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CALCIUM IONS AND THEIR RELATION TO SYMPATHETIC GANGLIONIC TRANSMISSION AND TO CYCLIC AMP LEVELS IN ISOLATED SUPERIOR CERVICAL GANGLION OF THE RAT IN VITRO

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Summary. The rat superior cervical ganglion was investigated in vitro by means of extracellularly recorded compound action potentials and of cyclic AMP content in relation to Ca^{2+}-concentrations in the extracellular fluid. Threshold concentrations for the appearance of the potentials were lower than 1 mM. Raising the Ca^{2+}-content in the Krebs solution above 40 mM led to an increase of the slow excitatory negative potential and to the appearance of the slow inhibitory positive wave, while the fast excitatory compound action potential disappeared. This effect was calcium-specific.
On the other hand, cyclic AMP accumulation in the ganglion seems to be calcium-independent. While high Ca^{2+}-concentrations (60 mM) in the medium led to a significant decrease of the cyclic AMP content, neurotransmitter-induced cyclic AMP accumulation occurred regardless from varying the extracellular calcium content. High external Ca^{2+} antagonized the ability of the β-adrenergic blocker propranolol in preventing isoproterenol-induced cyclic AMP accumulation to some extent. It was concluded that calcium ions are necessary for the appearance of fast and slow postganglionic compound action potentials in the ganglion. These events seemed to be independent from drug-induced changes in the ganglionic cyclic AMP content.

Introduction. There is considerable evidence that calcium ions are directly involved with many molecular events in the nervous system (1-4). The most prominent role of this alkaline earth ion is its direct association with the release of transmitter molecules from nerve endings during nerve cell excitation (5-7). The link between nervous membrane excitation, release of the neurotransmitter ACh and calcium was first demonstrated in the superior cervical ganglion (SCG) of the cat by Harvey and MacIntosh (5). They showed that removal of calcium from the perfusion medium abolished the release of ACh from preganglionic nerve terminals. The essen-

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tial requirement of calcium for synaptic transmission has been established over the last three decades (3).
Cyclic AMP is believed to mediate the postsynaptic effects of certain neurotransmitters (4, 7-9). A relationship between calcium and cyclic AMP in the excitation-secretion process of neurotransmitter molecules has been proposed (8,9). But in spite of cumulating evidence in favour of such a link the complexicity of the responses of neurohumoral agents in the nervous system has as yet defied any simple interpretation (10,11).
Returning to the original system - the superior cervical ganglion - where the first effects of calcium ions were described, it is of special interest to see if there is any causal relationship between ganglionic transmission, calcium and cyclic AMP.
The rat SCG in vitro was used for extracellular recordings of ganglionic transmission and for concurrent measurements of cyclic AMP. Variation of calcium in the extracellular fluid led to substantial changes in the transmission process both of fast and slow components of the compound action potential (CAP). It is shown, however, that these variations of calcium concentrations in the bathing fluid do not affect drug-induced cyclic AMP accumulations in the ganglion. A preliminary account of this work has been presented (12).

Materials and Methods. Male Wistar rats (250 - 300 g) were killed by cervical dislocation and the ganglia were rapidly removed and desheathed. Special care was taken to keep the attached nerve trunks as long as possible. After the dissection procedure the ganglia were pre-incubated for one hour in oxygenated Krebs-Henseleit solution (95% O_2/5% CO_2) at 37° C. For extracellularly recorded CAPs the ganglia were placed in a perspex chamber and superfused with the oxygenated saline at 37° C. Postganglionic CAPs were recorded via a suction electrode from the internal carotid nerve as described (13,14). Recording and amplification equipment was conventional. Photographs were taken with a Polaroid camera from a storage oscilloscope.
Cyclic AMP was measured according to the method of Gilman (15) as described in detail earlier (16). For these determinations ganglia were pre-incubated in Krebs-solution for one hour at 37° C and then, unless otherwise stated, for a further 30 minutes in Krebs-solution containing 10^{-5} M 3-isobutyl-1-methylxanthine (IBMX) in order to inhibit degradation of cyclic AMP by endogenous phosphodiesterases (17). For statistical analysis the Student's t-test was used.
Verapamil was kindly provided by Knoll, Ludwigshafen and Nifedipin by Bayer, Leverkusen.

Results

1) Extracellularly recorded compound action potentials and their relation to Ca^{2+} in the external medium from rat superior cervical ganglion *in vitro*.

In electrophysiological experiments the postganglionic response (recorded at the internal carotid nerve) to supramaximal preganglionic electrical stimulation consisted of a fast compound action potential followed by a slow negative after potential (Fig. 1a). When Ca^{2+} was omitted from the bathing fluid the ganglia did not respond to electrical stimulation. Raising the Ca^{2+}-concentration to 1 mM resulted in a dose-dependent appearance of fast and slow CAPs in response to preganglionic stimulation (n = 15; data not shown). Optimal Ca^{2+}-concentrations for maximal CAP-amplitudes ranged from 1 mM to 5 mM (Fig. 1a). Within this concentration range, the shape and magnitude of CAPs were not changed (n = 8). A gradual increase of Ca^{2+}-concentration above 10 mM led to a progressive decrease in the amplitude of fast and slow potentials. At Ca^{2+}-concentrations above 20 mM no further potentials were recorded (n = 8). Raising the Ca^{2+}-concentration in the external Krebs-Henseleit solution beyond 40 mM resulted in a disappearance of the fast CAP within 30 seconds (Fig. 1b, upper trace). Concomitantly with this decrease of the fast potential, the amplitude of the slow negative wave increased by up to 50% and time course increased by 45% (Fig. 1b, lower trace). These effects occurred rapidly. Within 30 seconds the reduction of the fast CAP and the increase of the slow potential could be readily seen (Fig. 1b, upper trace) and within 1 minute the fast CAP disappeared completely (Fig. 1b, lower trace). After 5 minutes of incubation with the elevated Ca^{2+}-concentrations a new slow positive wave appeared (Fig. 1c). Stable recordings could be achieved for more than 45 minutes from beginning with the superfusion of elevated Ca^{2+}-concentrations (Fig. 1c). The effects of high Ca^{2+} in the bathing solution on the CAPs were readily reversible as shown in Fig. 1d. Even 1 minute after superfusion with Krebs-saline containing the normal amount of Ca^{2+} (i.e. 2.2 mM) the fast CAP appeared again nearly completely (Fig. 1d-e). The slow positive wave disappeared as soon as the

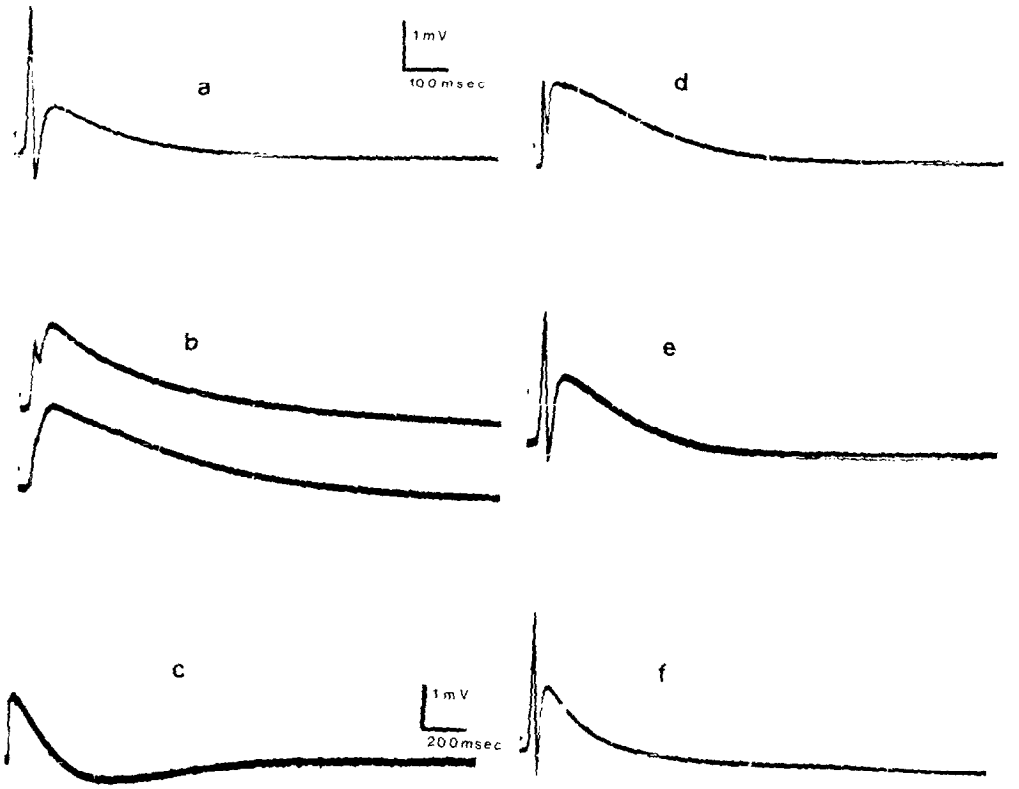

Figure 1:
Time course of the effects of high Ca^{2+}-concentrations (40 mM) in the medium and their reversibility on extracellularly recorded CAPs from rat SCG in vitro.
a) Control recording (Ca^{2+}: 2.2 mM)
b) Upper trace: Recording after 30 sec of superfusion with 40 mM Ca^{2+}
Lower trace: Recording after 1 min of superfusion with 40 mM Ca^{2+}
c) Recording after 10 and 45 min of superfusion with 40 mM Ca^{2+}
d) Recording after 1 min of returning from high (40 mM) to control (2.2 mM) concentrations of Ca^{2+}
e) Same conditions as d) after 5 min
f) Same conditions as d) after 1 hr
Calibration marks: a,b,d-f: 1 mV,100 msec; c: 1 mV,200 msec.

fast CAP returned. Activation of the slow negative wave was demonstrable even 1 hr after superfusion with normal Krebs-solution (Fig. 1f). Although the dynamics of the activation of the slow negative wave and the disappearance of the fast CAP caused by high Ca^{2+}-concentrations were not studied in detail, it should be noted that these effects on ganglionic

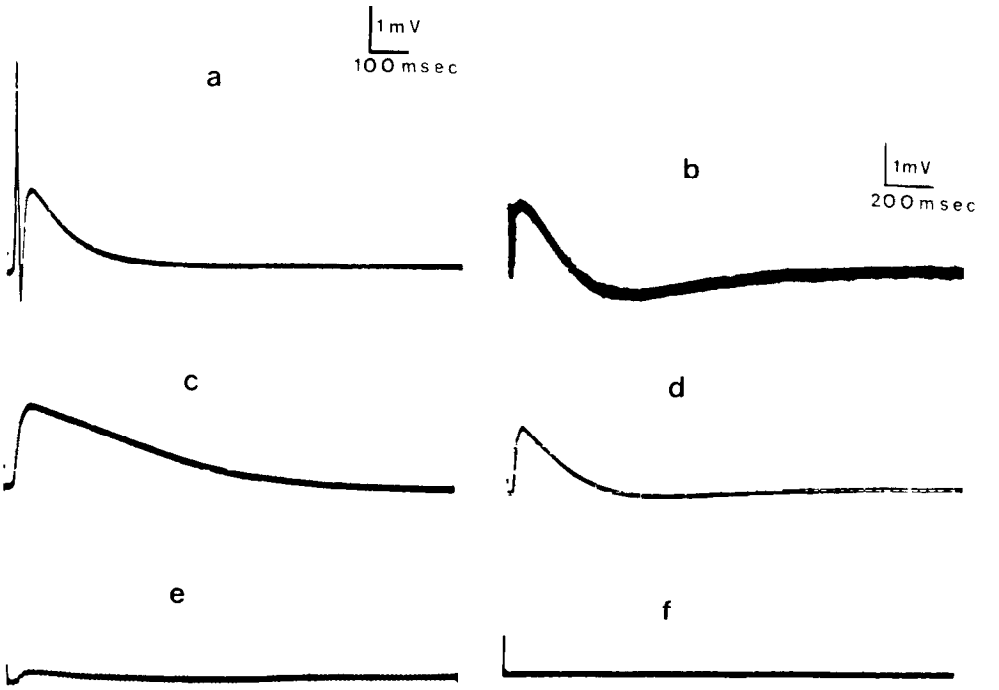

Figure 2:
Effects of Ca^{2+}-antagonists, atropine and of antidromic stimulation on calcium-induced effects of extracellularly recorded CAPs from rat SCG in vitro. Ca^{2+}-conc.: 40 mM.
a) Control recording (Ca^{2+}: 2.2 mM)
b) 40 mM Ca^{2+}
c) Ba^{2+} or Sr^{2+} (10 mM)
d) Verapamil or Nifedipin (10^{-5} M)
e) Atropine (10^{-5} M)
f) Antidromic electrical stimulation
Ca^{2+}-conc. in b-f: 40 mM; incub.-time: 10 min; pre-incub.-time with the blockers c)-e): 20 min.
Calibration marks: a,c-f: 1 mV, 100 msec; b: 1 mV, 200 msec.

transmission were sensitive to prior incubation with saline in which calcium concentrations were varied. This could be readily demonstrated when ganglia were superfused with Ca^{2+}-free saline and then switched immediately to high Ca^{2+}-concentrations. Under these experimental conditions only 30 mM Ca^{2+}-concentrations were needed for the activation of the slow negative wave and for inhibition of the fast CAP (Fig. 1b-d).

Prior incubation of ganglia with 10 mM Ba^{2+} or with 10 mM Sr^{2+} did not influence the effects of high Ca^{2+} on the fast and slow negative waves. But under these conditions there

Table 1:

Effects of various Ca^{2+}-concentrations in the incubation medium on cyclic AMP content in superior cervical ganglia of the rat in vitro. Values are expressed as picomoles cyclic AMP per ganglion (mean ± S.E.M.). n = 3 - 8.

Incubation conditions	time of incubation 4 min	15 min	30 min
Control: 2.2 mM Ca^{2+}	9.8 ± 2.1	11.7 ± 1.8	10.9 ± 2.6
- Ca^{2+}	10.4 ± 0.9	12.8 ± 2.8	11.9 ± 3.0
- Ca^{2+}; + 1 mM EGTA	12.4 ± 2.0	13.6 ± 2.3	16.9 ± 1.5 §
- Ca^{2+}; + 12 mM Mg^{2+}	10.3 ± 1.0	14.8 ± 2.2	15.8 ± 0.6 §
20 mM Ca^{2+}	8.9 ± 2.9	7.8 ± 1.7	8.0 ± 3.1
60 mM Ca^{2+}	5.9 ± 0.9 §	4.9 ± 1.2 §	3.3 ± 1.7 §

significantly different from control: § $p < 0.05$

was no slow positive wave present (Fig. 2c, n = 5). Verapamil and Nifedipin, two selective Ca^{2+}-antagonists, in concentrations of 10^{-5} M failed to influence the effects of elevated Ca^{2+} (Fig. 2d versus 2b). Atropine at low concentrations (10^{-5} M) was able to suppress the slow negative wave nearly completely after 10 minutes of incubation in the presence of high Ca^{2+} (Fig. 2e). This is interpreted as an indication for participation of muscarinic cholinergic receptors in this Ca^{2+}-dependent process. Antidromic electrical stimulation of the ganglion showed no electrical activity in the preganglionic nerve indicating the absence of electrotonically mediated Ca^{2+}-effects (Fig. 2f). Ba^{2+} and Sr^{2+} at high concentrations (20-60 mM) caused the disappearance of all recorded CAPs (Fig. 2f).

2) Effects of variations of Ca^{2+}-concentrations in the incubation medium on cyclic AMP content in rat superior cervical ganglion in vitro.

In contrast to the electrophysiological observations where calcium-effects were prominent, cyclic AMP accumulation was not affected by Ca^{2+} to the same extent. Table 1 shows that the levels of cyclic AMP actually decreased when ganglia were incubated in saline containing high amounts of calcium

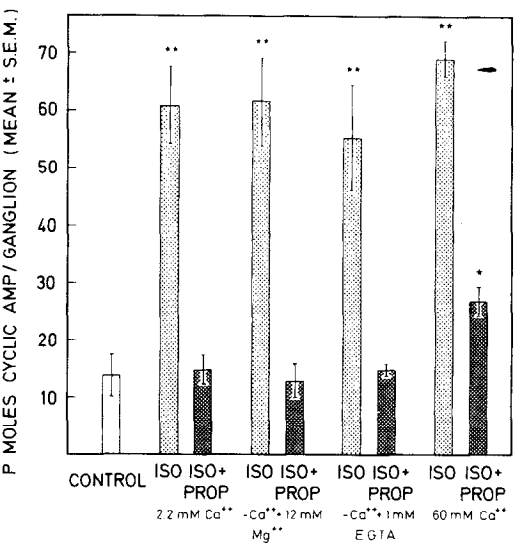

Figure 3:
Effects of propranolol (PROP, 10^{-5} M) on isoproterenol (ISO, 10^{-4} M)-induced accumulation of cyclic AMP in rat SCG in vitro under different calcium-conditions in the external medium. Incub.-time: 4 min (n = 5).
Significantly different from control: $^{*}p<0.05$; $^{**}p<0.01$.

ions. Omission of Ca^{2+} from the incubation medium did not significantly influence cyclic AMP levels in ganglia. Incubation of the tissue in Ca^{2+}-free medium and addition of either 1 mM EGTA or 12 mM Mg^{2+} increased cyclic AMP levels within 30 minutes.

Isoproterenol-induced cyclic AMP accumulation, via activation of ganglionic adenylate cyclase, was unchanged when ganglia were incubated in saline with different Ca^{2+}-concentrations (Fig. 3). In connection with the unaffected amine-induced response, due to activation of the β-adrenergic receptor of the enzyme, the blocking ability of propranolol, a potent β-blocking agent (18,19), was not significantly different under the various calcium concentrations except at high Ca^{2+}-concentrations. 60 mM Ca^{2+} in the incubation medium reduced the blocking ability of propranolol significantly (Fig. 3).

The effect of other biogenic amines and related neurotransmitters which are capable of activating ganglionic adenylate cyclase under control conditions (14,18,19) with the addi-

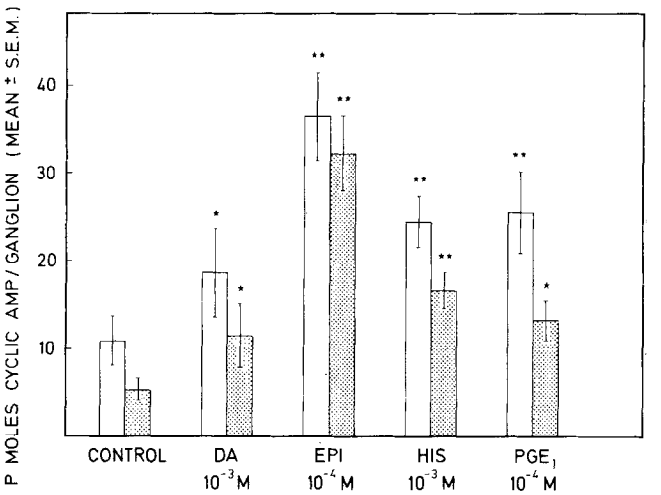

Figure 4:
Effects of dopamine (DA), epinephrine (EPI), histamine (HIS) and prostaglandin E_1 (PGE_1) on accumulation of cyclic AMP in rat SCG in vitro in the presence of low (2.2 mM = open bars) and of high (60 mM = dotted bars) calcium concentrations. Incub.-time: 4 min (n = 5).
Significantly different from control: $^{*}p<0.05$, $^{**}p<0.01$.

tion of 60 mM Ca^{2+} were investigated further. Although cyclic AMP concentrations fell markedly under high Ca^{2+}-additions in the incubation medium, accumulation of cyclic AMP caused by the neurotransmitters was almost the same as under control conditions (Fig. 4).

Discussion.

There is growing evidence that Ca^{2+}-induced potentials from nerve cells are mediated via special Ca^{2+}-channels in the membrane (20-22). It is also known that other divalent alkaline earth ions are capable of replacing Ca^{2+} at low concentrations (1-4). Ba^{2+} and Sr^{2+} are among the most potent of these while Mg^{2+} and Mn^{2+} are ineffective or even inhibitory (24,25). In contrast to their effects at low divalent ion concentrations, Ca^{2+} cannot be replaced at high concentrations by Ba^{2+} or by Sr^{2+}. In addition, these two alkaline earth ions are unable to antagonize the Ca^{2+}-induced changes in the transmission properties of the ganglion.
Verapamil and Nifedipin failed also to influence the Ca^{2+}-

effects although it was shown that these drugs could antagonize Ca^{2+}-influx in the myocardium effectively (26-28). It must be noted however that both drugs showed no influence on any potentials recorded after electrical stimulation in the ganglion.

Cholinergic participation in this process is suggested because of the sensitivity to pre-incubation of ganglia with atropine of the Ca^{2+}-induced activation of the slow negative wave. From this and earlier studies (13,29) it may be assumed that ACh is the mediator of this excitatory negative wave. This wave was characterized previously by Dunant as the slow EPSP in the rat SCG (13). The activation of the slow EPSP by high Ca^{2+} in the extracellular fluid may reflect an indirect action of ACh. Elevated Ca^{2+}-concentrations in the preganglionic nerve endings would be helpful for a further release of ACh and this enhanced release of the neurotransmitter may be the reason for the activation of this excitatory potential. A similar activation pattern of the slow EPSP could be achieved after prolonged stimulation of ganglia (13). It is known that electrical stimulation enhances Ca^{2+}-influx in both preganglionic and postganglionic elements (30-32). It is also known that the amount of ionized Ca^{2+} within the nerve cell is relatively low compared with the outside of the cell (1,31,32). It is possible therefore, that the increased amounts of ionized Ca^{2+} in the nerve cells are able to trigger the observed effects on ganglionic transmission. The appearance of the slow positive wave may be the consequence of inhibition of the fast postganglionic CAP. The same phenomenon was observed after superfusion of ganglia with low concentrations of mecamylamine, a ganglionic blocker (13). From biochemical studies it has been suggested that calcium is able to elevate cyclic AMP concentrations in nervous tissues suggesting a direct connection of cyclic AMP, Ca^{2+} and the transmission process (33). Findings from the present study show that elevated Ca^{2+}-concentrations in the extracellular fluid lowered the cyclic AMP content significantly. In addition the rise in cyclic AMP accumulation when ganglia are incubated in the absence of Ca^{2+} is consistent with this observation. It is possible, however, that the internal Ca^{2+}-content even after one hour of incubation in Ca^{2+}-free solu-

tion is still sufficient to preserve the full sensitivity of ganglionic adenylate cyclase to neurotransmitter activation. On the other hand, the inverse relationship between calcium and cyclic AMP content suggested rather a total independence of the enzyme from the divalent alkaline earth ion. The lowering of cyclic AMP content in ganglia due to high Ca^{2+}-concentrations in the extracellular fluid may be the consequence of activation of Ca^{2+}-dependent phosphodiesterase, which probably overcame the inhibition by IBMX (19,34). The failure of Ca^{2+} in influencing the sensitivity of ganglionic adenylate cyclase to neurotransmitters is probably the consequence of a complex relationship between Ca^{2+} and the receptor protein, which is responsible for binding and for the specificity of the activating transmitter. It is assumed that there is no causal relationship between the two processes, first the influx of Ca^{2+} into nerve cells due to activation by a preganglionic electrical pulse and secondly the activation of ganglionic adenylate cyclase by the neurotransmitter. The only significant interaction between calcium and the ganglionic adenylate cyclase in the intact ganglion is the apparent decreased blocking ability of propranolol in preventing cyclic AMP accumulation due to activation of the β-adrenergic receptor system (18,19,35).

In summary it is concluded that, besides the direct effects of low Ca^{2+} in the external fluid on ganglionic neurotransmission, this alkaline earth ion caused at high extracellular concentrations three readily demonstrable effects: Firstly, high Ca^{2+}-concentrations inhibited the fast postganglionic compound action potential probably due to its well known anaesthetic effects on nervous membranes (36). Secondly, both the amplitude and time course of the slow negative wave increased under these conditions. This effect seemed to be calcium-specific although its mechanism is unclear. Thirdly, regarding neurotransmission, the consequence of high Ca^{2+} and the inhibition of the fast CAP is the appearance of a slow positive wave which can be tentatively interpreted as the slow IPSP (29).

In contrast to these biophysical effects on the neuronal membrane the biochemical data present little evidence for a di-

rect and causal relationship between ganglionic transmission, Ca^{2+}-concentrations and the second messenger system. Therefore, interpretations of data which suggest a direct relationship between cyclic AMP and neurotransmission must be considered suspect (8,9). Other cell functions which are not directly linked with the transmission process cannot be overlooked. These functions include nerve cell metabolism, fast and slow axonal transport and the interrelationship between nonneuronal cells and nerve cell function (37).

References

1. Baker, P.F. Progress Biophys. Molec. Biol. 24, 177-223 (1972)
2. Baker, P.F. In "Calcium in biological systems" SEB Symposia 30, 67-88 (1976)
3. Baker, P.F. and Reuter, H. Calcium movement in excitable cells. Pergamon Press, Oxford, 1975
4. Rassmussen, H. and Goodmann, D.P.B. Physiol. Rev. 57, 421-509 (1977)
5. Harvey, A.M. and MacIntosh, F.C. J. Physiol. (London) 97, 408-416 (1940)
6. Hutter, O.F. and Kostial, K. J. Physiol. (London) 124, 234-241 (1954)
7. Rubin, R.P. Calcium and the secretory process. Plenum Press, New York (1974)
8. Greengard, P. and Kebabian, J.W. Fed. Proc. 33, 1059-1067 (1974)
9. Greengard, P. Nature 260, 101-108 (1976)
10. Cramer, H. and Schultz, J. Cyclic 3',5'-nucleotides: Mechanism of action. J. Wiley & Sons, London, 1977
11. Schwabe, U., Ohga, Y. and Daly, J.W. Naun. Schmdbg. Arch. Pharmacol. 302, 141-151 (1978)
12. Lindl, T. Hoppe Seyler's Zschr. f. Physiol. Chemie 359, 296 (1978)
13. Dunant, Y.J. Physiol. (London) 221, 577-587 (1972)
14. Lindl, T. Neuropharmacology, in press
15. Gilman, A.G. Proc. Natl. Acad. Sci. (USA) 67, 305-312 (1970)
16. Lindl, T. and Cramer, H. Biochim. Biophys. Acta 343, 182-191 (1974)
17. Lindl, T., Teufel, E. and Cramer, H. Hoppe Seyler's Zschr. f. Physiol. Chemie 357, 983-989 (1976)
18. Cramer, H., Johnson, D.G., Hanbauer, I., Silberstein, S.D. and Kopin, I.J. Brain Res. 53, 97-104 (1973)
19. Phillis, J.W. J. Canad. Sci. Neurol. 4, 151-195 (1977)
20. Tomita, T. Progress Biophys. Molec. Biol. 30, 185-203 (1975)
21. Reiser, G., Heumann, R., Kemper, W., Lautenschlager, E. and Hamprecht, B. Brain Res. 130, 495-504 (1977)
22. Schwartzkroin, P.A. and Slawsky, M. Brain Res. 135, 157-161 (1977)
23. Douglas, W.W., Lywood, D.W. and Straub, R.W. J. Physiol. (London) 156, 515-522 (1961)

24. McLachlan, E.M. J. Physiol. (London) 267, 497-518 (1977)
25. Silinsky, E.M. J. Physiol. (London) 274, 157-171 (1978)
26. Fleckenstein, A. Drug Res. 22, 22-33 (1972)
27. Kolhardt, M., Bauer, B., Krause, H. and Fleckenstein, A. Experientia 28, 288-289 (1972)
28. Vater, W., Kroneberg, G., Hoffmeister, F., Kaller, H., Oberdorf, A., Meng, K., Schlossmann, K., Puls, W. and Stoepel, K. Drug Res. 22, 1-14 (1977)
29. Libet, P. Fed. Proc. 29, 1945-1956 (1970)
30. Lipicky, R.J., Hertz, L. and Shanes, A.M. J. Cell. Comp. Physiol. 62, 233-242 (1963)
31. Robison, J.D. and Kabela, E.J. Neurobiol. 8, 511-522 (1977)
32. Blaustein, M.P. Science 172, 391-393 (1971)
33. Roth, R.H. Naun. Schmdbg. Arch. Pharmacol. 289, 327-343 (1975)
34. Lindl, T. and Chapman, G. Biochem. Biophys. Res. Commun. 71, 1272-1282 (1976)
35. Lindl, T. and Cramer, H. Biochem. Biophys. Res. Commun. 65, 731-739 (1975)
36. Blaustein, M.P. J. Pharmacol. Exp. Ther. 196, 80-86 (1976)
37. McIlwain, H. Neuroscience 2, 357-372 (1977)