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A TRANSIENT RELEASE OF POTASSIUM MEDIATED BY THE ACTION OF SUBSTANCE P ON RAT PAROTID SLICES

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SUMMARY

Substance P (a peptide of eleven amino acids) caused a Ca-dependent release of K^+ from rat parotid slices. The response to substance P differed from the α-adrenergic and the cholinergic responses in that it was transient, of smaller extent, and was not inhibited by phentolamine and atropine. Substance P caused little, if any, amylase secretion. Successive additions of the peptide to the slice system maintained the effect of K^+ release indicating that the transient response to a single addition of the peptide was due to inactivation of substance P and not due to a decline in the response of the tissue.

INTRODUCTION

The presence of a hypotensive and smooth muscle contracting agent ('substance P') in equine brain and intestinal extracts was first described by von Euler & Gaddum (1931). Later studies by Lembeck revealed high concentrations of a vasodilator and intestinal contracting substance in the dorsal root of the bovine spinal cord (Lembeck, 1953). Lembeck recognized that this agent closely resembled von Euler & Gaddum's 'substance P' and he suggested that it might act as a sensory neurotransmitter in the spinal cord (Lembeck, 1953; Lembeck & Zetler, 1962). More recently, Leeman and her co-workers found that a bovine hypothalamic extract contained a component possessing potent sialogenic effects in rats (Leeman & Hammerschlag, 1967). Using saliva production as a bioassay for the extract, Chang & Leeman (1970), Chang, Leeman & Niall (1971), were able to isolate a peptide of eleven amino acids in pure form and determined its amino acid sequence. This peptide was then shown to have the same biological activity as Euler & Gaddum's substance P (Leeman & Mroz, 1974). The introduction of a chemically defined substance P led to a proliferation of research on its function in the central nervous system. Recent experimental evidence suggests that substance P acts as the excitatory transmitter of primary sensory neurones in the spinal cord (Otsuka & Konishi, 1975). Despite the fact that the *in vivo* test of salivary secretion was efficiently used both for the isolation of substance P and for testing the success of its chemical synthesis, relatively little work was done on characterization of the actions of substance P on salivary glands *in vitro*.

Studies on the control of secretion in the rat parotid have shown that each acinar cell possesses α-adrenergic, β-adrenergic and muscarinic cholinergic receptors

(Schramm & Selinger, 1974). The requirements for eliciting each specific response, the action of inhibitors, the identity of second messengers and the accompanying morphological changes have been extensively studied *in vitro* using the parotid slice preparation (Schramm & Selinger, 1975). This easily accessible and well defined system thus appeared to offer a suitable preparation on which to analyse the mode of action of substance P on salivary secretion. While this work was in progress we learned of the study of Rudich & Butcher in which the effects of substance P were tested on parotid slices incubated in the presence of ouabain (Rudich & Butcher, 1976). Although ouabain increases the sensitivity of the system to substance P, it also masks some important features of its action. The present work shows that substance P causes a transient release of K^+ from rat parotid slices. The transient nature of the response appears to be due to a mechanism which inactivates the peptide since the ability of the slices to respond to repeated applications of substance P remains unchanged. As far as can be tested with the parotid slice system substance P does not cause any significant amylase secretion.

METHODS

Parotid slice system. Rat parotid slices were prepared and incubated as described by Batzri & Selinger (1973) but with the following modifications: the medium was a modified Krebs Ringer (Schramm & Selinger, 1975) in which the bicarbonate had been replaced by 25 mM-HEPES buffer pH 7·4 and the gas phase was 100 % O_2 (KRH medium). Slices were prepared immediately from each pair of glands since incubation of whole glands resulted in insufficient oxygen supply with a concomitant loss of K^+ from the cells. Slices from eight to twelve glands were pooled and incubated for 5 min, washed with fresh medium and then divided into individual systems. Each system consisted of a scintillation vial equipped with a rubber cork and contained slices equivalent to one gland suspended in 2 ml. oxygenated KRH medium. The depth of the medium and the amount of tissue were found to be critical for proper oxygenation of the slices. The vials were always kept closed except when flushed with O_2 which was performed every 5 min and during sampling. Determination of K^+ release, amylase secretion and expression of the results are as described by Schramm & Selinger (1975). The results shown in the Figures are typical experiments, each repeated for at least four times.

Chemicals. Substance P (bovine) was purchased from Beckman. Bacitracin and phenylmethylsulphonylfluoride from Sigma and L-1-tosyl-amido-2-phenyl-ethyl chloromethyl ketone from Cyclo. The sources of other chemicals are given in previous papers by Batzri & Selinger (1973), Schramm & Selinger (1975) and Selinger, Batzri, Eimerl & Schramm (1973).

RESULTS

The time course of substance P induced K^+ release. Addition of substance P (0·07–3·5 μM) to the parotid slice system initiated a rapid and transient release of K^+ from the cells. The response to substance P was not inhibited by the presence of propranolol (10^{-6} M) together with phentolamine (10^{-5} M) and atropine (10^{-6} M). Maximal K^+ release was achieved within 2 min, after which time the amount of K^+ in the medium decreased, presumably due to reuptake of K^+ into the cells (Fig. 1*A*). The transient release of K^+ caused by substance P was thus different from the sustained release elicited by epinephrine or carbamylcholine (Schramm & Selinger, 1974). The pattern of K^+ reuptake, following addition of substance P was essentially unaltered when the concentration of substance P was increased tenfold. It was therefore suspected that the transient release of K^+ was due to desensitization of the system responding to

substance P. To test this hypothesis we applied successive additions of substance P to the slice system. Although these multiple additions of the peptide did not increase the amount of K^+ released from the slices, a slightly lower than maximal level of K^+ in the medium was maintained as long as substance P was repeatedly added (Fig. 1*B*). After the last addition of the peptide K^+ was taken back into the slices, although at a somewhat slower rate than after a single addition of substance P. A second addition of the peptide after K^+ was taken back into the cell gave the same pattern of K^+ release as that caused for the first time. These experiments appeared to indicate that there was a continuous inactivation of substance P since the slices remained capable of responding to a second challenge of the peptide.

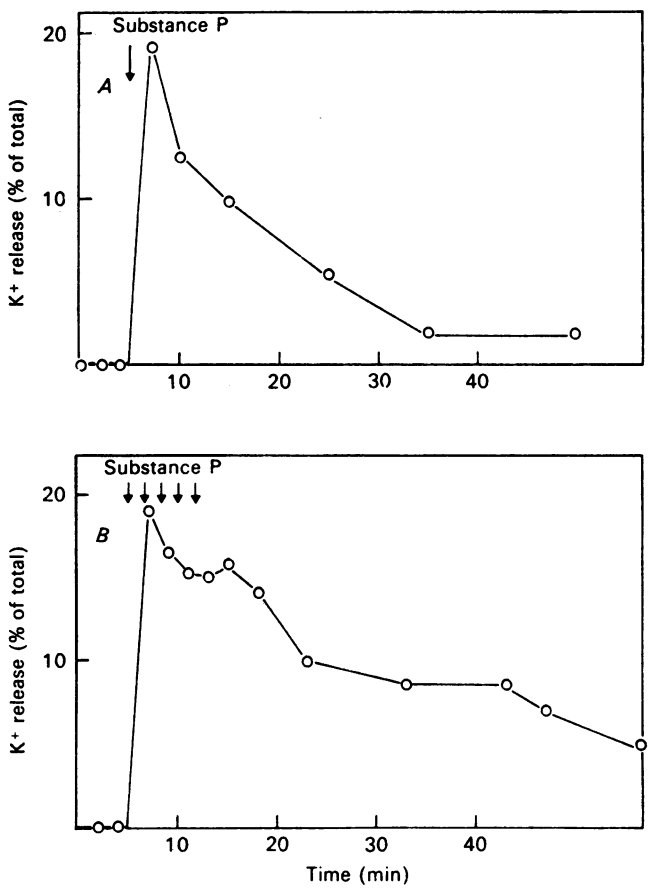

Fig. 1. Time course of K^+ release from rat parotid slices caused by a single addition (A) and multiple additions (B) of substance P. The standard incubation medium contained also propranolol (10^{-6} M), phentolamine (10^{-5} M) and atropine (10^{-6} M). Addition of substance P to give a final concentration of 70 nM in the medium is indicated by arrow.

Substance P induced K^+ release from parotid slices incubated in the presence of ouabain. We have previously shown that addition of the Na^+, K^+-ATPase inhibitor ouabain to the parotid slice system blocks K^+ uptake and greatly potentiates the epinephrine mediated response on K^+ release (Selinger *et al.* 1973). Since the K^+ loss

induced by substance P was transient, the amount released being only 15–20% of the K^+ initially present in the slices, it was of interest to test the effects of ouabain on the response to this peptide. The presence of a 1 mM concentration of the glycoside by itself caused a very slow release of K^+ (0·2% per min). However, the addition of substance P to slices which had been preincubated in the presence of ouabain (1 mM) resulted in a rapid release of 40% of the K^+ initially present in the slices. It should be pointed out that after reaching a peak, the K^+ in the medium was slowly taken back into the slices, suggesting that perhaps the Na^+, K^+-ATPase was not totally blocked by this concentration of ouabain (Fig. 2).

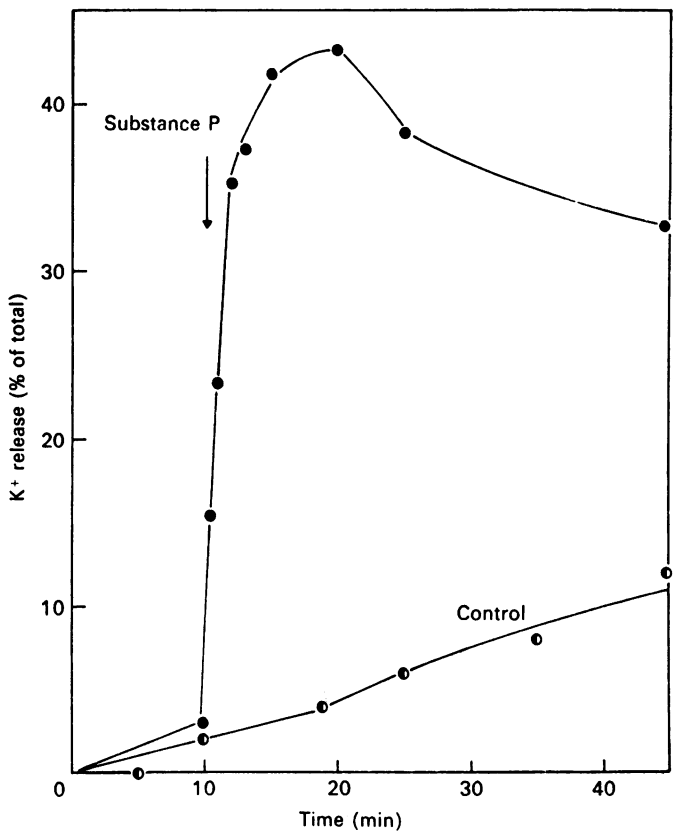

Fig. 2. The effect of ouabain on substance P mediated K^+ release from rat parotid slices. Ouabain 1 mM was added to both systems. At 10 min substance P (70 nM) was added to the system marked with filled symbols.

The effect of protease inhibitors and other agents on substance P induced K^+ release. Proteolytic degradation of substance P has been described in various preparations of nervous tissue (Benuck & Marks, 1975). Protease activity was therefore considered as a possible inactivating mechanism for substance P in the rat parotid system. However, the following protease inhibitors were without effect on the transient kinetics of the substance P induced K^+ release: soybean trypsin inhibitor (10 μg/ml.) bacitracin (50 u./ml.), phenylmethylsulphonylfluoride (10^{-5} M) diisopropylfluoro-

phosphate (10^{-4} M). L-1-tosylamido-2-phenylethyl chloromethyl ketone(10^{-3} M) benzamidine (10^{-4} M) and kallikrein inhibitor (1 mg/ml.). Inactivation of substance P was found to be dependent on the interaction of the peptide with the parotid slices since control experiments showed that the peptide retained its full biological activity when incubated in KRH medium without slices for 60 min under 100% oxygen atmosphere. The possibility of protecting substance P by the addition of excess concentrations of different proteins to the slice system was also tested. However, bovine serum albumin (2%), haemoglobin (0·5%), histone (0·2%) as well as freshly prepared rat plasma (20%) did not prolong the action of substance P. These experiments on the preservation of the activity of substance P in the parotid slice system led to the conclusion that if a protease was acting as the inactivating mechanism for substance P in this tissue, then the protease must be highly specific towards the peptide.

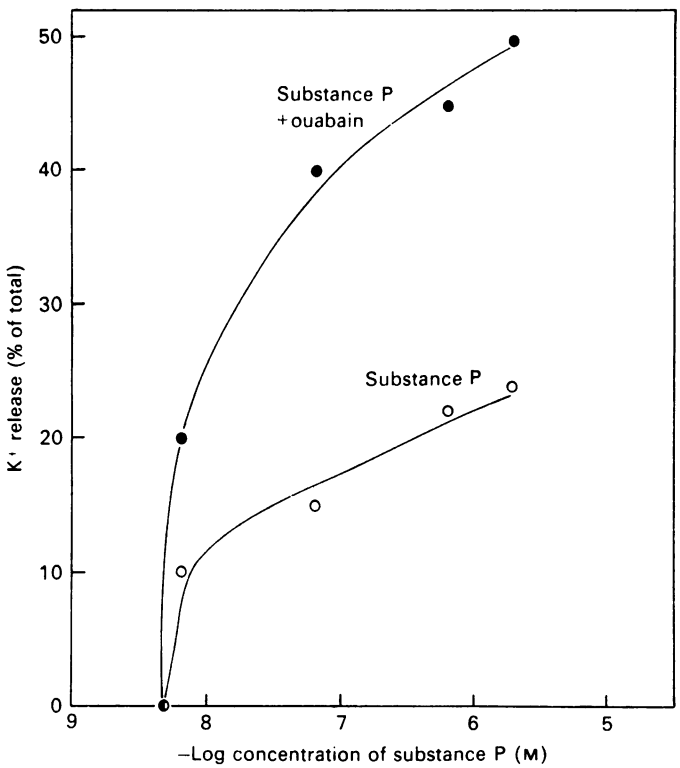

Fig. 3. Dose–response curve of substance P mediated K^+ release in presence and absence of ouabain. The systems marked with filled symbols were incubated for 10 min with 1 mM-ouabain before addition of the indicated concentrations of substance P. The time course of K^+ release was followed in each system and values shown are maximal extent of K^+ release which were usually obtained 2 min from the addition of substance P.

Dose–response relationship. The lowest concentration of substance P which gave a measurable K^+ release from the parotid slices was 7 nM (Fig. 3). At this low concentration substance P caused the loss of 10% of the K^+ initially present in the slices. On the other hand, a 200-fold increase in the concentration of substance P caused only a

two-fold increase in the amount of K^+ release. At each concentration of substance P the maximal amount of K^+ was released into the medium within two min of the addition of the peptide and thereafter the concentration of K^+ in the medium declined. Therefore, the amounts of K^+ released within two min were used to construct the dose–response curve. While ouabain at 1 mM did not essentially change the minimal concentration of substance P causing K^+ release, it did considerably increase the total amount of K^+ released (up to 40–50% of the amount which was initially present in the slices) (Fig. 3). The low concentration of substance P sensed by the

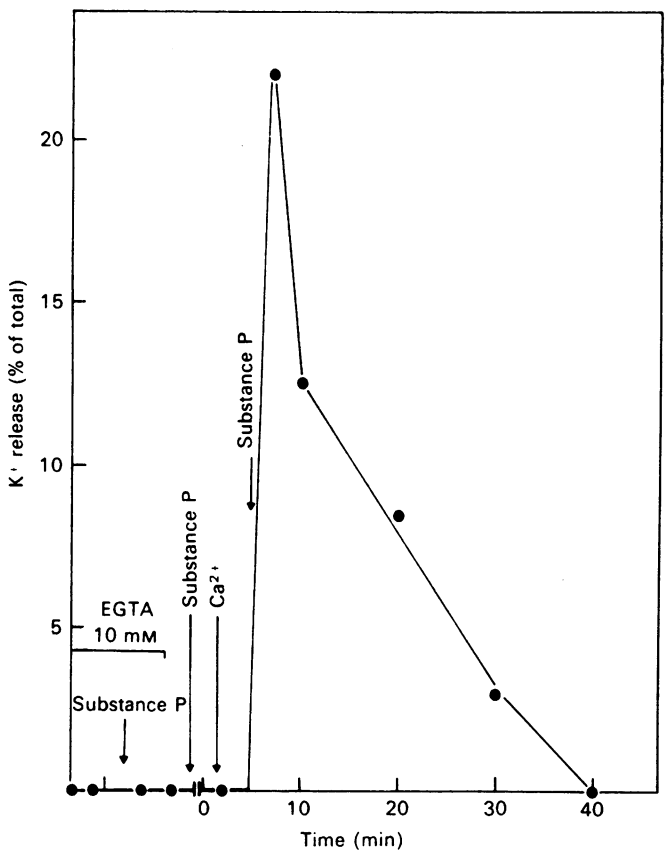

Fig. 4. Calcium dependence of substance P mediated K^+ release. Slices were incubated four times, each incubation for 10 min in the presence of 10 mM-EGTA. During the fourth incubation, shown in the Figure, substance P was added to give a concentration of 70 nM. Thereafter EGTA was removed by washing, and the slices were tested for their response to substance P (70 nM) in the absence of EGTA (second arrow). Thirty min after the second addition of substance P, Ca^{2+} was added to a concentration of 2·5 mM, followed by addition of substance P, 70 nM (third arrow of substance P).

parotid was within the same concentration range as that found for its action on the spinal motor neurones of the rat (Otsuka & Konishi, 1975).

Ca dependence. The parotid gland is known to have a very high content of cellular calcium (Feinstein & Schramm, 1970). Therefore, in order to test whether the response

to substance P is Ca-dependent, the slices had to be treated with four successive washes each of which involved incubation for 10 min in the presence of 10 mM-EGTA. Following this treatment and removal of EGTA by washing the response to substance P was lost but could be restored to its previous value by addition of calcium to the medium (Fig. 4). It should be pointed out that less extensive depletion of Ca^{2+} than that described above gave either partial inhibition of the substance P induced K^+ release, or no inhibition at all. It was not possible to test the requirement for calcium

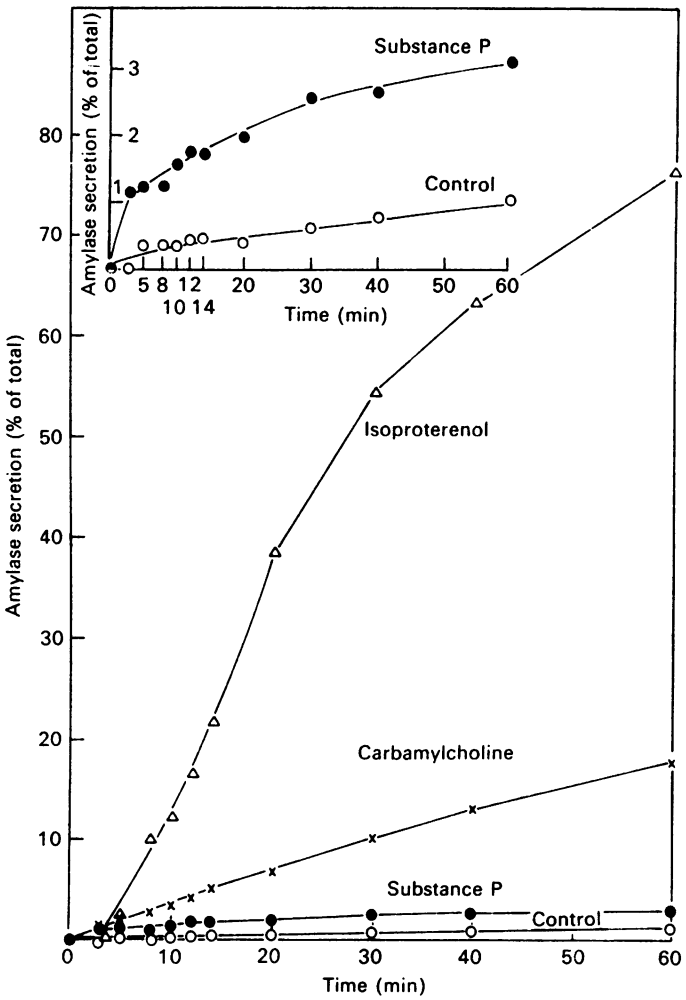

Fig. 5. Effect of substance P, carbamylcholine and isoproterenol cn amylase secretion from rat parotid slices. Substance P 70 nM, carbamylcholine 0·01 mM, and isoproterenol 0·1 mM were added to the slice system at zero time. The inset shows enlarged scale of amylase secretion in the system containing substance P and control with no additions.

in the presence of ouabain since the combination of ouabain and EGTA led to extensive leakage of K^+ from the slices (cf. Rudich & Butcher, 1976). The reason for this leakage is not known.

The effect of substance P on amylase secretion. Compared with isoproterenol and even with carbamylcholine, substance P caused negligible secretion of amylase (Fig. 5). Kinetic studies showed that substance P caused a small burst of amylase secretion during the first few minutes after which the rate of secretion declined and became close to that of the control system incubated in the absence of secretagogue (Fig. 5 inset). The initial rate of amylase secretion was not retained even if multiple additions of substance P were made (not shown). The most probable explanation for the initial burst of amylase secretion is that the peptide caused K^+ release and water secretion, thus sweeping out any amylase which was present in the parotid ducts.

DISCUSSION

The finding that the undecapeptide 'substance P' caused K^+ release from rat parotid slices is consistent with the pronounced sialogenic effect of this peptide in the whole rat (Leeman & Hammerschlag, 1967). The response to substance P in the rat parotid was apparently mediated via a specific receptor since none of the antagonists to the α-adrenergic, the β-adrenergic and the muscarinic cholinergic receptor, nor a combination of the three antagonists inhibited the substance P mediated response on K^+ release. The affinity of the putative receptor for substance P in the parotid system was similar to the affinity found for the peptide in the rat spinal cord preparation (Otsuka & Konishi, 1975). On a molar basis substance P was much more effective than adrenaline or carbamylcholine, both of which act on specific receptors to cause K^+ release from the parotid slices. On the other hand, addition of a saturating dose of substance P to the parotid slice system produced only half of the extent of K^+ release as that elicited by maximal doses of carbamylcholine or adrenaline. It is not likely that this partial response of substance P was due to the short-lived effect of the peptide in the parotid slice system, since successive additions of substance P only maintained the effect but did not increase its extent. Previous work has shown that the amount of K^+ release is determined by two opposite reactions; K^+ efflux as a result of receptor activation and K^+ influx mediated by the Na^+, K^+-ATPase. When the ATPase is blocked by ouabain the K^+ flux becomes unidirectional and even weak stimulation will now be able to release the maximal amount of K^+ available in the cell. Indeed, in the presence of ouabain, substance P caused a release of 50% of the K^+ initially present in the slices. Since the percent of ductal cells is not more than 15%, this experiment indicates that in order to account for the amount of K^+ released substance P must act either on acinar cells or on both acinar and ductal cells.

Cells which respond to hormones and neurotransmitters have various mechanisms for terminating the response. In the case of acetylcholine and noradrenaline there are specific enzymes which inactivate the neurotransmitters. In addition, catecholamines are also known to be actively taken back into the nerve terminals, thus their action at the synapse junction is terminated (Whitby, Axelrod & Weil-Malherbe, 1961; Iversen, 1973). Another common mechanism which is classified as desensitization or refractoriness involves a decrease in the response of the receptors to a continuous or repeated stimulation. The transient nature of the substance P mediated K^+ release reveals that the parotid has an efficient mechanism for terminating the action of the peptide. The fact that the slices readily responded to a second challenge of substance

P rules out desensitization of the receptors and points to inactivation of the undecapeptide as a more likely alternative. A proteolytic activity has been described in brain homogenate whereby substance P is hydrolysed to inactive products (Benuck & Marks, 1975). The inability of protease inhibitors or large amounts of non-specific proteins to prolong the transient response to substance P in the parotid does not prove or refute a proteolytic inactivation of substance P. Equally possible are inactivation mechanisms by specific inhibitors which bind to the peptide, or non-specific absorption of the highly basic substance P to the membrane. The questions of how substance P is inactivated, whether it is present in nerve terminals supplying the gland and how it is released will require more quantitative measurements of the undecapeptide. It is hoped that further studies in the parotid system will shed light on the functions of substance P in the more complex central nervous system.

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