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ROLE OF CALCIUM IN THE FADE OF THE POTASSIUM RELEASE RESPONSE IN THE RAT PAROTID GLAND

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SUMMARY

1. The ^{86}Rb release response in the parotid due to α-adrenergic (epinephrine), muscarinic (carbachol) or peptide (Substance P) receptor activation exhibited 'fade': a return of efflux to control levels despite the continuing presence of agonist.

2. The time course of fade of the response to all three agonists was independent of the concentration of the agonist.

3. After fade was fully developed to one agonist, the response to an agonist acting on a different receptor was either absent or greatly diminished.

4. Removal of carbachol from muscarinic receptors with atropine 10 min prior to Substance P partially restored the ability of Substance P to produce a response.

5. Fade of the response with all three agonists was greatly retarded by the omission of Ca.

6. Release of α-amylase did not appear to fade following exposure to carbachol or Substance P.

7. It is concluded that the K^+ release response may be inactivated with time due to diminution in responsiveness of the K^+ channel to an increase in internal Ca^{2+}.

INTRODUCTION

The parotid gland of the rat has served as an extremely useful tool for the study of receptor mechanisms because of the existence of several distinct receptors in the acinar cells (Butcher, 1978). Three of these, muscarinic, α-adrenergic and peptide (Substance P) receptors, probably act by regulating the activity of a single population of Ca^{2+} channels (Putney, 1977, 1978). The effects of agonists on K^+ efflux can be monitored by using ^{86}Rb as an isotopic indicator of K^+ movements (Putney, 1976*a*). The response to an agonist activating one of these receptors is a biphasic increase in ^{86}Rb release interpreted to signify a biphasic increase in membrane permeability to K^+ (Putney, 1976*a*, 1977, 1978). A transient increase occurs, lasting 2–4 min, which is independent of Ca^{2+} in the bathing medium; then follows a sustained (or slowly falling) phase that requires extracellular Ca^{2+}. The early transient phase has been suggested to result from the release of bound Ca (Putney, 1977) and thus derives its transient nature from the limited size of this membrane Ca pool. The later phase of ^{86}Rb release is believed to be mediated by influx of Ca^{2+} through receptor-activated channels and thus, according to the simplest model, should be a sustained response.

In fact, however, the response appears to 'fade'; that is, efflux of ^{86}Rb returns to normal despite the continued presence of agonist.

In other systems, it has been difficult to determine whether the fade (or desensitization) is due to receptor phenomena or events subsequent to receptor activation (Waud, 1968; Magaznik & Vyskocil, 1973). Resolution of these possibilities should prove simpler with the parotid because of the common Ca^{2+} influx pathway shared by the three receptor mechanisms, and because of the divergent consequences of Ca^{2+} influx.

METHODS

The procedure for measuring the K^+ release response with ^{186}Rb has been described previously (Putney, 1976*a*, 1977). Briefly, rat parotid slices were preincubated for 30 min in a mammalian Ringer solution containing 2–10 μCi ^{86}Rb/ml. The composition of the medium was: NaCl, 120 mM; KCl, 5·0 mM; $CaCl_2$, 1·0 mM; $MgCl_2$, 1·0 mM; tris(hydroxymethyl) aminomethane, 20·0 mM; β-hydroxybutyrate Na, 5·0 mM; pH, 7·40; temperature, 37 °C; gas phase, 100% O_2. After preincubation, the slices, contained in glass and nylon net tissue baskets, were transferred through a series of 2 min incubations for a total of 60 min. Radioactivity emerging from the tissue during each 2 min incubation plus that remaining in the tissue after 60 min was used to calculate apparent first order rate coefficients (%/min). In some experiments, the Ringer solution contained no added Ca and 10^{-4} M-ethyleneglycol(bisaminoethylether)-N,N'-tetraacetic acid (EGTA).

Release of α-amylase was measured as described previously (Leslie, Putney & Sherman, 1976). The protocol was similar to that for the ^{86}Rb experiments, except that the slices were transferred every 10 min and the rate of release expressed as units released per g of tissue per min.

Carbachol, atropine and epinephrine were purchased from Sigma Chemical Co., St Louis, Mo. Substance P was obtained from Beckman Instrument, Inc., Palo Alto, Calif. The ^{86}Rb was purchased from New England Nuclear Corp., Boston, Mass. and had a specific activity of 400–800 Ci/mole.

Statistical analyses were performed with one- and two-way analyses of variance. The maximum level of P to which significance was attributed was 0·05.

RESULTS

Fig. 1 shows the time course of the ^{86}Rb release response of parotid slices activated via α-adrenergic, muscarinic or peptide receptors. In an earlier report (Putney, 1976*a*) when experiments were terminated at 40 min, the increased level of ^{86}Rb efflux between 30 and 40 min was termed 'a sustained (or sometimes gradually falling) response' (only α-adrenergic and muscarinic agonists were employed). Extending the time course of these experiments by 20 min reveals that by 50 min (30 min after drug), ^{86}Rb efflux has returned to control levels following exposure to supramaximal concentrations of epinephrine or carbachol, despite the fact that the drugs are still present.

This return to baseline of the permeability is termed *fade* in this report. Ariens & Simonis (1964) distinguish the term *fade* (decline in response level in the continued presence of agonist) from *desensitization* (decrease in magnitude of response with successive exposures to agonist), and this distinction will be preserved herein. This is not meant to imply, however, that the mechanism(s) of fade and desensitization need be different.

In the case of Substance P, the response returns to control levels more rapidly than with epinephrine or carbachol (by 35–40 min; see also Fig. 3), perhaps because

the magnitude of the Ca-dependent phase is less (Marier, Putney & Van de Walle, 1978). This relative time course did not appear to be dependent on dose for any of the three agonists. The results obtained with carbachol (as representative of all three) are given in Fig. 2.

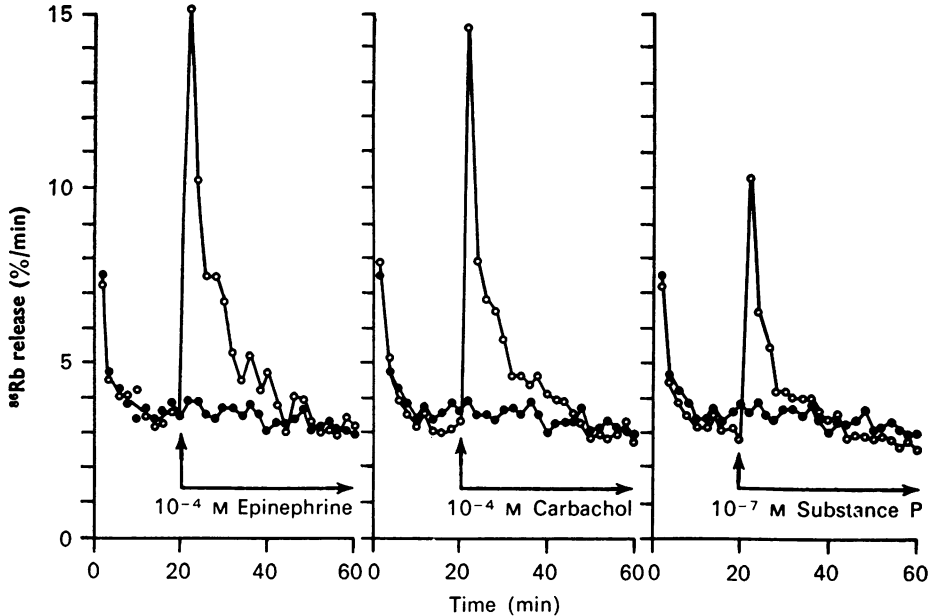

Fig. 1. Time course (fade) of the ^{86}Rb release response in rat parotid slices due to epinephrine, carbachol or Substance P. The filled circles represent control experiments; the open circles, experiments in which the agonists were employed. The agonists were present, in the specified concentrations, from 20–60 min. Each curve is the mean from four separate experiments. The standard error for each data point was generally less than 10% of the mean.

In order to determine the level of inactivation involved in the fade response, effects of the fade of response due to one agonist on the ability of another to elicit a response was investigated. These results are summarized in Fig. 3. In each case, a supramaximal concentration of one agonist was presented to the tissue at 20 min and remained until the end of the experiment. After the response had faded ($t = 50$ min), a supramaximal concentration of a second agonist was added. In all cases, the response to the second agonist was either absent or markedly less than that obtained prior to exposure to the first agonist. The largest second response was always obtained with carbachol or epinephrine following substance P.

The experiments summarized in Fig. 4 were designed to determine the roles of agonist concentration and Ca^{2+} concentration on the desensitization of response to one agonist by prior exposure to another. Decreasing Ca^{2+} concentration to 0·3 mM did not affect the ability of carbachol to prevent a subsequent response to epinephrine. This concentration of Ca^{2+} is still sufficient to produce a Ca-dependent phase of ^{86}Rb release (Marier *et al.* 1978) but the Ca-dependent phase appears to terminate

very quickly under these conditions. In this respect, the fade of the response to carbachol at a decreased level of extracellular Ca^{2+} resembles the apparent rapid fade seen with Substance P at 1·0 mM-Ca^{2+} (Fig. 1).

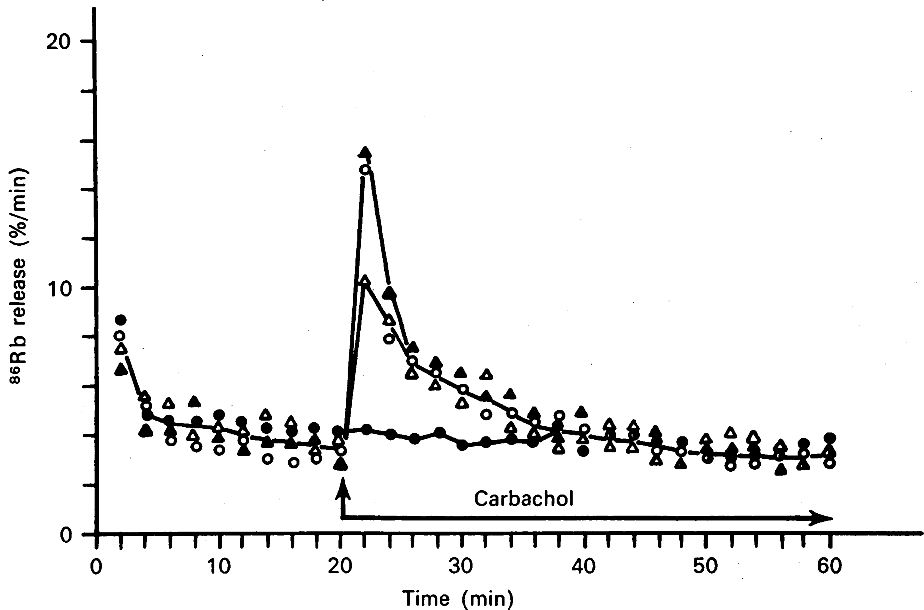

Fig. 2. Fade of ^{86}Rb release due to various concentrations of carbachol, as indicated. The drug was present from 20–60 min. The carbachol concentration (M) was: ●, none; ○, 10^{-4}; ▲, 10^{-5}; △, 10^{-6}. The standard error for each data point (mean of four) was generally less than 10 % of the mean.

When the carbachol concentration was submaximal (3×10^{-7} M), a small but significant response to epinephrine was apparent (Fig. 4).

Inactivation of the Substance P response by carbachol was further investigated in the experiments summarized in Fig. 5. Here an attempt was made to restore the response to Substance P by blocking the muscarinic receptors with atropine. As before, Substance P, 30 min after carbachol, failed to produce any response. When preceded 10 min by atropine, however, a significant response to Substance P was obtained. This response was significantly less than that obtained with Substance P when not preceded by drugs (Fig. 5).

Because of the suspected role of Ca^{2+} in desensitization of nicotinic receptors (see Discussion), the potential role of this divalent cation in the fade of the responses observed here was investigated. Two protocols were employed; the first is illustrated by Fig. 6. In these experiments, the Ringer medium contained no added Ca^{2+} and 10^{-4} M-EGTA. Under these conditions, all three agonists produced Ca-independent, transient responses when added at 20 min. Receptor activity was tested by addition of 1·1 mM-Ca (ionized Ca^{2+} = 1·0 mM), for two minutes only at 30 and 50 min. The responses at 30 min represented greater levels of efflux than those obtained at 30 min if Ca^{2+} were present throughout. More important, significant elevation of ^{86}Rb release was obtained with this protocol for all three receptors at 50 min, a time

when fade of all three responses is complete in Ca-containing media. Though the responses at 50 min appeared less than those at 30 min, these differences were not statistically significant. Earlier experiments have demonstrated that the later addition of Ca^{2+} to media lacking Ca^{2+} will not increase ^{86}Rb efflux if no agonist is present (Putney, 1977).

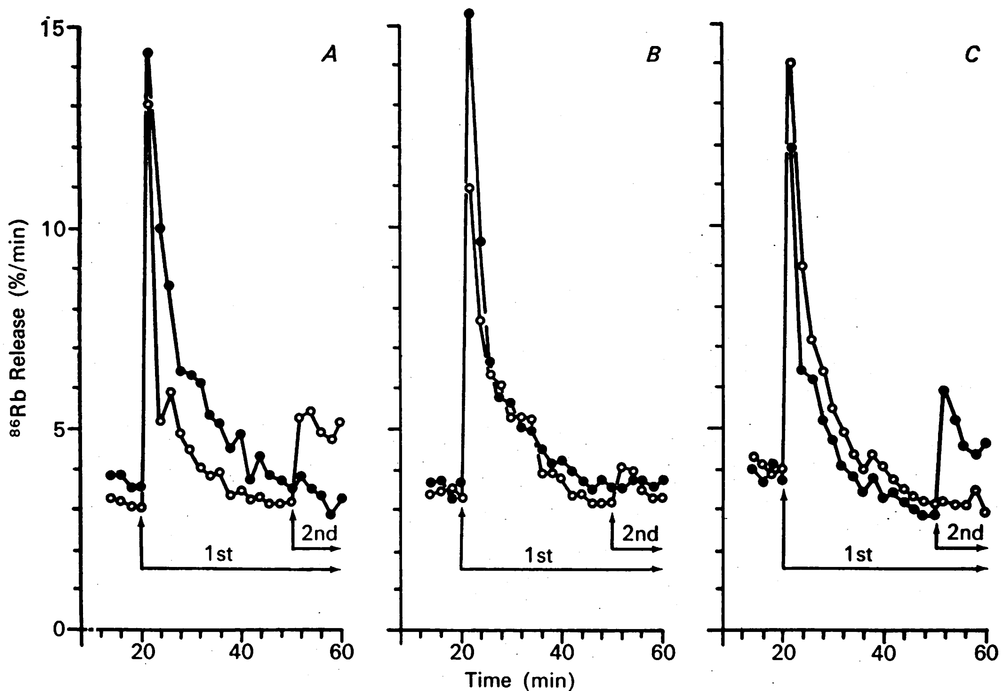

Fig. 3. Fade of ^{86}Rb release due to carbachol (10^{-5} M), Substance P (10^{-7} M) and epinephrine (10^{-4} M), and cross-desensitization. One agonist was added from 20–60 min, and the second from 50–60 min. *A*: ●—●, carbachol followed by Substance P; ○—○, Substance P followed by carbachol. *B*: ●—●, carbachol followed by epinephrine; ○—○, epinephrine followed by carbachol. *C*: ●—●, Substance P followed by epinephrine; ○—○, epinephrine followed by substance P. Each curve is the mean from four separate experiments. The standard errors of the individual data points were generally less than 10% of the mean.

Another protocol similarly demonstrated the role of Ca^{2+} in the fade of the ^{86}Rb release response. These experiments are summarized in Fig. 7. As before, when experiments were performed in the absence of Ca^{2+}, responses to the readdition of Ca^{2+} 30 min after the application of carbachol or substance P could be obtained. If, however, Ca^{2+} was present for all but the last 10 min preceding reintroduction of Ca^{2+}, the responses were significantly less (Fig. 7).

These experiments suggested that Ca^{2+} might be involved in the development of desensitization. In order to determine if the influx of Ca^{2+} was diminished or if the effect of internal Ca^{2+} on K^+ permeability was in some manner affected, the time course of stimulation by carbachol and substance P of another response presumably mediated by Ca^{2+} influx, α-amylase release, was examined. The results are

summarized in Fig. 8. Both total secretion and net secretion due to the secretagogues is presented. The temporal protocol is similar to that for Fig. 3 (add 10 min to the times in Fig. 8 for comparison with Fig. 3). No fade in the enzyme secretion rate

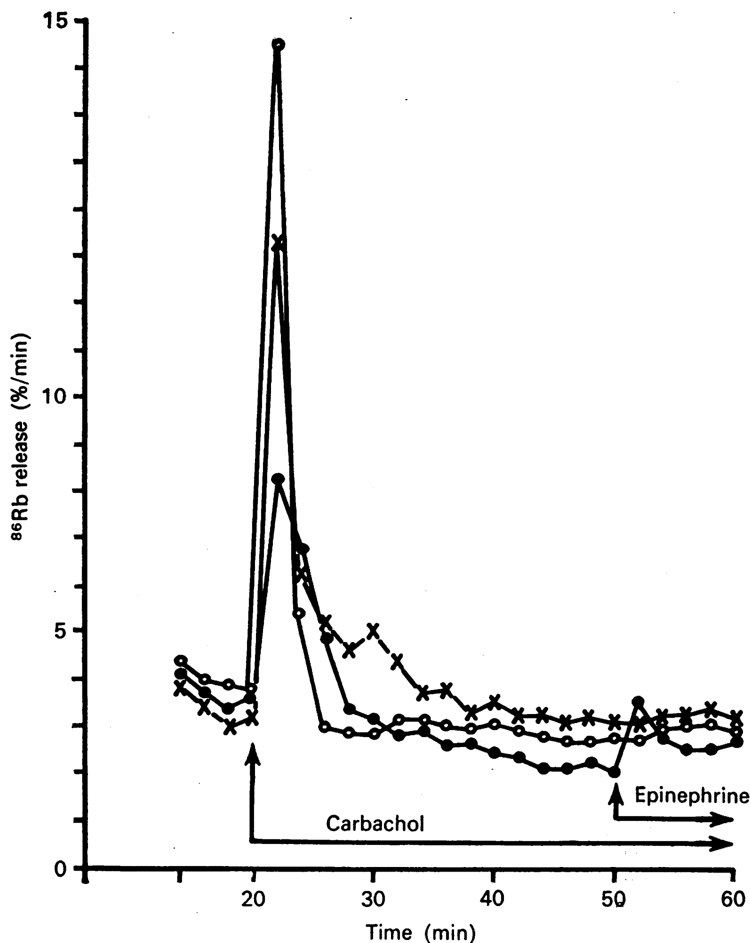

Fig. 4. Effects of decreasing the calcium or carbachol concentration on desensitization of the response to epinephrine. In all experiments, 10^{-4} M-epinephrine was present 50–60 min. Carbachol was present 20–60 min at 10^{-5} M (○—○, ×—×) or 3×10^{-7} M (●—●). Ca concentration (0–60 min) was either 1·0 mM (●—●, ×—×) or 0·3 mM (○—○). Each curve is the mean of three separate experiments. The standard errors of the individual data points were generally less than 10% of the mean.

was noted during the interval where ^{86}Rb release had returned to normal (i.e., compare 30–40 min in Fig. 8 with 40–50 min in Fig. 3). Addition of another agonist (40 –50 min in Fig. 8) failed to increase α-amylase release. This might have been expected, since both carbachol and Substance P presumably act through activation of the same Ca^{2+} channel (Putney, 1977, 1978).

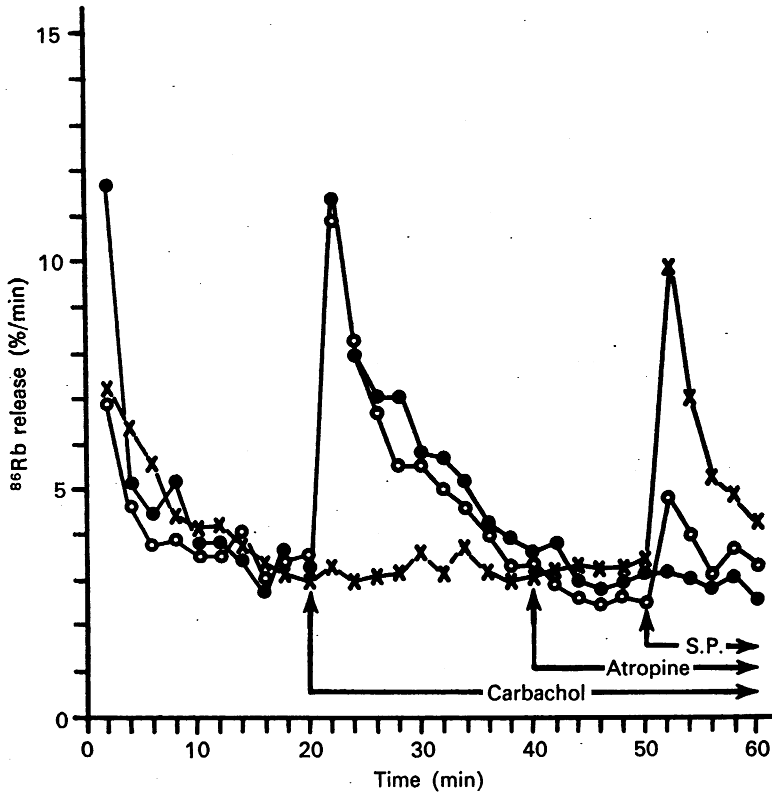

Fig. 5. Densitization of the Substance P response by carbachol, and partial resensitization by atropine. ●—●, carbachol (10^{-5} M), 20–60 min; Substance P (10^{-7} M), 50–60 min. ○—○, carbachol (10^{-5} M), 20–60 min; atropine (10^{-4} M), 40–60 min; Substance P (10^{-7} M), 50–60 min. ×—×, Substance P (10^{-7} M), 10–60 min. S.P. = Substance P. Each curve is the mean from four separate experiments. The standard errors of the individual data points were generally less than 10% of the mean.

DISCUSSION

Burgen (1956) first described the characteristic transient release of cellular K that occurs upon stimulation of the salivary glands. Petersen (1970) found that this release was not inhibited by g-strophanthin or by dinitrophenol and concluded that agonists acted to increase membrane permeability to K^+. When net K^+ release was measured from slices of parotid gland, the response was found to require extracellular Ca^{2+} (Selinger, Batzri, Eimerl & Schramm, 1973), and could be mimicked by a Ca^{2+} ionophore (Selinger, Eimerl & Schramm, 1974). These, and other observations, have led several authors to conclude that activation by muscarinic, α-adrenergic or peptide (Substance P) receptors of the parotid acinar cell leads to a stimulation of Ca^{2+} influx. The resulting elevation in intracellular Ca^{2+} in some manner activates a potassium channel leading to an apparent increase in membrane permeability to K^+ (Selinger, Eimerl & Schramm, 1974; Putney, 1976*a*, *b*, 1977, 1978; Martinez, Quissel & Giles, 1976; Martinez & Quissel, 1976; Butcher, 1978). Actually, by using isotopic techniques (as in this study), the K^+ release response appears to occur in

two phases. One, transient in nature and lasting 2–4 min, is believed to be triggered by the release of a membrane-bound pool of Ca (Putney, 1977). The other, a slowly falling phase lasting 20–30 min, has been proposed to result from the influx of Ca^{2+} ions (Putney, 1977).

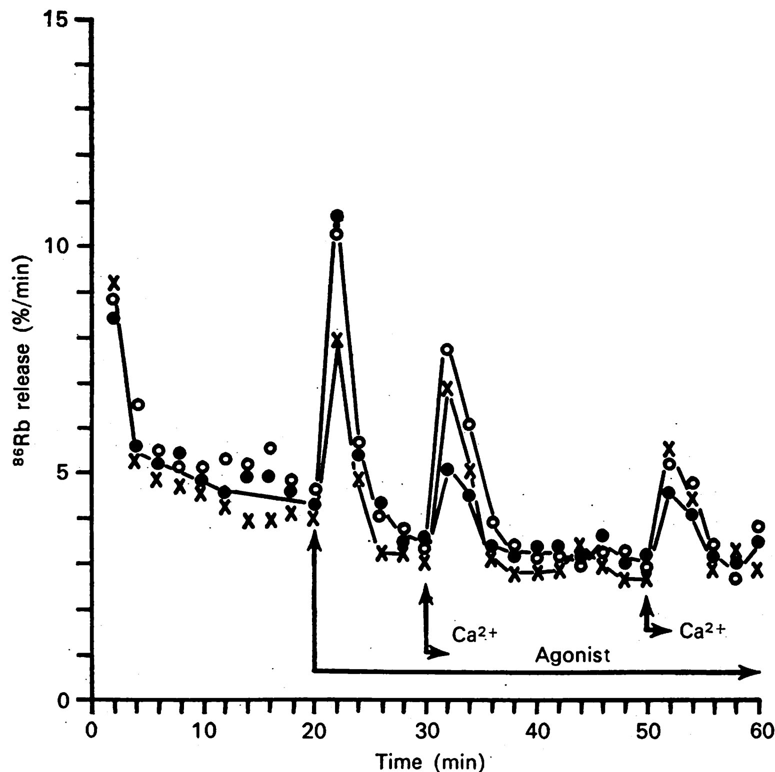

Fig. 6. Maintenance of response sensitivity in the absence of Ca. Experiments were performed in media containing no added Ca and 10^{-4} M-EGTA. Each of the three agonists were present from 20–60 min. ●—●, Substance P (10^{-7} M); ○—○, carbachol, 10^{-5} M; ×—×, epinephrine (10^{-5} M). $CaCl_2$ (1·1 mM) was added from 30–32 min and from 50–52 min. Each curve is the mean from four separate experiments. Standard errors averaged less than 10% of the mean.

The transience of the early phase of K^+ (or ^{86}Rb) release can be explained by assuming a limited pool of releasable Ca^{2+}. The purpose of this investigation was to determine why the Ca-dependent phase of ^{86}Rb release is not permanent (Figs. 1 and 2), despite the continued presence of agonist and Ca^{2+} (note that with this protocol, agonist was replaced freshly every 2 min). This phenomenon has been termed 'fade' or desensitization (Waud, 1968). While most theories attempt to explain such phenomena by assuming disappearance or perturbation of receptors, there is no *a priori* reason why a step (or steps) subsequent to receptor activation could not be involved. Waud (1968) suggested that, in the case of tissues with more than one receptor (as in the parotid), the specificity of the desensitization may serve to distinguish these possibilities. The phenomenon observed here appears primarily

non-specific. Desensitization of the response to carbachol, for example, rendered the tissue incapable of responding to the peptide agonist, Substance P or to epinephrine, an α-agonist. Other sequences of agonists produced qualitatively similar results although substance P could not completely inactivate subsequent responses to carbachol or epinephrine (Fig. 3). These effects were at least partially reversible (Fig. 5), and probably fully reversible with longer wash periods.

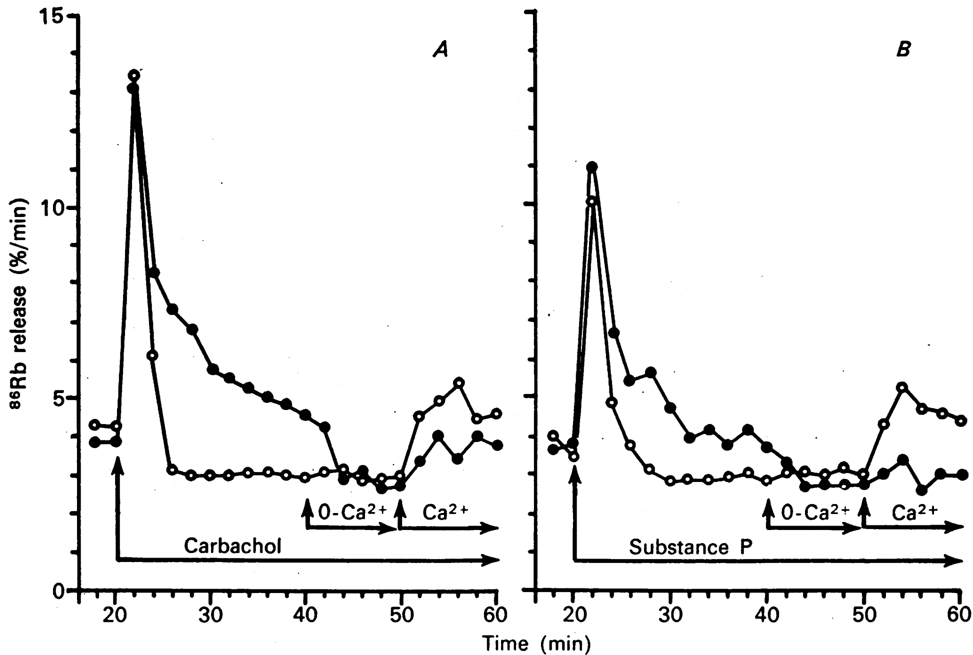

Fig. 7. Role of Ca in the fade of the carbachol and Substance P response. Either carbachol (10^{-5} M; *A*) or Substance P (10^{-7} M; *B*) was present from 20–60 min. ●—●, 1·0 mM-Ca, 0–40 min; 0 Ca + 10^{-4} M-EGTA, 40–60 min. ○—○, 0 Ca + 10^{-4} M-EGTA, 0–60 min. Both: 1·1 mM-Ca, 50–60 min. Each curve is the mean from four separate experiments. Standard errors averaged less than 10 % of the mean.

The failure of Substance P completely to inactivate a subsequent response to carbachol or epinephrine is probably due to its lesser efficacy in producing Ca-dependent K^+ release. In support of this, a submaximal concentration of carbachol did not fully inactivate a subsequent epinephrine response (Fig. 4). Reducing the Ca rather than the carbachol concentration, however, did not produce a similar effect if the epinephrine response was also tested in the low Ca medium (Fig. 4).

In the case of smooth muscle, one possible explanation for non-specific desensitization is fatigue (Waud, 1968), or an inability to produce ATP at a rate sufficient to maintain tension. These considerations probably do not pertain to the K^+ permeability response of the parotid, since, as discussed above, activation of K^+ channels by internal Ca^{2+} probably does not require cellular energy.

The desensitization of ^{86}Rb efflux in the parotid was dependent on the presence of Ca^{2+} in the bathing medium (Figs. 6 and 7). In this respect, the phenomenon

resembles that observed for the nicotinic response at the neuromuscular junction (Katz & Thesleff, 1957; Manthey, 1966) and the response of crab muscle fibres to γ-aminobutyric acid (Sarne, 1976). Since these agents act to stimulate Ca^{2+} influx (Putney, 1978; Butcher, 1978), it is conceivable that a rise in cytosolic Ca^{2+} may serve to trigger the desensitization. Accordingly, Ca^{2+} could act at any of three general

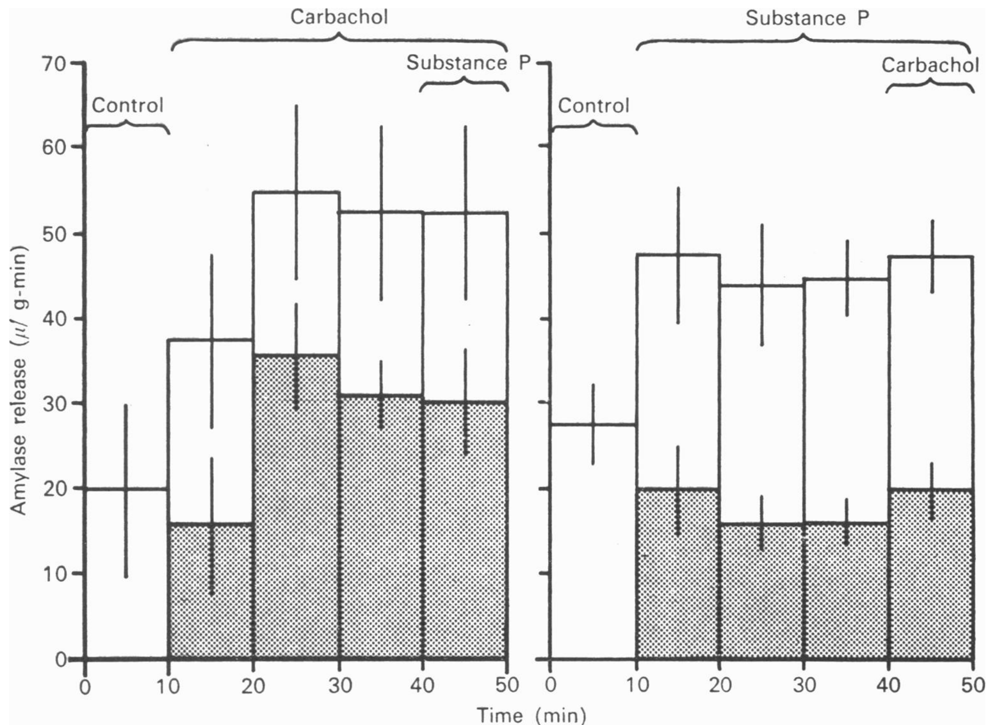

Fig. 8. Time course of release of α-amylase in response to carbachol (10^{-5} M) or Substance P (10^{-7} M). Carbachol (left) or Substance P (right) was added to the incubation media from 10–50 min. In both cases, the other agonist was also present from 40–50 min. The rate of release of α-amylase during each interval is indicated by the open bars. The stippled bars represent the mean paired difference in the release between the control period and each of the periods of drug exposure. Each value is the mean ($\pm$ 1 S.E. of mean) from three replications.

loci: Ca^{2+} could (1) indiscriminantly inactivate all three receptors, (2) inactivate the Ca^{2+} channels or (3) inactivate the K^+ channels. Alternatives (1) and (2) would be difficult to distinguish from one another without ligand binding measurements to determine receptor numbers. However, the results of the experiment summarized in Fig. 8 serve to deny alternatives (1) and (2) in favour of (3). The sustained nature of the α-amylase release due to carbachol or Substance P, throughout the time required for complete desensitization of the K^+ permeability response, suggests that receptors, Ca^{2+} channels and Ca^{2+} influx do not fail within this time frame. Rather, it is suggested, the K^+ channels may become refractory to activation by intracellular Ca^{2+}. If this is so, the ^{86}Rb release response should desensitize even if activated by

Ca^{2+} introduced into the cell by a pathway not involving receptors or endogenous Ca channels. Inspection of the time course of response to the divalent cationophore, A-23187, in previous reports suggests that this is indeed the case (Putney, 1976*a*; Marier *et al.*, 1978).

The classical model for study of the Ca^{2+}-regulated K^+ channel has been the erythrocyte (Gardos, 1956; Lew, 1971; Romero & Whittam, 1971; Lew & Ferreira, 1976; Simons, 1976). Ca^{2+} may also act to modulate K^+ permeability in excitable cells as well (Krjnevic & Lisiewicz, 1972; Meech, 1974; Isenberg, 1975). Until now, evidence has not been obtained with these systems suggesting a desensitization of the K^+ channel to Ca^{2+} with time. Whether this reflects a basic difference in the mechanisms operating in salivary glands or simply to differences in experimental design cannot be clearly determined at present. The results reported here suggest that a similar mechanism is possible in other systems and could be considered in future studies of this nature.

In summary, non-specific desensitization (fade) of the ^{86}Rb release response in the rat parotid gland occurs in response to α-adrenergic, muscarinic or peptide receptor activation. The inactivation is suggested to be mediated by Ca^{2+} influx and may be due to a development of insensitivity of the K^+ channel for activation by internal Ca^{2+}. Whether both or either of these mechanisms play a role in controlling tissue responsiveness in the intact organism must await the results of further investigation.

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