. Further, it has now been shown in vertebrate and invertebrate neurons that the late potassium conductance that generates the long-lasting after-hyperpolarization may be caused by an inward calcium current during the action potential (23, 30–35, cf. ref. 36). Moreover, a slow, noninactivating, inward calcium current has recently been reported

in snail neurons (37–39), which may be related to the pacemaker activity of these cells.

Finally, it has been shown that calcium is important in the regulation of the intracellular level of guanosine 3′:5′-cyclic monophosphate (cGMP) (40). In the cerebellum of mice it has been found that depolarizing agents such as veratridine and ouabain or an increase in extracellular potassium raise the level of cGMP only in the presence of extracellular calcium (41, 42). These data are supported by experiments in the bovine superior cervical ganglion (43). There too the magnitude of the acetylcholine-induced increase in cGMP was considerably reduced by incubation of the tissue in calcium-free Ringer's solution prior to the addition of acetylcholine. Though it is not clear whether depolarization alone is sufficient to cause an increase in cGMP (cf. ref. 43), conditions leading to depolarization (such as repetitive activation of the climbing fiber–Purkinje cell synapse) increase the intracellular cGMP concentration (44), while those increasing the level of γ-aminobutyric acid tend to decrease cGMP intracellularly.

It is quite conceivable, therefore, that entry of calcium following activation of a potential-dependent calcium channel may be the link between electrical activity of the neuronal membrane and increased levels of intracellular cGMP, leading, among other changes, to alterations in the molecular matrix of the plasma membrane.

Research was supported by USPHS Grants NS-09916 and NS-05748 from the National Institute of Neurological and Communicative Disorders and Stroke.

1. Llinás, R., Nicholson, C., Freeman J. & Hillman, D. (1968) *Science* **160**, 1132–1135.
2. Nicholson, C. & Llinás, R. (1971) *J. Neurophysiol.* **34**, 509–531.
3. Llinás, R. & Nicholson, C. (1971) *J. Neurophysiol.* **34**, 532–551.
4. Llinas, R., Hillman, D. E. & Precht, W. (1970) in *The Cerebellum in Health and Disease*, eds. Fields, W. S. & Willis, W. D., Jr. (Warren H. Green Inc., St. Louis, Mo.), pp. 269–291.
5. Eccles, J. C., Llinás, R. & Sasaki, K. (1966) *J. Physiol. (London)* **182**, 316–345.
6. Llinás, R. & Nicholson, C. (1969) in *Neurobiology of Cerebellar Evolution and Development*, ed. Llinás, R. (American Medical Assc., Chicago, Ill.), pp. 431–465.
7. Kandel, E. R. & Spencer, W. A. (1961) *J. Neurophysiol.* **24**, 243–259.
8. Narahashi, T., Moore, J. W. & Scott, W. R. (1964) *J. Gen. Physiol.* **47**, 965–974.
9. Blankenship, J. E. & Kuno, M. (1968) *J. Neurophysiol.* **31**, 195–209.
10. Narahashi, T. (1974) *Physiol. Rev.* **54**, 813–889.
11. Wald, F. (1972) *J. Physiol. (London)* **220**, 267–281.
12. Kleinhaus, A. L. & Prichard, J. W. (1976) *Brain Res.* **102**, 368–373.
13. Hagiwara, S. & Takahashi, K. (1967) *J. Gen. Physiol.* **50**, 583–601.
14. Baker, P. F., Hodgkin, A. L. & Ridgway, E. B. (1971) *J. Physiol. (London)* **218**, 709–755.
15. Pelhate, M. & Pichon, Y. (1974) *J. Physiol. (London)* **242**, 90–91P.
16. Yeh, J. Z., Oxford, G. S., Wu, C. H. & Narahashi, T. (1976). *Biophys. J.* **16**, 77–81.
17. Llinás, R., Walton, K. & Bohr, V. (1976) *Biophys. J.* **16**, 83–86.
18. Katz, B. & Miledi, R. (1967) *J. Physiol. (London)* **192**, 407–436.
19. Baker, P. F., Meves, H. & Ridgway, E. B. (1973) *J. Physiol. (London)* **231**, 511–526.
20. Standen, N. B. (1975) *J. Physiol. (London)* **249**, 241–252.
21. Kleinhaus, A. L. & Prichard, J. W. (1975) *J. Physiol. (London)* **246**, 351–361.
22. Koketsu, K. & Nishi, S. (1969) *J. Gen. Physiol.* **53**, 608–623.
23. Barrett, E. F. & Barrett, J. N. (1976) *J. Physiol. (London)*, **255**, 737–774.
24. Reuter, H. (1973) *Prog. Biophys. Mol. Biol.* **26**, 1–43.
25. Katz, B. & Miledi, R. (1969) *J. Physiol. (London)* **203**, 459–487.
26. Purpura, D. P. (1967) in *The Neurosciences: A Study Program*, eds. Quarton, G. C., Melnechuk, T. & Schmitt, F. O. (Rockefeller Univ. Press, New York), pp. 372–393.
27. Llinás, R. (1975) "Physiology and pathology of dendrites," in *Advances in Neurology*, ed. Kreutzberg, G. W. (Raven Press, New York), Vol. 12, pp. 1–13.
28. Garcia-Ramos, J. & Ibarra, B. H. (1973) *Acta Physiol. Lat. Am.* **23**, 202–212.
29. Shepherd, G. M. (1972) *Physiol. Rev.* **52**, 864–917.
30. Meech, R. W. (1972) *Comp. Biochem. Physiol. A* **42**, 493–499.
31. Meech, R. W. (1974) *J. Physiol. (London)* **237**, 259–277.
32. Krnjevic, K. & Lisiewicz, A. (1972) *J. Physiol. (London)* **225**, 363–390.
33. Jansen, J. K. S. & Nicholls, J. G. (1973) *J. Physiol. (London)* **229**, 635–655.
34. Nishi, S. & North, R. A. (1973) *J. Physiol. (London)* **231**, 471–491.
35. Clusin, W., Spray, D. C. & Bennett, M. V. L. (1975) *Nature* **256**, 425–427.
36. Lux, H. D. & Schubert, P. (1975) "Physiology and pathology of dendrites," in *Advances in Neurology*, ed. Kreutzberg, G. W. (Raven Press, New York), Vol. 12, pp. 29–44.
37. Meech, R. W. & Standen, N. B. (1975) *J. Physiol. (London)* **249**, 211–239.
38. Eckert, R. & Lux, H. D. (1976) *J. Physiol. (London)* **254**, 129–151.
39. Eckert, R. & Lux, H. D. (1975) *Brain Res.* **83**, 486–489.
40. Schultz, G., Hardman, J. G., Schultz, K., Baird, C. E. & Sutherland, E. W. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3889–3893.
41. Ferrendelli, J. A., Kinscherf, D. A. & Chang, M. M. (1973) *Mol. Pharmacol.* **9**, 445–454.
42. Ferrendelli, J. A., Chang, M. M. & Kinscherf, D. A. (1974) *J. Neurochem.* **22**, 535–540.
43. Kebabian, J. W., Steiner, A. L. & Greengard, P. (1975) *J. Pharmacol. Exp. Ther.* **193**, 474–488.
44. Guidotti, A., Biggio, G. & Costa, E. (1975) *Brain Res.* **96**, 201–205.