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COMMENTARY

DOES Na,K-ATPase PLAY A ROLE IN THE REGULATION OF NEUROTRANSMITTER RELEASE BY PREJUNCTIONAL α-ADRENOCEPTORS?

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Historical perspective

The idea that there are, on the nerve cell membrane, receptors whose action when stimulated is to modulate the release of neurotransmitter from that nerve cell has now gained widespread acceptance. With regard to sympathetic, adrenergic nerves it is considered that among other receptor sites on the prejunctional membrane there are α-adrenoceptors, stimulation of which reduces and blockade of which enhances the release of noradrenaline evoked by nerve impulses. This mechanism is considered to constitute a physiological measure which serves to regulate the output of neurotransmitter from the nerve terminal to suit local requirements: a high concentration of noradrenaline in the synaptic cleft will reduce subsequent release by the adjacent nerve terminals by virtue of its stimulation of the α-adrenoceptors on these terminals.

Several excellent reviews have been written on the subject [1–5] and from these a comprehensive picture can be obtained of the development of the idea since the pioneering days of the mid-1950s. Briefly, Brown and Gillespie [6], using the isolated, perfused cat spleen to study neurotransmitter release, reported that noradrenaline output was related in a linear manner to the frequency of nerve stimulation. In addition they found that after exposure of the tissue to either of a pair of drugs known to block α-adrenoceptors—phenoxybenzamine and dibenamine—a dramatic increase in noradrenaline output occurred during subsequent nerve stimulation. To explain their findings Brown and Gillespie suggested that under normal circumstances the noradrenaline released from the nerve terminals is taken up by those post-junctional α-adrenoceptors at which it exerts its effect and is subsequently metabolised. However, following blockade of these post-junctional α-adrenoceptors this process is prevented and the released noradrenaline thus overflows in increased amounts. Numerous investigators have since confirmed the ability of α-adrenoceptor antagonists to enhance the overflow of noradrenaline evoked by nerve stimulation but as the phenomenon occurs also in tissues such as the guinea pig atrium [7] where the post-synaptic adrenoceptors are predominantly of the β type the increase in noradrenaline overflow after phenoxybenzamine cannot be due solely to post-synaptic α-adrenoceptor blockade.

In 1960 Paton [8] suggested that it might not be only a post-junctional site which takes up and metabolises noradrenaline. He considered that, in addition, released noradrenaline could be "sucked back (into the nerve), recovered and returned to (the intraneuronal) store when the events of excitation are over". This laid the foundations for the concept of neuronal uptake of noradrenaline, support for which was provided by those subsequent investigators who looked for, and found, an increase in neuronal stores of noradrenaline after administration of large amounts of this catecholamine [9]. The use of radioactively labelled noradrenaline in recent years has proved beyond doubt that adrenergic nerve endings are able to take up and store the compound. When, subsequent to Paton's suggestion, it was observed that phenoxybenzamine inhibits neuronal uptake of noradrenaline, the suggestion was made [10] that the increase in noradrenaline overflow evoked by nerve stimulation in the presence of phenoxybenzamine was not so much related to the ability of the drug to block the post-junctional α-adrenoceptors but rather was due to reduced neuronal uptake of the released noradrenaline. Subsequent investigation has shown this hypothesis to be untenable. In this regard it was found that inhibitors of neuronal uptake more potent than phenoxybenzamine, such as cocaine or desipramine, caused only a slight augmentation of noradrenaline overflow during subsequent nerve stimulation compared with that which followed phenoxybenzamine treatment. Furthermore, after effective blockade of the uptake mechanism with cocaine, addition of α-adrenoceptor antagonists (in doses themselves insufficient to block neuronal uptake) produced a further increase in noradrenaline release evoked by nerve stimulation [see Ref. 4].

When it emerged that noradrenaline released from adrenergic nerves is accumulated at extraneuronal sites in addition to the presynaptic location and that phenoxybenzamine was able to reduce this extraneuronal accumulation it was suggested that this might be the way in which phenoxybenzamine allowed the greater overflow of noradrenaline during subsequent nerve stimulation. This theory collapsed very shortly after its elaboration under the weight of two main lines of contradictory evidence. On the one hand it was found that the other major α-adrenoceptor antagonist then available, phentolamine, over a wide concentration range, had virtually no extraneuronal uptake blocking ability, yet at these doses it was as effective as phenoxybenzamine in

enhancing noradrenaline overflow [4]. On the other hand non-α-adrenoceptor antagonist inhibitors of extraneuronal noradrenaline accumulation such as normetanephrine or corticosterone were found to be ineffective in augmenting the nerve stimulation evoked released of noradrenaline [4].

Finally, in the early 1970s the hypothesis was advanced that phenoxybenzamine might cause enhancement of noradrenaline overflow by some direct effect on the release process from the nerve terminal rather than by affecting disposition of the neurotransmitter subsequent to its release. Since then it has been demonstrated repeatedly and convincingly that phenoxybenzamine and other α-adrenoceptor antagonists do exert such an augmenting effect on the noradrenaline release process from sympathetic nerve endings. In support of the implication that it is an α-adrenoceptor on the nerve ending which mediates this effect, it has been shown consistently that exposure of the nerve ending to α-adrenoceptor agonists causes a reduction of the quantity of noradrenaline released by nerve stimulation [1–5].

Although during the last 10 years considerable evidence has accumulated to support this α-adrenoceptor mediated mechanism for the regulation of neurotransmitter release, the pathway of intermediate steps which links prejunctional α-adrenoceptor stimulation to the subsequently regulated noradrenaline release remains obscure.

What is known of the noradrenaline release regulating mechanism?

Several investigations have been undertaken to determine the sequence of events between prejunctional α-adrenoceptor stimulation and the resulting change in evoked noradrenaline release, but most of the working hypotheses generated have been shown to be untenable.

(a) It is unlikely that α-adrenoceptor agonists act like local anaesthetic agents to reduce further release of neurotransmitter by depressing the excitability of the neuronal cell membrane for, contrary to what would be predicted if this were the case, even supramaximal concentrations of such agonists cannot completely prevent evoked release of neurotransmitter [11, 12]. A further argument against altered membrane excitability playing a part in the process is the finding that several α-adrenoceptor antagonists which effectively deregulate neurotransmitter release have no effect on the electrophysiological properties of noradrenergic nerve endings [13, 14].

(b) Interference with intraneuronal noradrenaline metabolism has been advanced as a possible mode of action of the α-adrenoceptor agonists and antagonists subsequent to their entry into the nerve ending [see Ref. 2]. However, most of the experimental evidence suggests that in their regulatory role these agents act at the neuronal membrane surface. In this regard, substances which block the uptake of noradrenaline into the nerve ending (e.g. cocaine) do not prevent the inhibition of nerve impulse evoked neurotransmitter release. On the contrary the effectiveness of noradrenaline as an inhibitor of neurotransmitter release is increased under such conditions presumably because of the resultant increase in concentration of this substance in the synaptic cleft.

(c) It appears that the α-adrenoceptor mediated regulation mechanism does not operate under all circumstances which culminate in the release of noradrenaline; a modulating effect is only exerted on those release pathways which depend on the availability of extracellular Ca^{2+} [2–4]. The release of noradrenaline evoked by indirect sympathomimetic agents such as tyramine occurs in the absence of extracellular Ca^{2+}: this is not influenced by α-adrenoceptor agonists or antagonists. Consequently it is considered that the prejunctional α-adrenoceptor modifies noradrenaline release from the adrenergic nerves by regulating the influx of the extracellular Ca^{2+} required for the release process [1–4]. Additional experimental evidence has been adduced to support this hypothesis [see 2, 15] but the way in which α-adrenoceptor activation limits the availability of this cation is still unknown.

Na,K-ATPase and the regulation of neurotransmitter release

It has been suggested that the sodium, potassium activated ATP-phosphohydrolase (Na,K-ATPase) of neuronal cell membranes may play a role in the release mechanism of neurotransmitters. Amongst others, Vizi [5, 16] has suggested that inhibition of the activity of this enzyme enhances release while stimulation of the enzyme reduces release. In this respect, Lorenz *et al.* [17] have reported an increase in noradrenaline release from sympathetic nerves following their exposure to cardiac glycosides—compounds which are presumed to act by inhibition of the Na,K-ATPase. Bonaccorsi *et al.* [18, 19] have used K^+-free bathing solutions to inhibit this enzyme in adrenergically innervated tissue. They too reported an increase in the release of noradrenaline. They also found that readmission of K^+ to preparations previously exposed to K^+-free solutions caused an abrupt termination of noradrenaline release, and argued that this was a consequence of Na,K-ATPase stimulation.

The Na,K-ATPase is considered to be the biochemical basis for the sodium pumping function of cell membranes [20–22]. Its activity results in removing to the extracellular space those Na^+ ions which have entered the cell and recovering from the extracellular space those K^+ ions which have left the cell. The energy for the transport process derives from cleavage of the terminal phosphate group of ATP. The pump operates slowly during quiescent periods in excitable tissues to counteract cation leakage, but assumes greater importance to cellular homeostasis in the period immediately succeeding membrane depolarization which results in an action potential, when comparatively large fluxes of Na^+ and K^+ occur.

Although it is by no means entirely clear how an action potential triggers the Ca^{2+}-dependent release of neurotransmitter, there are compelling arguments relating the process to the increased intracellular Na^+ concentration consequent upon membrane depolarization. In this regard it is suggested that the Na^+ which is present in the neurone in increased concentration following depolarization is exchanged

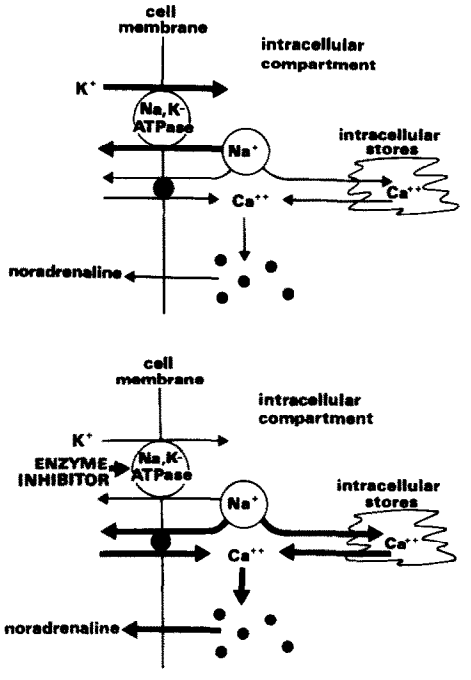

Fig. 1. Upper panel: Intracellular Na^+ removed by Na,K-ATPase driven pump or exchanged for Ca^{2+} which promotes noradrenaline release. Lower panel: Inhibition of Na,K-ATPase results in increased amount of intracellular Na^+ available for exchange with Ca^{2+}. Consequently noradrenaline release is augmented.

for extracellular Ca^{2+} across the nerve cell membrane. Whereas this exchange process has obtained experimental support in smooth muscle and in cardiac muscle, the evidence for its existence in nervous tissue is less clear [23, 24]. However, if such a mechanism does exist then it would be presumed that inhibition of the Na,K-ATPase by any means would result, *pari passu*, in a decreased rate of extrusion of intracellular Na^+, hence an increased and prolonged intracellular availability of Na^+ and a consequent increased capacity to take up Ca^{2+} (Fig. 1).

It would follow, on the other hand, that stimulation of the Na,K-ATPase would result in more rapid reduction of the intracellular Na^+ concentration by virtue of an increased rate of Na expulsion from the cell. Consequently less Na^+ would be available for exchange with extracellular Ca^{2+} and less Ca^{2+} would enter the cell.

The final link between an increased concentration of intracellular Ca^{2+} and neurotransmitter release is still speculative. Recent evidence obtained by de Lorenzo and his colleagues [25] has implicated the Ca^{2+} binding protein calmodulin in the process, and these investigators suggest accordingly that a combination of Ca^{2+} with calmodulin forms the moiety which promotes neurotransmitter release.

With the foregoing in mind it would appear that the neural cell membrane Na,K-ATPase is a good candidate for the cellular regulator of neurotransmitter release. However, if it is to be considered seriously for a role in that regulation pathway which stems from the prejunctional α-adrenoceptor it would have to be shown convincingly (a) that α-adrenoceptor agonists stimulate the activity of the enzyme and (b) that this stimulation is prevented by antagonists, agents which are presumed merely to block the effects of the noradrenaline or other agonists on prejunctional α-adrenoceptors and by this means prevent their stimulation of the Na,K-ATPase. It would not be a requirement to show that α-adrenoceptor antagonists actually inhibit the Na,K-ATPase: inhibition would lead to augmented neurotransmitter release and such antagonists do not characteristically have this effect on adrenergic nerve endings.

The evidence for a link between prejunctional α-adrenoceptors and Na,K-ATPase

Since the first papers on the subject appeared in the early 1970s, at least 15 groups of investigators using various experimental preparations derived from nervous tissue (e.g.) brain homogenates, synaptosomes and synaptic membranes have claimed to demonstrate a stimulation of Na,K-ATPase by noradrenaline [26–45]. Some, in addition, have demonstrated attenuation of the stimulation by α-adrenoceptor antagonists [29, 31, 39, 41, 43–45]. Such effects as these are entirely consistent with the contention of a direct link between α-adrenoceptor and Na,K-ATPase. The case for the direct link between adrenoceptor and enzyme would be strengthened however if it could be shown, for example, that substances other than noradrenaline, but which have been shown to stimulate prejunctional α-adrenoceptors, likewise stimulate the Na,K-ATPase. Such evidence would be more convincing still if it were shown in addition that these agonists stimulate the enzyme in the same rank order of potency as they affect the prejunctional α-adrenoceptor. In this context Starke, Endo and Taube [46] have evaluated the relative potencies of various α-adrenoceptor agonists on prejunctional α-adrenoceptors in rabbit pulmonary artery and found that adrenaline $\gg$ noradrenaline $\gg$ phenylephrine >methoxamine. They note that the latter two compounds may be regarded as exerting an action predominantly on postjunctional α-adrenoceptors. The relative ineffectiveness of phenylephrine and methoxamine on prejunctional α-adrenoceptors has been noted in other tissues also [see Table 6, pp. 48–51 Ref. 2]. With this information in mind the results of Wu and Phillis [44] can be evaluated. They reported that noradrenaline stimulates the activity of neuronal membrane Na,K-ATPase but that methoxamine is equieffective in this regard and while phenylephrine exerts no stimulatory action on the enzyme, the β-adrenoceptor agonist isoprenaline does. In light of the findings of Starke and of others [2, 45], it is difficult to reconcile these results of Wu and Phillis with a stimulation of Na,K-ATPase mediated specifically by prejunctional α-adrenoceptors particularly when account is taken of the results obtained by the latter investigators with antagonists. Here they found that the stimulation of Na,K-ATPase evinced by noradrenaline is blocked by the α-adrenoceptor antagonist phentolamine or with the β-adrenoceptor antagonist propranolol while that evinced by isoprenaline is attenuated by propranolol

and that caused by certain other catechol compounds is blocked by neither antagonist.

A similar pattern for stimulation of Na,K-ATPase by these sympathomimetic agents was obtained by Hexum using beef brain homogenates [36]. Hexum reported that adrenaline and noradrenaline stimulate the enzyme each to an equivalent degree while isoprenaline was yet more effective and phenyleprine less so—a rank order of potency that is more characteristic of a β-adrenoceptor mediated effect. In this regard, Hexum examined the effects of propranolol on the stimulatory action of isoprenaline and found this to be attenuated.

On the one hand, the essential consistency between the results of the two investigators reported above and on the other the discrepancies between these results and those of Starke *et al.* [45] are of concern to the proponents of the α-adrenoceptor–Na,K-ATPase hypothesis; the data support equally the suggestion of a β-adrenoceptor mediated stimulation of the enzyme.

It is at this point that the literature on the subject of adrenoceptor mediated effects on Na,K-ATPase becomes very confusing. A bewildering variety of conflicting and apparently irreconcilable results have appeared in print during the past few years.

In this regard, the 1975 report of Gilbert, Wyllie and Davidson [31] indicates that noradrenaline stimulates Na,K-ATPase of rat brain synaptosomes in a dose-dependent manner and that this stimulation is prevented by phentolamine, an antagonist which itself has no direct effect on the activity of the enzyme. Confirmation that the noradrenaline induced stimulation of Na,K-ATPase activity can be abolished by the α-adrenoceptor antagonist phentolamine is afforded by both Logan and O'Donovan [43] who used rabbit synaptic membranes for their experiments and by Cohen *et al.* [45] who used adrenal medullary cells. However, Wolleman [32], reporting the results of his student Toth, who, like Gilbert's group used rat brain preparations, states that the stimulation of Na,K-ATPase activity by noradrenaline is unaffected by the α-adrenoceptor antagonist phentolamine, but is prevented by the β-adrenoceptor blocking agent propranolol. Limas and Cohn [30] likewise found that propranolol effectively prevented catecholamine induced stimulation of Na,K-ATPase activity, though it should be noted that they used preparations of sympathetically innervated vascular tissue in their experiments. Yet a different result was reported by Iwangoff, Enz and Chappuis [29]. With homogenates of cat brain they found that *both* α- and β-adrenoceptor antagonists prevented the noradrenaline mediated stimulation—a finding that has been reported also by Wu and Phillis for rat cerebral cortex synaptosomes [39] and for rat cortex homogenates [41, 44].

Contrary to the finding above that both α- and β-adrenoceptor antagonists prevent the stimulatory action of noradrenaline on neural Na,K-ATPase activity, Arnaiz and de Pacheco [38] reported that this action on the activity of the enzyme was attenuated by neither the α-adrenoceptor antagonists phenoxybenzamine and phentolamine nor the β-adrenoceptor antagonist propranolol. Hexum [36] using beef cortex synaptosomes found that phentolamine was ineffective at preventing the noradrenaline evoked stimulation.

Taken as a whole, the papers in this extended series present arguments for all possible combinations of adrenoceptors which mediate the stimulatory effects of noradrenaline on Na,K-ATPase activity: α- but not β-; β- but not α- and both α- and β-adrenoceptors. In addition some investigators failed to implicate adrenoceptors in the phenomenon at all. No reason for such fundamental differences is apparent from the individual reports; the experimental methods used by the investigators were standard biochemical techniques similar in all but minor ways to each other. The only common ground between investigations is the agreement that noradrenaline exerts a stimulatory effect on the activity of Na,K-ATPase.

Before the debate on the participation of an adrenoceptor in the pathway through which catecholamines stimulate the activity of Na,K-ATPase is closed, however, there is another relevant finding that should be mentioned. There has been a report recently which describes experiments of a different type to the above and which strongly supports the notion of a direct linkage between α-adrenoceptor and the Na,K-ATPase of neural tissue. Lorenz *et al.* [17] found that cardiac glycosides release noradrenaline from sympathetic nerve endings and they argue that this is a consequence of Na,K-ATPase inhibition. However, they found unexpectedly that the α-adrenoceptor antagonists phentolamine and phenoxybenzamine were highly effective at preventing this glycoside evoked release of noradrenaline.

Adrenoceptor or not?

The previous section, taken as a whole, must raise considerable doubt about the genuine participation of an α-adrenoceptor in the process of noradrenaline evoked stimulation of Na,K-ATPase activity. Indeed, on the evidence summarised so far there is ample justification for the opinion that if an adrenoceptor is involved at all it is just as likely to be a β-adrenoceptor.

The controversy which is aroused by the above results concerning α- or β-adrenoceptors may be developed a stage further. The hypothesis which forms the subject of this commentary states that one step in the pathway between α-adrenoceptor stimulation and negatively regulated noradrenaline release is Na,K-ATPase stimulation. The results of some of the investigations reported above apparently indicate equally strong support for a β-adrenoceptor mediated Na,K-ATPase stimulation. In this regard it should be noted that the presence of β-adrenoceptors on sympathetic nerve terminals has been supported [see Ref. 3]. Stimulation of these receptors is said to enhance subsequent noradrenaline release evoked by depolarizing stimuli (i.e.) precisely the opposite of prejunctional α-adrenoceptor stimulation. Consequently it seems to be obvious that should both α- and β-adrenoceptors be shown convincingly to mediate stimulation of Na,K-ATPase then the position of this enzyme in the noradrenaline release regulation pathway would be seriously in doubt.

Before the adrenoceptor hypothesis is discarded, however, we should at this point evaluate the pos-

sible alternative mechanisms proposed to explain the observed stimulatory action of noradrenaline.

It is noteworthy that many investigators have found the demonstration of a noradrenaline induced stimulation of Na,K-ATPase to be far from straightforward. In order to obtain a consistent stimulation the literature indicates that a set of experimental conditions must be produced which are in excess of merely bringing noradrenaline into contact with the enzyme. One of the earliest papers published on the subject is that of Schaefer, Unyi and Pfeifer [26]. They reported that noradrenaline will only stimulate the membrane bound enzyme when the soluble fraction of the cell is present. Their preparations of rat brain synaptic membranes had high specific Na,K-ATPase activity which could not be increased further by catecholamines. Fractionation of rat brain homogenates in such a way as to keep the soluble fraction present, however, lowered basal Na,K-ATPase activity and allowed an enhancement to be demonstrated when noradrenaline was subsequently added. The maximum degree of enhancement obtained, however, did not result in enzyme activity which exceeded that measured in the absence of the soluble fraction. These investigators concluded that the soluble fraction contains an inhibitor of Na,K-ATPase which is "neutralised" by noradrenaline and other catecholamines. In a later publication, Schaefer and his colleagues [47] develop their hypothesis and on the basis of the finding that the chelating agents EGTA and EDTA have a stimulating effect on Na,K-ATPase activity similar to that of noradrenaline, they suggest that the substance ultimately responsible for the inhibition of the enzyme might be a divalent cation. The results of Godfraind and his colleagues [28] using EGTA and also of Lee and Phillis [37] who used EGTA and EDTA in their experiments permit similar conclusions to be drawn. Each group found that noradrenaline caused stimulation of the Na,K-ATPase of neural tissue and that the maximum degree of stimulation of the enzyme obtained with the chelating agents alone was similar to that obtained with noradrenaline. In addition they found that in the presence of the chelating agents noradrenaline was ineffective in causing stimulation of Na,K-ATPase.

There is no shortage of evidence to show that divalent cations are potent inhibitors of Na,K-ATPase [47, 48] and thus an obvious interpretation of the experimental results reported above would be that EGTA and EDTA do not so much stimulate the enzyme but rather, by binding divalent cations remove a source of inhibition. This explanation for Na,K-ATPase stimulation by EGTA/EDTA could be applied equally to noradrenaline for this catecholamine is able to chelate divalent cations also and forms both binary chelates and, in the presence of ATP, stable ternary chelates [49–51]. Hence it is possible that the apparent stimulatory effects of noradrenaline on Na,K-ATPase may be due rather to removal of divalent cation inhibition, than to a direct stimulatory effect on the enzyme mediated by adrenoceptors.

Various candidates for the role of principal inhibitory cation have been proposed. Hexum [48], for example, noting the presence of Cu^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+} in nervous tissue has determined that in the concentrations found all exert significant inhibitory effects on Na,K-ATPase. The results of his later experiments [36], however, showed that noradrenaline was able to reverse only the inhibition produced by Fe^{2+}. Schaefer and his colleagues [47] having studied a range of metallic divalent cations had also favoured Fe^{2+} as the ultimate inhibitor of Na,K-ATPase although their scheme for noradrenaline relief of such inhibition was more complex. Schaefer states that the cell membrane contains Fe^{3+}, an ion which itself has no inhibiting effect on the Na,K-ATPase. However, the soluble fraction of the cell contains a reducing agent—they suggest ascorbic acid—which reduces the ferric ion to the ferrous form which is strongly inhibitory towards Na,K-ATPase. Noradrenaline, or for that matter, any divalent cation chelating agent, by removing the ferrous ion removes also the inhibition of the enzyme. The resulting increased activity is interpreted as a stimulation of the Na,K-ATPase.

While implicating ferrous ion as the intracellular inhibitor of Na,K-ATPase whose effects can be prevented by noradrenaline, both Schaefer's group [47] and Hexum [48] considered the possible role of Ca^{2+}, but on the basis of their experimental results discounted its likely participation. This failure to demonstrate noradrenaline reversal of Na,K-ATPase inhibition caused by Ca^{2+} is in direct contrast, however, to the findings of Godfraind and his colleagues [28] and of Lee and Phillis [37]. The results of these investigators confirm that noradrenaline, like EGTA or EDTA cause an increase in Na,K-ATPase activity by removal of a pre-existing inhibition but both of these groups favour Ca^{2+} as the cationic enzyme inhibitor.

Noradrenaline evoked stimulation of Na,K-ATPase: physiological effect or artefact?

In their paper of 1974, Schaefer *et al.* [47] reached the conclusion that noradrenaline increased the activity of neurally derived Na,K-ATPase by chelating Fe^{2+} thereby removing the inhibitory effect of this cation on the enzyme. In their experiments commercial ATP from a number of sources was used as the substrate for the Na,K-ATPase. The marked differences in results obtained with the different ATP preparations were ascribed by these investigators to contaminants in the ATP and analysis indicated that Fe^{3+} was a significant contaminant in three out of four commercial preparations. More recently, Josephson and Cantley [52] reported another contaminant of commercial ATP derived from equine muscle which at concentrations of between 10^{-8}–10^{-7} M caused a 50 per cent inhibition of the Na, K-ATPase. They noted that such inhibition could be reversed by noradrenaline. In a note added in the proof to their paper they reported that vanadium in its 5+ oxidation state constituted the major inhibitory contaminant of ATP prepared from equine muscle.

Recent publications by Cantley and his colleagues and by Hudgins and Bond [53, 54] indicate that noradrenaline is able to form complexes with VO_4^{3-} and can by this means attenuate the inhibitory effects of

the latter on the activity of Na,K-ATPase. These findings, though interesting in their own right, hardly add weight to the contention that the phenomenon of noradrenaline stimulation of Na,K-ATPase is a physiologically significant effect. On the contrary there appears to be sufficient grounds here to support more strongly the alternate suggestion that the noradrenaline evoked stimulation obtained by these and other investigators was due to removal, or neutralisation, by this catecholamine of an "artificial" exogenous inhibitor added unwittingly by way of substrate ATP to the experimental system.

Before it is concluded that the stimulatory effect of noradrenaline on Na,K-ATPase is a mere artefact, however, the experimental results of Ádám-Vizi *et al.* [42] and by Schaefer *et al.* [40] are of direct relevance. The first paper [42] shows that rat neural tissue does in fact contain normally vanadate in quantities sufficient to produce significant Na,K-ATPase inhibition. These investigators show also that noradrenaline can produce stimulation of the activity of the enzyme even when commercial ATP free of vanadium is used for the experiments. The report of Schaefer *et al.* argues that the stimulatory effects of noradrenaline on Na,K-ATPase can occur in the absence of inhibition produced by a contaminant in the substrate ATP: they reaffirm that such stimulation is due to reversal of inhibition of the enzyme by an *endogenous* heavy metal ion.

Concluding remarks

The proposition which forms the basis of this commentary states that noradrenaline acting via α-adrenoceptors stimulates neuronal membrane Na,K-ATPase and thereby exerts a negative regulating influence on neuronal neurotransmitter release. In other words, the suggestion is made that Na,K-ATPase forms part of the presynaptic α-adrenoceptor initiated neurotransmitter release regulating pathway.

Before the ultimate acceptance of this proposition, three facets require thorough investigation:

1. that alteration of Na,K-ATPase activity, *per se*, can influence neurotransmitter release;
2. that noradrenaline can stimulate Na,K-ATPase activity;
3. that such stimulation is mediated specifically by an α-adrenoceptor.

There is reasonable evidence to support the first statement [see Ref. 5] and it seems clear from the foregoing text that statement number two can be regarded as proven. However, it is clear also from the text that the mechanisms through which noradrenaline stimulates the enzyme are still far from being understood.

Two general mechanisms can be identified, however, but only the first suggests that noradrenaline acts on Na,K-ATPase via interaction with an adrenoceptor. The second states that Na,K-ATPase activity is increased by virtue of the ability of this catecholamine to chelate an inhibitor of the activity of the enzyme, an action which must be presumed to be independent of adrenoceptors. Only the first mechanism therefore is compatible with the hypothesis that forms the basis of this commentary. Among those who have concluded that the major effects of noradrenaline on Na,K-ATPase derive from the second mechanism: the ability of the amine to chelate certain inhibitory ions, there is no concensus as to the identity of the inhibitor, but there is, perhaps, sufficient data available to be able to implicate a substance endogenous to nervous tissue [40, 42, 48] and hence to return the phenomenon of noradrenaline stimulation of Na,K-ATPase to the sphere of possible physiological relevance. But it is necessary to re-emphasis that if inhibitor chelation is the sole mechanism whereby noradrenaline stimulates Na,K-ATPase; a process in which an α-adrenoceptor is not involved, then the conclusion that must be drawn is that the presynaptic α-adrenoceptor which is sensitive to noradrenaline cannot work through Na,K-ATPase to regulate neurotransmitter release.

The mechanism of Na,K-ATPase stimulation: Do catecholamines act via adrenoceptors or do they chelate inhibitors of the enzyme?

As stated above, the minimum requirement which has to be fulfilled to be able to implicate an α-adrenoceptor into the pathway that results in Na,K-ATPase stimulation is that conventional α-adrenoceptor agonists should be shown to stimulate the enzyme. The majority of those studies reported above have indicated that noradrenaline will stimulate consistently the Na,K-ATPase. Some have shown in addition that dopamine or adrenaline are also effective in this regard. However, since all three of these agents have been shown able to chelate divalent cations which inhibit Na,K-ATPase, the demonstration that these conventional α-adrenoceptor agonists can stimulate Na,K-ATPase is not sufficient to enable specific implication of an α-adrenoceptor in the process. α-Adrenoceptor participation in the stimulation of Na,K-ATPase by noradrenaline could be more readily accepted if it could be shown that α-adrenoceptor antagonists could prevent consistently the effect. Only a few investigators have so far demonstrated this [31, 43, 45]. Those data which indicate that β-adrenoceptor antagonists in addition to [29, 39, 41, 44], or instead of [30, 32, 36], α-adrenoceptor antagonists can prevent the stimulation of the enzyme by noradrenaline detract from the hypothesis of specific α-adrenoceptor involvement. Hence on the more rigorous criterion of specific antagonism, no conclusions can yet be drawn about α-adrenoceptor mediation in noradrenaline evoked Na,K-ATPase stimulation.

An approach which might have proved productive in demonstrating that the presynaptic α-adrenoceptor which regulates neurotransmitter release works through Na,K-ATPase was that which tried to reconcile the degree of Na,K-ATPase stimulation with the rank order of potency of presynaptic α-adrenoceptor agonists. The results obtained to date have not been encouraging: of those who have studied the effects of a range of catecholamines on Na,K-ATPase activity there is little agreement between degree of stimulation of the enzyme and the recorded ability of the agonist to regulate neurotransmitter release. For example methoxamine and phenylephrine are accepted as having low presynaptic α-adrenoceptor affinity or efficacy [2, 46] yet both have been shown by some [44, 45] to have marked stimu-

latory effects on Na,K-ATPase activity. Equally the β-adrenoceptor agonist isoprenaline would be expected to be ineffective at presynaptic α-adrenoceptors yet this amine too has been shown to have a marked stimulatory effect on Na,K-ATPase [26, 30, 36, 39, 41, 44].

The thought that springs to mind at this point is that perhaps the inconsistencies which arise when trying to explain the effectiveness of these amines on Na,K-ATPase by the adrenoceptor hypothesis can be resolved if explanation is attempted in terms of the inhibitor chelation hypothesis. In other words, can the effectiveness of the biological amines to cause Na,K-ATPase stimulation be correlated with their inhibitory ion chelating capacity?

In respect of the vanadate ion, one of the foremost contenders for the role of principal intracellular Na,K-ATPase inhibitor, Hudgins and Bond [54] have determined the structural features of the catecholamine or related agent required to form complexes with it. They note that two adjacent hydroxyl groups on the phenyl ring are an absolute requirement for vanadate chelation. Hence noradrenaline, adrenaline, dopamine and isoprenaline would all be able to fulfil the function. However, phenylephrine and methoxamine should be ineffective, the former compound having but one hydroxyl group the latter having none associated with the phenyl ring.

Wu and Phillis [44] would probably not have been aware of these findings when their most recent paper was in preparation but their results indicate general agreement with this proposed structural requirement for a catecholamine or related compound to be able to counteract the effects of the inhibitor on Na,K-ATPase activity. However, these authors found that methoxamine, which as noted above is an ineffective chelating agent was equieffective with noradrenaline as a stimulator of Na,K-ATPase. Likewise, Gutman and his colleagues have recently shown [45] that phenylephrine, another poor chelator of inhibitory ions can stimulate Na,K-ATPase to a significant degree.

Besides these inconsistencies there is another recurring observation in the data currently available which detracts from the proposition that inhibitor chelation alone is the sole mechanism through which catecholamines bring about an increase in cellular Na,K-ATPase activity. This is the finding by so many investigators that adrenoceptor antagonists effectively prevent the stimulation of the enzyme [29–32, 36, 39, 41, 43–45]. These compounds which interact with catecholamines at the receptor level have no predictable effect on the purely chemical combination which is embodied by inhibitor chelation.

The evidence available at this time therefore does not give unequivocal support for either mechanism being the sole modulator of the effects of noradrenaline on Na,K-ATPase. That which is adduced by the proponents of the α-adrenoceptor initiated pathway contains inconsistencies which are more readily explained in terms of the inhibitor chelation hypothesis. Similarly some of the data presented by those who suggest that noradrenaline increases the activity of Na,K-ATPase solely by virtue of its ability to chelate inhibitory ions are more consistent with the notion that an adrenoceptor is involved. These statements need not be regarded pessimistically for they reveal another possibility that has not been considered seriously so far, but has been alluded to directly or indirectly in some recent publications: that *both* mechanisms could act simultaneously in the cell to promote Na,K-ATPase activity in the presence of catecholamines [40, 44].

This hypothesis is immediately attractive and, on the whole, appears adequate to reconcile the data of the majority of the investigations cited above but, before it is developed, there is one factor of fundamental importance which must be noted here. In this regard, it has been found, *in vivo*, that the amounts of noradrenaline which are effective in regulating prejunctionally the release of neurotransmitter are some 1000 × lower [see Ref. 2 for example] than those concentrations which need to be employed *in vitro* to obtain consistent effects on Na,K-ATPase activity. So, although the phenomenon of stimulation (or disinhibition) of Na,K-ATPase activity of neural tissue can now apparently be explained adequately in terms of the mechanism, it would appear that the amounts of amine that need to be used to demonstrate the effect are outside the physiological range. So once again one is led to speculate whether the Na,K-ATPase activity changes caused by the addition of noradrenaline and related compounds is more a phenomenon of the test tube than a physiologically relevant effect.

It will not have passed unnoticed that in almost all of the experiments described above, the experimental tissue used comprised homogenates or more pure subfractions of either neural or otherwise heavily innervated tissues: in practically no instance was intact tissue used. This is perhaps very relevant to the current discussion.

For example, if an adrenoceptor is involved in the pathway by which noradrenaline stimulates the activity of Na,K-ATPase, then in view of the unquestioned assumption that such receptors are situated on the outer aspect of the cell membrane, its likely specific location can be in one of only two places. Of these, it is unlikely that the adrenoceptor would constitute part of the enzyme molecule itself. Therefore such an adrenoceptor would be situated on the neuronal membrane at some point distant from the Na,K-ATPase but linked in such a way that it could influence the activity of the enzyme. In this situation membrane integrity would be of extreme importance if adrenoceptor mediated effects on the enzyme are to be observed.

With this in mind it could be suggested that the failure of many of the investigators to implicate an α-adrenoceptor in the noradrenaline initiated Na,K-ATPase stimulation pathway is attributable to the destruction by homogenisation, or other preparative techniques used, of the delicate linkage between adrenoceptor and enzyme. Assuming for the moment that this speculative proposal is correct there is at least one unifying hypothesis, consistent with the experimental results cited in this paper so far, that can be advanced. This may be cast in the following form.

It is suggested that at physiological concentrations of noradrenaline an α-adrenoceptor mediates stimulation of Na,K-ATPase activity in neural cell mem-

branes. The failure of most investigators to demonstrate specifically this interaction is because they used tissue homogenates or other experimental systems in which the membrane linkages between adrenoceptor and enzyme were largely destroyed. Under these circumstances all that these investigators would achieve subsequently in terms of Na,K-ATPase stimulation is that which derives at high concentrations of catecholamines from chelation of cellular ionic inhibitors.

Developing this hypothesis further, one could suggest that in the experiments of some investigators there were a few intact, but maybe functionally compromised, α-adrenoceptor-enzyme linkages. The residual effects of these on Na,K-ATPase activity might only become apparent at high concentrations of noradrenaline; concentrations sufficiently high to allow also chelation of cellular ionic inhibitors of Na,K-ATPase. The overall effect observed in terms of Na,K-ATPase activity would, in this instance, be a composite and would be explicable by neither "adrenoceptor" nor "inhibitor chelation" hypothesis alone.

This unifying hypothesis is probably not unreasonable: there is in the literature data which is supportive. For example Wu and Phillis [44] failed to demonstrate noradrenaline evoked stimulation of Na,K-ATPase following exposure of their experimental preparations to Triton X-100, a substance which would interfere with membrane structure. In addition, it has already been noted that Lorenz *et al.* [17] could attenuate with α-adrenoceptor antagonists the noradrenaline release from sympathetic nerve *in intact tissues* consequent upon Na,K-ATPase inhibition with cardiac glycosides. However, it has not yet proved possible to reduce consistently the degree of glycoside inhibition of Na,K-ATPase activity of *homogenates* of the same tissue with α-adrenoceptor antagonists (Powis, unpublished observations). Again, in this case absence of membrane structural integrity could account for the discrepancy.

Summary

It has been suggested that the α-adrenoceptor situated on the adrenergic nerve cell membrane and whose effect when stimulated by noradrenaline or by other specific agonists is to regulate neurotransmitter release, acts via modulation of membrane Na,K-ATPase activity.

Ultimate acceptance of this proposition requires proof of the following subordinate phenomena:

1. that alteration of Na,K-ATPase activity, *per se*, can influence neurotransmitter release;
2. that noradrenaline can stimulate Na,K-ATPase activity;
3. that such stimulation is mediated specifically by an α-adrenoceptor.

There is, perhaps, sufficient evidence available to support statements 1 and 2; statement No. 3, however, is contentious.

Of the 15 or so groups [26–45] who have studied the phenomenon of noradrenaline induced stimulation of Na,K-ATPase activity, only three [31, 34, 45] have claimed to implicate specifically an α-adrenoceptor in the pathway. Of the others most have attributed the increase in enzyme activity to chelation by noradrenaline of endogenous inhibitors of Na,K-ATPase.

Three points are noteworthy. Firstly that all these experiments were conducted with homogenates or similarly disrupted preparations of neural tissue. Secondly, that the concentrations of noradrenaline required to produce significant stimulation of enzyme activity were considerably in excess of those normally considered to be within the physiological range. Thirdly, few of the published results are entirely consistent with Na,K-ATPase stimulation being brought about by either mechanism alone; most of the data are explained more satisfactorily in terms of both mechanisms working simultaneously and each mediating part of the effect.

It is possible that the use of structurally disrupted neural tissues for such experiments might hold the key to explain the discrepancies and inconclusive results; a recent investigation performed with intact tissues has given more compelling—but still circumstantial—evidence for functional links between α-adrenoceptors and Na,K-ATPase [17].

With the foregoing in mind, the following working hypothesis is advanced as a basis for further discussion and study:

The stimulatory effect of noradrenaline on neural Na,K-ATPase activity is mediated by an α-adrenoceptor located at some point distinct but still linked physically to the enzyme on the nerve cell membrane. The failure of most investigators to produce stimulation of Na,K-ATPase activity with a physiological concentration of noradrenaline could be due to their having destroyed the majority of such linkages by homogenisation or similar treatment of the experimental tissue used. Under these circumstances adrenoceptor mediated stimulation of Na,K-ATPase activity is only obtained at a concentration of noradrenaline high enough to stimulate the remaining, but possibly functionally compromised, α-adrenoceptor/enzyme linkages. Such concentrations of noradrenaline, however, are sufficient to chelate the endogenous ions which tend normally to inhibit Na,K-ATPase. The resultant stimulation of Na,K-ATPase activity is the composite of the dual effect of noradrenaline: it can be explained in terms of neither adrenoceptor stimulation nor inhibitor chelation alone.

It must be emphasised that this hypothesis is highly speculative; the participation of an α-adrenoceptor in the Na,K-ATPase stimulation pathway is by no means proven. Perhaps though those experiments required for such proof to be obtained are those which utilize intact tissue where membrane integrity is preserved rather than homogenates of neural tissue in which the postulated delicate functional linkage between adrenoceptor and enzyme may have been compromised.

If, with intact tissues, convincing data are obtained then it will be quite appropriate to suggest that regulation by α-adrenoceptors of neurotransmitter release involves the Na,K-ATPase.

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