Hyperpolarization by Cyclic AMP (Activation of Diphosphoinositide Kinase)

During activity the cyclic AMP content of neurons may increase[1-3]. This increase seems to generate membrane hyperpolarization[1-3]. cAMP usally acts by first forming a complex with one of its specific receptors. This specific receptor has been identified in the present study as the regulatory subunit of intrapostsynaptic diphosphoinositide kinase (DPIK).

In vitro experiments. DPIK is a soluble enzyme[4] that phosphorylates DPI (diphosphoinositide) to TPI (triphosphoinositide). The enzymic activity of DPIK was studied by measuring[5] the nmoles of TPI formed during 5 min (37°C) incubation of an enzyme preparation with the essential cofactors ($MgCl_2$, ATP), and DPI. DPIK was purified from rat or rabbit brain[5]. The catalytic and regulatory subunits of DPIK were obtained by weakening the bonds through a 20 min exposure to [^{3}H] cAMP. The mixture was chromatographed on a Sepharose 4B column[6] (Pharmacia, Sweden) previously equilibrated with 10 m*M* 2 [N-morpholino] ethane sulfonic acid buffer (pH 6.0) and 2 m*M* EDTA. The column was eluted by a linear gradient of NaCl in the same buffer (Figure 1). The proteins were measured by means of an LKB ultraviolet adsorptiometer uvicord at 280 nm, [^{3}H] cAMP with a dioxane based scintillant. 9–26 ml of the eluate contained all protein-bound [^{3}H] cAMP (fraction A). 27–190 ml of the eluate contained other proteins (fraction B). Fractions A and B were separately dried on a rotary evaporator at 4°C collection temperature and were diluted with *Tris*-HCl buffer (pH 7.2) before use.

The enzymic activity was linear for 10 min in the presence of ATP (5–8 m*M*) and $MgCl_2$ (0.6–2 m*M*). DPIK activity was proportional with the protein concentration (range of 0.4 mg). The K_m was $1.43 \times 10^{-4} M$ for DPI, and $2.5 \times 10^{-5} M$ for ATP. The pH optimum was 7.0–7.5. Purified DPIK yielded 6.6 nmoles of TPI/min/mg of protein. It was unaffected by –SH, Ca^{++}, Na^+, K^+, and was activated by cAMP. Fraction A lacked enzyme activity. Fraction B yielded 111 nmoles of TPI/min/mg of protein, and was unaffected by –SH, Ca^{++}, Na^+, K^+, and cAMP.

Fraction A and B seemend to function as the regulatory and catalytic subunits of DPIK. When incubated together, the enzymic activity of fraction B decreased in proportion with the amounts of fraction A. Further incubation with added cAMP reversed the inhibition (in proportion with the amount of cAMP bound to fraction A of the fraction A–B compound formed during the incubation). cAMP increased the enzymic activity only in those mixtures where fractions A and B formed a complex (e.g. purified DPIK, mixture of fractions A and B), but did not affect the activity of fraction B alone. Therefore, the regulatory subunit of DPIK may serve as a specific receptor of cAMP (Table).

In vivo experiments. Observations on membrane hyperpolarization. The intracellular bioelectric process of the superior cervical ganglion of rabbit[7,8] were recorded with glass microelectrodes (0.5 nm tip diameter, 10 *M*Ohm tip resistance, filled with 4 *M* K citrate), on a dual oscilloscope (using a cathode ray follower). The presynaptic neuron was stimulated by constant voltage rectangular pulses (1/min) through platinum wire electrodes. Hyperpolarizing currents were given through the intracellular microelectrodes. Intrapostsynaptic microinjections were given by combining pressure with iontophoresis (40 nA for 3–30 msec). The subunits of DPIK were prepared by the above method described for preparation of fraction A (without cAMP) and fraction B. 1 Unit of fraction B (catalytic subunit) was arbitrarily chosen as the amount of enzyme that yielded 1 ng of TPI/min. 1 Unit of the regulatory subunit inhibited 1 U of fraction B. The substances were injected in increasing amounts, until biolelectric changes were observed. Less than 5 U always sufficed. Spontaneous recovery always occurred in less than 30 min.

Intrapostsynaptic microinjections of DPIK with DPI or cAMP with DPI caused hyperpolarization that was comparable to the effect of hyperpolarizing electical stimuli. The effects of weak electrical stimuli and low concentrations of the chemicals (cAMP and DPI or DPIK and DPI) were additive. Since the intracellular space lacks free DPI[4], DPI had to be simultaneously injected. Since cAMP acted only when its specific intrapostsynaptic phosphodiesterase[9] was inhibited by theophylline, and since cAMP did not generate full hyperpolarization without added DPI, one may assume that cAMP induced hyperpolarization through activation of DPIK. Inhibition of DPIK resulted in significant decrease of hyperpolarization (regardless of whether it was induced by electrical or chemical means). The activity of DPIK was inhibited by lack of DPI or by the regulatory subunit of DPIK. These experiments suggest that hyperpolarization by cAMP may be induced through activation of DPIK. Intrapostsynaptic cAMP increase during preynaptic stimulation is due to the activity of dopaminergic interneurons[10,11] (Figure 2).

Discussion and conclusions. The results fully support the assumption of DURELL et al.[12] and of KAI et al.[5] that hyperpolarization results from synthetic and not from

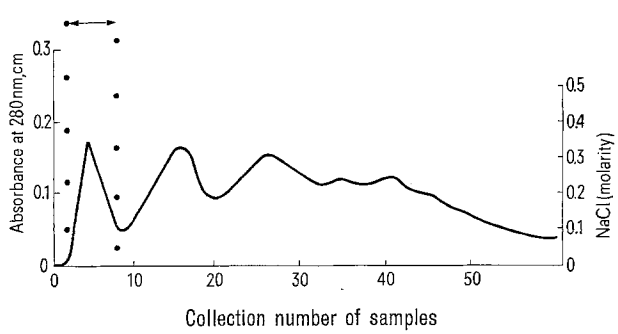

Fig. 1. Chromatography of DPIK on sepharose 4B column. Each sample contained 3.5 ml of eluate. The number of the samples was indicated on the horizontal axis. The protein contents of the samples was recorded on the vertical axis. All [^{3}H] cAMP content appeared between the dotted lines (9–26 ml of eluate).

[1] *Advances in Biochemical Psychopharmacology*, 1970) vol. 3.
[2] C. A. ROBISON, R. W. BUTCHER and E. W. SUTHERLAND, *Cyclic AMP* (Academic Press, New York 1971).
[3] C. TORDA, Res. Commun. Chem. Biol. Pharmac. *2*, 483 (1971).
[4] J. N. HAWTHORNE and M. KAI, *Handbook of Neurochemistry* (Ed. A. LAJTHA; Plenum Press, N.Y. 1970), vol. *3*, p. 491.
[5] M. KAI, J. G. SALWAY and J. N. HAWTHORNE, Biochem. J. *106*, 791 (1968).
[6] E. M. REIMANN, C. O. BROSTROM, J. D. CORBIN, C. A. KING and E. G. KREBS, Biochem. biophys. Res. Commun. *42*, 187 (1971).
[7] R. M. ECCLES and B. LIBET, J. Physiol., Lond. *157*, 484 (1961).
[8] B. LIBET, Fedn. Proc. *29*, 1945 (1970).
[9] N. T. FLORENDO, R. J. BARRNETT and P. GREENGARD, Science *173*, 745 (1971).
[10] H. MCLENNAN, *Synaptic Transmission* (Saunders, Philadelphia 1970).
[11] D. A. MCAFEE, M. SCHRODERET and P. GREENGARD, Science *171*, 1156 (1971).
[12] J. DURELL, J. T. GARLAND and R. O. FRIEDEL, Science *165*, 862 (1969).

Observations on the DPIK-activity of the various enzyme preparations ± cAMP

Type of enzyme preparation used for incubation	No. of experiments	Incubation time (min)	Amounts of [^{32}P] TPI formed from DPI and [^{32}P] APT during incubation of the various enzyme preparations with DPI and the essential cofactors ($MgCl_2$, [^{32}P] ATP)	
			nmoles TPI/min/mg of enzyme protein followed by the S.E. of mean	Control (%)
Purified DPIK	25	5	6.6 ± 0.18	100
Same and cAMP ($1 \times 10^{-7} M$)	25	5	39.6 ± 0.20	600
Fraction B (catalyt. subu.)	30	5	111.0 ± 0.15	100
Same and cAMP ($1 \times 10^{-7-3} M$	25	5	107.7 ± 0.10	96.4
Same and fraction A (reg. sub.) (prepared with cAMP)	25	5	92.8 ± 0.12[a]	82.9
Same and fraction A (no cAMP)				
($^1/_4$ of Fr.B[b])	20	5	85.7 ± 0.14	77.2
($^1/_2$ of Fr.B)	20	5	57.2 ± 0.16	51.5
($^3/_4$ of Fr.B)	20	5	26.0 ± 0.17	23.4
Same and fraction A ($^3/_4$ of Fr.B)	20	5	26.0 ± 0.17	23.4
Same and same and cAMP (a)[a]	20	5	39.6 ± 0.21	35.7
(2a)	20	5	52.7 ± 0.25	47.5
Fraction A (with or without cAMP)	30	5	0	0

[a] Some inhibition of the enzymic activity of DPIK (fraction B) occurred due to redistribution of fraction A between cAMP and fraction B according to their relative affinity, during the incubation period. [b] Unit of fraction B was arbitrarily chosen as the amount of enzyme that yielded 1 ng of TPI formed/min. Fraction B was expressed in U. Fraction A was added in various amounts, e.g. $^3/_4$; $^1/_2$; or $^1/_4$ amounts in U of the fraction B content. 1 U of fraction A inhibited 1 U of fraction B. [c] (a) is an arbitrarily chosen amount of cAMP that did bind to fraction A during the 5 min incubation of cAMP and fraction A and fraction B.

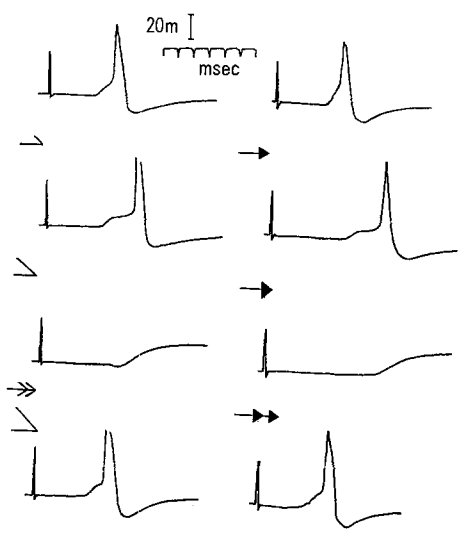

Fig. 2. Effects of activated and inhibited DPIK on membrane hyperpolarization. Column 1. Effects of hyperpolarizing currents and inhibition of DPIK. Row 1: Postsynaptic response to supramaximal presynaptic pulses. Row 2 and 3: Hyperpolarizing currents of increasing intensity were applied (see half arrows). Response decreased due to hyperpolarization. Row 4: Reversal of hyperpolarization after microinjections of the regulatory subunit of DPIK. Column 2. Effects of the activated and inhibited DPIK on membrane hyperpolarization. Row 1: Postsynaptic response to supramaximal presynaptic pulses. Row 2 and 3: Added microinjections of the catalytic subunit of DPIK (and DPI) induced hyperpolarization that decreased the response of the postsynaptic neuron. Row 4: Reversal of hyperpolarization after microinjections of the regulatory subunit of EPIK. (Similar hyperpolarization was generated by either the catalytic subunit of DPIK (and DPI or cAMP and DPI).

hydrolytic processes. The in vitro experiments suggest that the regulatory subunit of DPIK (or a closely related substance) is one of the specific receptors of cAMP. The in vivo experiments suggest that activation of DPIK by cAMP may couple in time and space the formation of cAMP-receptor complex, ion transport, and membrane hyperpolarization: On combination with cAMP, the regulatory subunit ceases to inhibit the catalytic subunit of DPIK, and DPI is phosphorylated to TPI. Since TPI is a stronger chelating agent than DPI[13,14], membrane-bound Ca^{++} increases, ion transport decreases to that of Cl^- and K^+. The sum of the equilibrium potentials of the migrating Cl^- and K^+ is expressed as membrane hyperpolarization[15].

Zusammenfassung. In-vitro-und In-vivo-Versuche beweisen, dass ein molekulärer Mechanismus für das Zustandekommen der Membran-Hyperpolarisation verantwortlich ist.

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27 March 1972.

[13] R. M. C. DAWSON, Biochem. J. *97*, 134 (1965).
[14] H. S. HENDRICKSON and J. L. REINERTSEN, Biochem. biophys. Res. Commun. *44*, 1258 (1971).
[15] R. D. KEYNES and F. R. LEWIS, J. Physiol., Lond. *114*, 151 (1951).
[16] Presented in part at the Annual Meeting of the Biophysical Society, Toronto, Canada, February 1972.