0014-4754/94/040342-04$1.50 + 0.20

'Action substances' of peripheral nerve re-visited

S. Weidmann

Physiological Institute, University of Bern, Bühlplatz 5, CH-3012 Bern (Switzerland)

Dr. Silvio Weidmann is Emeritus Professor of Physiology at the University of Bern, Switzerland, and was a member of the Editorial Board of EXPERIENTIA from 1974 to 1982.

Abstract. Fifty years ago, four 'action substances' were described as being 'liberated' from peripheral nerve in the course of activity. An attempt has been made to update this information. Confirmatory evidence is available that acetylcholine and thiamine do play a role in the course of activity. However, the question of the relationship in time between their release and the electrical events, and the possible effect on specific ionic gating mechanisms, remains unclarified. The 'liberation' of potassium has been found to be a transmembrane K efflux, and the question of the existence of another substance similar to thiamine has not been pursued.

Volume 1 of EXPERIENTIA (1945) contains a review article entitled 'Vagusstoffe' (vagal substances), by Alexander von Muralt[1], one of the founding editors of EXPERIENTIA. The review was followed in 1946 by a more extensive monograph[2], which gave details of four chemical agents that had been shown to be 'liberated' during nerve activity: acetylcholine, thiamine, potassium and an unidentified fourth substance. The present author wrote his doctoral thesis from 1943 to 1946 and joined the staff of von Muralt's laboratory in 1951. This paper summarizes the work on 'action substances' done in von Muralt's and other laboratories, and looks at the way in which these topics have developed since.

Acetylcholine

The idea that transmission between frog vagal fibres and cardiac muscle was humoral, first suggested by Otto Loewi in 1921[3], had been generally accepted by 1945 and had been extended to other nerve-muscle and nerve-nerve junctions (see ref. 1). There were several reports by 1936 pointing to the 'liberation of a substance resembling acetylcholine', as shown by biological tests. The substances concerned seemed to diffuse out of a fresh cross-section of a nerve, especially when the latter was stimulated. The method of collecting fluid from a cross-section was deservedly criticized in 1937[4]. Von Muralt thought of a method known to him from muscle physiology, namely to fix the chemical state of excitation by rapid freezing. Nerves were vertically mounted in a holder and 'shot' into a bath of liquid air. Using one animal, one sciatic nerve was stimulated at 50/s while the corresponding nerve was 'shot in' when at rest. Pieces of the frozen nerves were ground using sea sand and solid CO_2, then extracted by adding Ringer's solution with an enzyme added to inhibit the splitting of acetylcholine. Tonic contractions of the dorsal muscle of the leech were recorded and compared to the contractions obtained with known concentrations of pure acetylcholine. Average equivalent values found were 0.15 µg/g and 0.24 µg/g for the resting and stimulated sciatic nerves, respectively[5,6]. There were some problems in finding the proper speed for shooting nerves into liquid air. Rapid cooling in itself would stimulate, yet impulse propagation would be too slow to let the wave of excitation travel any appreciable distance. With a nerve stimulated at 50 Hz the time interval between two spikes is 20 ms, with a spike duration of the order of 1 ms. At a conduction velocity of 20 ms^{-1} two successive spikes would be separated by 2000 cm $s^{-1} \times 20 \times 10^{-3}$ s or 40 cm. With nerve lengths of 5 cm, and with no synchronization between the dropping of the nerves into the liquid air and the stimuli, the chance of losing the spike and freezing the nerve during the restitution phase would be considerable. A compromise for the falling speed (100 cm s^{-1}) was found, for which it could be anticipated that 2–3 full cycles would be frozen. If indeed 'liberation' and the splitting of acetylcholine were strictly confined to the electrical spike, the ratio reported between active and resting nerves (160% to 100%) seems to be rather large.

Dropping any object into liquid air will result in the transient formation of a layer of gas surrounding the object. This acts as an insulator against the flow of heat, and precludes a truly instant fixation of excitation waves. However, since gas formation is expected to have the same delaying action in excited and in resting nerve, this should not invalidate the conclusions. As to the role played by acetylcholine in the propagation of the impulse, v. Muralt[1] writes 'acetylcholine is connected with the process of excitation, while propagation of the impulse is due to the spread of the electrical field (in ref. 7).' In modern terms, he would have connected acetylcholine release in some way with open Na^+ channels.

A more detailed view of the role of acetycholine was presented by Nachmansohn[8,9]. He suggested that the substance is dissociated from a store by the approaching action potential and given the possibility of combining with a receptor. This combination would correspond to the opening of Na^+ channels. Dissociation of the complex resulting from a splitting of acetylcholine by choline/esterase would allow the channels to close. Following the spike, acetylcholine would be reconstituted enzymatically and bound to the store. A good argument in favour of Nachmansohn's hypothesis is the presence of choline/esterase close to the surface membrane of non-myelinated axons. A missing key argument is the possible opening of single Na^+ channels today, which might be demonstrated by recording current from single channels using the inside-out configuration of the patch. The nerve cell, as argued by v. Muralt, is a functional unit, whence the statement 'whatever is possible at the terminals (liberation of acetylcholine) must be possible along the whole length of the fibers'[2]. It seems strange that v. Muralt did not test the many pharmacological agents which were already known to increase or decrease cholinergic transmitter release at nerve terminals. He may well have anticipated that the sheath of a peripheral nerve would be a considerable diffusion barrier[10,11].

Much attention has been devoted over the past decades to acetylcholine as a transmitter at nerve terminals. Comparatively little effort has been made to clarify further the role of acetylcholine along the axon. There is good evidence in favour of a transport of acetylcholine and the enzymes choline/esterase and choline/acetyltransferase from cell bodies to terminals[12], but there seem to be no recent reports on acetylcholine in connection with membrane activity.

Thiamine (Vit. B_1)

Thiamine is an essential factor for nerve function, as is most convincingly demonstrated by the disease beriberi, which results from a B_1-deficient diet[13]. The discovery that thiamine is liberated from the cross-section of a stimulated nerve had been reported by Minz in 1938[14]. At this time v. Muralt was busy testing the effects of acetylcholine liberation on leech muscles, and was concerned about 'irregularities of results' which made him think that there might be a second humoral factor besides acetylcholine[15]. During the Second World War, when Captain v. Muralt spent much of his time with the artillery, his laboratory was busy exploring all sorts of tests to quantitate the thiamine liberation in frog sciatic nerves. These were bradycardia in the rat, the growth rate of Phycomyces, CO_2 production by yeast, and thiochrome fluorescence. It was realized that none of these tests could be claimed to be highly specific for thiamine. But v. Muralt felt confident that the results of all four tests, when taken together, did not leave any reasonable doubt as to the identity of the agent under investigation.

The thiochrome method also gave a clue concerning the localization of thiamine. Single fibers of the frog sciatic were treated by mild oxidation at an alkaline pH, fixed in methanol and preserved in glycerol[16]. This method is known to transform thiamine into a fluorescing substance (thiochrome) with an emission maximum near 358 nm. Much to everybody's astonishment, the nodes of Ranvier were found to be free of fluorescence, while the myelin sheath was intensely and evenly stained. This does not easily fit in with the idea that thiamine 'liberation' is intimately connected with the process of excitation, since the structure responsible for excitation is now recognised as the nodal membrane[17]. Thiamine has also been reported to be set free, as well as acetylcholine, by vagal nerve endings in the frog's heart[18]. Both agents are transported towards the endings of frog sciatic nerves, as is demonstrated by the accumulation of both acetylcholine[12] and ^{35}S-labelled thiamine[19] proximal to a crush or ligature.

In 1961, Gurtner administered ^{35}S-thiamine to rats and compared extracts from sciatic nerves frozen during activity or during rest[20]. He divided the total radioactivity into 4 fractions by paper electrophoresis and identified these fractions as thiamine triphospate (ThTP), diphosphate (ThDP), monophosphate (ThMP), and un-phosphorylated thiamine (Th). A quantitative comparison of the components by autoradiography suggested that ThTP and ThDP diminished as a result of stimulation, while the ThMP and Th fractions increased. 'Liberation' would thus mean 'dephosphorylation'. An alternative way of getting more information about Th was by way of anti-thiamines, which naturally occur in fern and carp intestines or have been synthesized and shown to compete with Th (pyrithiamine, neopyrithiamine). Isolated nodes of Ranvier (ridge method of Stämpfli, ref. 21) were used to test the antagonists. Kunz in 1952[22] found a characteristic action of neopyrithiamine on the time-course of the monophasic action current: depolarization was slowed and repolarization was delayed. There was no change of resting potential. Kunz interpreted the neopyrithiamine effect in terms of a partial inhibition of the rapid inward current, and thus saw the role of thiamine as an agent required for the proper operation of the Na^+ carrier (today: Na^+-channel). A more detailed analysis by a voltage-clamp method[23] disclosed that neopyrithiamine, besides depressing the peak amplitude of the rapid inward current, decreased both its rate of rise and its rate of fall.

Nodes of Ranvier are sensitive to UV irradiation in the sense that threshold increases as a function of time. A wavelength of 265 nm is most effective, and this coincides with the peak of absorption of thiamine. Using a stroboscopic method, v. Muralt and Stämpfli[26] applied

flashes of equal energy and lasting for 5 ms in different phases of the monophasic action potential. They observed a maximal effect of the flashes coincided with the peaks of the action potentials. Shorter flashes of 3 µs gave a more complicated picture, with a first maximum at 0.5 ms (i.e. before the end of the refractory phase) and a second maximum at 8 ms. In his short note v. Muralt[27] did not attempt a correlation between the state of ionic channels and UV absorption. The first maximum would coincide with the process of recovery from inactivation of Na^+ channels whilst the second maximum would seem to have no correlate.

The account on thiamine as an 'action substance' should not be closed before drawing attention to the finding that thiamine diphosphate is identical with cocarboxylase[24], an active coenzyme for pyruvate dehydrogenase (pyruvic acid to acetaldehyde). A competitive inhibitor of cocarboxylase is oxythiamine. Rats given oxythiamine develop bradycardia and weight loss, similarly to animals fed on a thiamine-deficient diet, while animals given neopyrithiamine develop cramps[28]. Von Muralt first pointed out this double action of thiamine in 1943[25]; however, the present survey deliberately focuses on the role of thiamine as an 'action substance'.

Potassium

In his monograph of 1946 (ref. 2, p. 302), v. Muralt reviews pre-existing reports about stimulation-induced loss of potassium into a bathing solution and concludes that in some way potassium must be 'liberated from its binding site'. It was well known at that time that muscle and nerve fibers contain much potassium and little sodium[28], also that there is a surface membrane about 100 Å in thickness.

In 1947 Hodgkin and Huxley[29] immersed non-myelinated single nerve fibers of crab in oil. This technique leaves a narrow aqueous space surrounding the fiber, within which any agent moving out through the surface membrane will accumulate. A burst of activity resulted in a reversible decrease of membrane resistance, and the same effect was observed if the nerve was dipped into a solution with a higher K^+ concentration and pulled up into oil again. The next step was to load nerve fibers of *Sepia* with ^{42}K and to compare the efflux of radioactivity at rest and during stimulation. The K^+-loss calculated by Keynes[30] is 4.7 pmol cm^{-2} $impulse^{-1}$, and this figure compares very satisfactorily with the expected K^+ loss calculated from voltage clamp data in giant axons of the squid[31]. Finally, by measuring the diffusion coefficient and electrophoretic mobility of $^{42}K^+$ injected into *Sepia* axons it could be concluded that at least 90% of the total potassium within the axoplasm is free to move[32]. This made it necessary to re-interpret v. Muralt's 1946 data in the sense that 'bound' potassium is actually K^+ moving freely within the intracellular space, and the 'liberation' of K^+, the third 'action substance', is simply transmembrane efflux.

An 'action substance' named A_4

The polarographic method introduced by Heyrovský[33] measures electrical current between a reference electrode and a capillary cathode from which mercury droplets are producted at a rate of about 1 per second. Positively charged inorganic ions and organic compounds are reduced at the mercury surface at well-defined potential differences, and this gives rise to a step when current is plotted against voltage. Thiamine shows up as a current step near 1900 mV, and there is a similar step in polarograms of tissue extracts. Comparing solutions from stimulated and from resting sciatic nerves seemed to indicate that the process of thiamine liberation was more complex than had been realized. A closer look at the properties of the 'thiamine step' revealed that the two substances behaved differently in at least two respects (ref. 2, pp. 304–309). The substance in nerve extracts resists boiling, whereas thiamine is thermolabile. Also, the substance from nerve shows a pH-sensitivity which is quite different from that of thiamine. I felt slightly uneasy when I had to tell my chief about these findings, but was relieved by his saying simply, 'so we have a new action substance, and let us call it A_4'. As far as I am aware, nobody has ever followed the traces of this ill-defined substance.

Epilogue

Von Muralt's experimental work drifted away from 'action substances' to nerve regeneration and to high-altitude physiology. In 1968, following his retirement, he concentrated his efforts on looking into physical changes in the course of nerve activity. He assured himself of the superiority of non-myelinated fibers (pike olfactory nerve) over frog sciatics, the former having much more excitable surface area per unit volume. With the monophasic action potential as a reference signal, he recorded 1) changes of light scattering and induced fluorescence[35], 2) heat production (with Howarth, Keynes and Ritchie[36]) and 3) changes of birefringence (with Weibel and Howarth[37]). It remains to be seen whether the 'chemical spike' or the 'optical spike' will turn out to have more impact on the future development of nerve physiology.

1 Muralt, A. von, Experientia *1* (1945) 136.
2 Muralt, A. von, Die Signalübermittlung in Nerven. Birkhäuser, Basel 1946.
3 Loewi, O., Pflügers Arch. *189* (1921) 239.
4 Gaddum, J. H., Kayyal, M. A., and Rydin, H., J. Physiol. *89* (1937) 9P.
5 Muralt, A. von, Proc. R. Soc. *B 123* (1937) 399.
6 Muralt, A. von, Pflügers. Arch. *245* (1942) 604.

7 Hodgkin, A. L., J. Physiol. *90* (1937) 183.
8 Nachmansohn, D., Circulation Res. *3* (1955) 429.
9 Nachmansohn, D., Science *134* (1961) 1962.
10 Krnjevic, K., J. Physiol. *123* (1954) 338.
11 Nicely, M., Experientia *11* (1955) 199.
12 Dahlström, A., Booj, S., Larsson, P. A., and McLean, W. G., Acta physiol. scand. *115* (1982) 493.
13 Eijkman, C., Virchows Arch. path. Anat. Physiol. *148* (1897) 523.
14 Minz, B., C.r. Séanc. Soc. Biol. *127* (1938) 1251.
15 Muralt, A. von, Naturwissenschaften *27* (1939) 265.
16 Muralt, A. von, Pflügers Arch. *247* (1943) 1.
17 Huxley, A. F., and Stämpfli, R., J. Physiol. *108* (1949) 315.
18 Wyss, A., and Wyss, F., Helv. physiol. pharmac. Acta *3* (1945) C30.
19 Bergquist, J. E., and Hansson, M., Expl Neurol. *79* (1983) 622.
20 Gurtner, H. P., Helv. physiol. pharmac. Acta. Suppl. XI (1961) 1.
21 Kilb, H., and Stämpfli, R., Helv. physiol. pharmac. Acta *14* (1956) 251.
22 Kunz, H. A., Helv. physiol. pharmac. Acta *14* (1956) 411.
23 Barchi, R. L., and Braun, P. E., J. biol. Chem. *247* (1972) 7668.
24 Lohmann, K., and Schuster, P., Biochem. Z. *247* (1937) 188.
25 Muralt, A. von, Schweiz. med. Wschr. *73* (1943) 1101.
26 Muralt, A. von, and Stämpfli, R., Helv. physiol. pharmac. Acta *11* (1953) 182.
27 Muralt, A. von, Colloques Int. CNRS *67* (1955) 201.
28 Steinbach, H. B., and Spiegelman, S., J. cell. comp. Physiol. *22* (1943) 187.
29 Hodgkin, A. L., and Huxley, A. F., J. Physiol. *106* (1947) 341.
30 Keynes, R. D., J. Physiol. *113* (1951) 99.
31 Hodgkin, A. L., and Huxley, A. F., J. Physiol. *117* (1952) 500.
32 Hodgkin, A. L., and Keynes, R. D., J. Physiol. *119* (1953) 513.
33 Heyrovský, J., Polarographie. Theoretische Grundlagen, praktische Ausführungen und Anwendungen der Elektrolyse mit der tropfenden Quecksilberelektrode. Springer, Wien 1941.
34 Weidmann, S., Experientia *1* (1945) 61.
35 Muralt, A. von, Phil. Trans. R. Soc. *B270* (1975) 411.
36 Howarth, J. V., Keynes, R. D., Ritchie, J. M., and Muralt, A. von, J. Physiol. *249* (1975) 349.
37 Muralt, A. von, Weibel, E. R., and Howarth, J. V., Pflügers Arch. *367* (1976) 67.