Relationship between latency and period for 5-hydroxytryptamine-induced membrane responses in the *Calliphora* salivary gland

Michael J. BERRIDGE

The Babraham Institute Laboratory of Molecular Signalling, Department of Zoology, Cambridge University, Downing Street, Cambridge CB2 3EJ, U.K.

Following stimulation with a calcium-mobilizing agonist there is often a distinct latency (L) preceding the onset of the first calcium spike. In the continued presence of the agonist, repetitive spikes appear separated by a variable period (P). The relationship between L and P has been investigated in an insect salivary gland responding to 5-hydroxytryptamine (5-HT). Both L and P were found to decrease as the concentration of 5-HT was increased over its physiological range of 1–10 nM. Lowering the concentration of external calcium from 1×10^{-3} M to 1×10^{-5} M increased both P and L. However, the effect on L was apparent only at low levels of 5-HT. Reducing the content of the internal

stores by repeated stimulation in a calcium-free medium resulted in a progressive prolongation of L. On the other hand, the effect on L decreased when glands were stimulated repetitively in normal calcium-containing medium. All these results are consistent with a hypothesis that calcium plays a critical role in determining the kinetics of calcium release during both L and P. An important component seems to be the entry of external calcium, which sets the stage for calcium release by loading up the internal stores. As these stores fill up with calcium, the $\text{Ins}(1,4,5)\text{P}_3$ receptors will initiate a calcium spike once they become sensitized to the ambient level of $\text{Ins}(1,4,5)\text{P}_3$.

INTRODUCTION

Much attention has been focused recently on the temporal aspects of calcium signalling. Following stimulation with a calcium-mobilizing agonist there is often a distinct latency preceding the onset of the calcium signal, which usually appears as a discrete spike. In the continued presence of the agonist, this initial spike is then repeated at periodic intervals to set up oscillations. Rooney et al. (1989) have suggested that events during latency (L) may resemble those taking place in the period (P) between transients within an oscillation. Of particular interest is that both L and P vary with agonist concentration.

Increasing the agonist concentration was found to reduce L in blood platelets (Sage and Rink, 1987), adrenal glomerulosa cells (Quinn et al., 1988), mast cells (Millard et al., 1988), rat lacrimal gland (Horn and Marty, 1989), *Xenopus* oocytes (Miledi and Parker, 1989), hepatocytes (Rooney et al., 1989), hamster eggs (Miyazaki et al., 1990) and smooth muscle (Kalthof et al., 1993; Iino et al., 1993). In all of these examples, the onset of calcium signals results from the release of internal calcium following the generation of $\text{Ins}(1,4,5)\text{P}_3$ (Berridge, 1993). It has been proposed that the generation of $\text{Ins}(1,4,5)\text{P}_3$ might be the major determinant of the latent period (Miledi and Parker, 1989; Lipinsky et al., 1993). Such an interpretation seemed to be supported by the finding that $\text{Ins}(1,4,5)\text{P}_3$ could mobilize calcium within milliseconds of its release from a caged precursor (Kao et al., 1989; Parker and Ivorra, 1993). However, when the amount of $\text{Ins}(1,4,5)\text{P}_3$ being released was reduced to threshold levels L became as long as 8 s (Parker and Ivorra, 1993). Similar long latencies were recorded following flash photolysis of caged $\text{Ins}(1,4,5)\text{P}_3$ in mouse oocytes (Peres, 1990). Therefore, in addition to the time required to generate $\text{Ins}(1,4,5)\text{P}_3$, L may also depend upon the time required for this second messenger to create conditions for the regenerative release of calcium that characterizes a typical calcium spike.

In this paper, the calcium-dependent membrane responses of

an insect salivary gland were used to examine the sequence of events which lead up to the onset of a calcium spike during either L or P. Fluid secretion by this salivary gland is controlled by 5-hydroxytryptamine (5-HT), which uses cyclic AMP to drive potassium pumps, whereas chloride follows passively through channels regulated by calcium (Prince and Berridge, 1973). When stimulated with 5-HT, the transepithelial potential across the salivary gland depolarizes due to the opening of the calcium-dependent chloride channels, which can thus be used as endogenous detectors for changes in intracellular calcium. The main conclusion of this study is that L may depend upon events occurring subsequent to the formation of $\text{Ins}(1,4,5)\text{P}_3$. In particular, the loading of the internal stores with calcium seems to play a critical role in determining both L and P.

MATERIALS AND METHODS

The abdominal regions of the salivary glands from adult female blowflies (*Calliphora erythrocephala*) were isolated and maintained in a medium with the following composition (mM): Na^+ , 155; K^+ , 10; Ca^{2+} , 1; Mg^{2+} , 2; Tris, 10; Cl^- , 156; malate, 2.7; glucose, 10 at pH 7.2.

For the electrical measurements, salivary glands were set up in a perspex perfusion chamber that had three parallel baths (Berridge and Prince, 1972). The two baths containing medium were insulated from each other by liquid paraffin in the middle chamber. A salivary gland was placed in a narrow groove connecting all three compartments so that the closed end was in the perfusion bath while the open end lay in the other outer bath. When switching from control medium to medium containing 5-HT, the delay before 5-HT reached the gland was 1 s (Berridge et al., 1984), and this time has been subtracted when estimating L. Each outer bath was connected to a calomel electrode through a KCl–agar bridge and the transepithelial potential was recorded as described previously (Berridge et al., 1984).

RESULTS

Effect of 5-HT on L and P

The procedure for measuring L and P is illustrated in Figure 1(a). As L is much shorter than P the two were recorded at different pen recorder rates. The stimulus artefact marks the point when the perfusion was switched from control to 2 nM 5-HT. The onset of the depolarizing response was sometimes preceded by a pacemaker, which is clearly evident in Figure 1(a). As this pacemaker was very variable, attention was focused on the rapid rising phase of the response. Therefore, L was taken as the time between the addition of 5-HT and the onset of the depolarizing response, arbitrarily determined as the point where the line through the maximum rate of rise intersected the basal level (Figure 1a, L). Variations in the rate of rise will affect this value, resulting in a small underestimation of L at low agonist concentrations. P was measured as the time between the peaks of the regular oscillations in membrane potential observed at the lower recorder rates (Figure 1a, P).

Because L varies with stimulation frequency (see later), a standard stimulus regime was adopted (Figure 1b). If glands were stimulated repeatedly at a set interval of 60 s, the responses

Figure 1 Transepithelial potential responses of the blowfly salivary gland to stimulation by 5-HT

Salivary glands were constantly perfused in a perspex bath set up to record the transepithelial potential (see the Materials and methods section). (a) Sequential measurement of latency (L) and period (P) in response to 2 nM 5-HT were made at two different pen-recorder speeds. The brief depolarizing spike at the beginning of the trace is a stimulus artefact which marks the beginning of the 5-HT perfusion. The broken lines indicate how L and P were measured. (b) Three consecutive responses to 4 nM 5-HT (solid bars). During the recovery interval between stimulations, the pen recorder was slowed down from 30 cm/min to 30 cm/h. The time bar refers to the timing during each period of stimulation with 5-HT.

Figure 2 Relationship between latency (L) and period (P)

(a) L (●) and P (○) were measured as described in Figure 1(a) at different concentrations of 5-HT. Each point represents the mean $\pm$ S.E.M. for six glands; where absent, the S.E.M. is smaller than the symbol. (b) In a separate experiment, L and P were measured sequentially over a range of 5-HT concentrations. Each point represents the values obtained from individual glands. The regression line intercepted the x-axis at 2.7 s and the correlation coefficient was 0.93.

became highly reproducible and L was constant for each application of 5-HT (Figure 1b).

Both P and L decreased as the concentration of 5-HT increased (Figure 2a). At the lower concentrations, L was much shorter than P, but the two curves intersect at approx. 10 nM 5-HT, at which point oscillations cease. At the higher concentrations of 5-HT, L stabilizes at a minimum value of 2.1 s. Throughout the range of 5-HT concentrations examined, L and P are linearly related to each other (Figure 2b). The regression line intersects the L axis at 2.7 s thus providing another estimate of the absolute L which is close to the value of 2.1 s measured directly in Figure 2(a).

Effect of varying the concentration of external calcium on L

The effect of external calcium on L varied with 5-HT concentration. At high agonist concentrations (i.e. above 10 nM 5-HT), L remained constant at both high (1×10^{-3} M) and low (1×10^{-5} M) calcium concentrations (Figure 3a). However, as the

Figure 3 Effect of varying the concentration of external calcium on latency

(a) L was measured at different concentrations of 5-HT in the presence of either 1×10^{-3} M Ca^{2+} (○) or 1×10^{-5} M Ca^{2+} (●). Each point represents the mean $\pm$ S.E.M. from six glands. (b) Typical transepithelial potential recordings of responses from a single gland to different 5-HT concentrations recorded first in the presence of 1×10^{-3} M Ca^{2+} (A) and later in 1×10^{-5} M Ca^{2+} (B). The two traces have been superimposed to illustrate how L is extended by lowering the level of Ca^{2+} . Recordings like these were used to construct the curves in (a).

concentration of 5-HT declined, L became progressively larger at the lower calcium concentration. A comparison of traces from individual glands clearly illustrates how the reduction in external calcium prolongs L at low, but not at high, 5-HT concentrations (Figure 3b). At the lower levels of stimulation some glands failed

Figure 4 Effect of varying the concentration of external calcium on the period (P) of transepithelial potential oscillations

P was measured at different concentrations of 5-HT in the presence of either 1×10^{-3} M Ca^{2+} (○) or 1×10^{-5} M Ca^{2+} (●). Each point represents the mean $\pm$ S.E.M. from six glands.

to respond to 1 nM 5-HT at the lower calcium level (e.g. bottom trace in Figure 3b). These experiments suggest little role for external calcium at high agonist concentrations, but its contribution becomes increasingly important as the level of stimulation declines.

Effect of varying the concentration of external calcium on P

Frequency was sensitive to a change in calcium concentration throughout the range of 5-HT concentrations which gave rise to oscillatory responses (Figure 4). At each dose of 5-HT, P increased when the concentration of calcium declined from 1×10^{-3} to 1×10^{-5} M. The two curves were not exactly parallel, suggesting that the effect on P may be greater at the lower doses of 5-HT as was observed for L (Figure 3a). Oscillation frequency responds immediately to a change in calcium concentration (Figure 5a). At 5 nM 5-HT, the shift to a lower concentration of calcium results in a large hyperpolarization which precedes the change to a lower frequency (Figure 5a). At the higher dose of 5-HT, there is little evidence of any oscillation at 1×10^{-3} M calcium but these appeared immediately and reversibly upon lowering the calcium concentration to 1×10^{-5} M (Figure 5b).

Depleting the internal store of calcium prolonged L

The *Calliphora* salivary gland contains internal stores of calcium which can be mobilized to maintain responses for a prolonged period in the absence of external calcium (Prince and Berridge, 1973). Gradual depletion of this internal store by repetitive stimulation in a calcium-free medium resulted in a progressive lengthening of L (Figure 6). Salivary glands perfused in a calcium-free medium were stimulated repeatedly with 10 nM 5-HT. As described earlier (Figure 1b), there was no change in L when glands were stimulated repeatedly in the presence of calcium (Figure 6D). In calcium-free solutions, however, L increased progressively during repeated stimulation (Figures 6A–6C). In order to avoid a rapid depletion of the store, 5-HT was quickly removed following each stimulation. The rate at

Figure 5 Effect of varying the concentration of external calcium on the period of transepithelial potential oscillations

External calcium concentration was lowered from 1×10^{-3} M to 1×10^{-5} M for the indicated periods during stimulation of the salivary glands with 5-HT at a concentration of either 5 nM (a) or 10 nM (b).

which L changed with time varied considerably. In those glands where L changed slowly (Figure 6C), the shape of each response remained relatively constant, whereas in those where L increased more quickly, the steepness of the depolarizing response gradually declined (Figures 6A and 6B).

L varies with stimulation frequency and strength

L was found to vary significantly with the frequency of stimulation, especially at low agonist concentrations. At 10 nM 5-HT, there was no change in L after repetitive stimulation at different frequencies (Figure 6D). However, when stimulated with repeated doses of 3 nM 5-HT, L declined from an initial value of 9.2 s to a stable level of 4.5 s (Figure 7a). Stimulating the gland with a much higher concentration of 5-HT (100 nM) for 1 min resulted in an immediate increase in L which once again returned to a stable value upon repeated stimulation with the lower doses. Periods of rest also resulted in temporary increases in L (Figure 7a).

The increase in L observed after a period of prolonged stimulation depended on the dose of 5-HT. A 1 min period of stimulation with 4 nM 5-HT had no effect on L, but this increased markedly following stimulation for the same interval with 100 nM 5-HT (Figure 7b) as noted previously (Figure 7a).

DISCUSSION

A major unsolved problem in calcium signalling concerns the characterization of the events which culminate in the initiation of a regenerative calcium spike. The depolarizing response of these insect salivary glands displays such a regenerative phase charac-

teristic of the calcium spikes recorded in many other cell types. The main conclusion to emerge from these studies is that, during both L and P, there are covert changes in calcium metabolism that set the stage for the initiation of a calcium spike. The pacemaker changes that presage the onset of a calcium spike represent the culmination of this covert activity, which probably results from the action of a localized elevation of $\text{Ins}(1,4,5)P_3$ immediately below the plasma membrane (Berridge, 1992). Release of calcium from stores near the plasma membrane and the resulting capacitative uptake of external calcium will then charge up the stores responsible for the regenerative release of calcium.

A calcium-induced calcium release (CICR) hypothesis attempts to explain the interval between repetitive calcium spikes as the time required to re-charge the internal stores sufficiently to induce the next spike (Goldbeter et al., 1990; Berridge, 1993). A similar process may contribute to L when cells are first stimulated. Some of this L must depend upon the time required to generate the messenger $\text{Ins}(1,4,5)P_3$. Unfortunately it is not possible to measure the time course of $\text{Ins}(1,4,5)P_3$ formation at the low agonist concentrations used in this study. In a previous study, using a high concentration of 5-HT (10 μM), the level of $\text{Ins}(1,4,5)P_3$ was found to increase with no apparent delay, whereas the depolarizing response was delayed by 1–2 s (Berridge et al., 1984). If the same kinetics of $\text{Ins}(1,4,5)P_3$ formation apply at the more physiological levels of 5-HT, it seems that the time required to initiate the formation of $\text{Ins}(1,4,5)P_3$ may not contribute significantly to L. Of more importance, however, might be the time taken for the intracellular stores to become sufficiently responsive to trigger the release of calcium in an all-

Figure 6 Depletion of calcium stores prolongs latency

Individual salivary glands were stimulated repetitively with 10 nM 5-HT either in the absence (A–C) or in the presence of 1×10^{-3} M Ca^{2+} (D). The change in L is indicated both by superimposing the successive transepithelial potential responses obtained from each gland (insets) and by plotting the L values against time of stimulation. Each stimulation with 5-HT lasted for 20 s.

or-none manner; and it is during this phase that calcium may influence its own release. It will be argued therefore that the onset of a calcium spike depends critically on the time taken to prime the internal stores such that they will initiate the process of regenerative calcium release. An important component of this priming event may be the loading of the internal stores with calcium.

When L and P were compared, the former was always found to be much shorter (Figures 3 and 4). A similar relationship was reported in hepatocytes where the P/L ratio was 3:1 (Rooney et al., 1989) which was comparable with the ratio of 3:5 for the insect salivary gland (Figure 2b). On the basis of the store-filling idea, this difference can be explained by assuming that the stores in the unstimulated gland already contain considerable quantities of calcium, whereas they are empty (or partially emptied) following a spike. The effect of varying external calcium on both L and P may also be explained in terms of store loading. Oscillation frequency was found to be sensitive to the level of external calcium over the entire oscillatory range of 5-HT concentrations (Figure 4). Similar effects of calcium on oscillatory frequency have been described in endothelial cells (Jacob et al., 1988), pancreatic cells (Zhao et al., 1990), fibroblasts (Kawanishi et al., 1989), and human sweat ducts (Pedersen, 1991). Even in *Xenopus* oocytes, which can oscillate for long periods in the absence of external calcium, the frequency of calcium spiking can be accelerated by enhancing the influx of external calcium by membrane hyperpolarization (Girard and Clapham, 1993).

L can also be influenced by modulating the entry of external calcium but this effect was only apparent at the lower concentrations of 5-HT (Figures 3a and 3b). Removing external calcium

Figure 7 Effect of repetitive stimulation on latency

(a) A single salivary gland was stimulated repetitively with pulses (20 s) of 3 nM 5-HT during which L was recorded as outlined in Figure 1 (the stimulus regime is indicated at the top of the figure). The responses shown were typical of those obtained in four similar experiments. (b) Similar protocol to that used in (a). In this case test pulses (20 s) of 4 nM 5-HT were used to measure L.

also lengthened L in hepatocytes (Rooney et al., 1989) and aortic smooth-muscle cells (Kalthof et al., 1993), and prevented the onset of spiking in rat chromaffin cells in response to low concentrations of bradykinin (Malgarelli and Meldolesi, 1991). Although not studied in detail, membrane depolarization was found to prolong both L and P in the insect salivary gland (results not shown). Similarly, L in endothelial cells was reduced by hyperpolarization but lengthened by depolarization (Nilius et al., 1993). These effects of membrane potential on L may result from changes in calcium entry because depolarization reduces both the driving force and the current flow through the calcium channels responsible for capacitative calcium entry (Fisher et al., 1992; Zhang and Melvin, 1993). It seems that the entry of external calcium may contribute to the initiation of a calcium spike, especially at sub-maximal agonist concentrations where the level of $\text{Ins}(1,4,5)\text{P}_3$ will be limiting.

Perhaps the strongest evidence to support a role for store loading as a determinant of L has come from experiments where the store was slowly depleted by repetitive stimulation in a

calcium-free medium (Figure 6). There was a progressive lengthening of L as the internal store was emptied. A similar prolongation of L has been reported in aortic smooth-muscle cells (Kalthof et al., 1993), *Xenopus* oocytes (Lipinsky et al., 1993) and in human umbilical vein smooth-muscle cells (Nicholls et al., 1993) following repeated stimulation in calcium-free conditions. Variations in store filling could also explain the changes in L when varying either the frequency or the strength of stimulation (Figure 7). The long L observed when cells are first stimulated, or if they are allowed to rest for a long period, may mean that the stores of resting cells have lower levels of calcium than those which are being stimulated regularly. L increased following a supra-maximal dose of 5-HT which would be expected to induce a large depletion of the internal stores.

All this evidence suggests that calcium plays a critical role in determining the kinetics of calcium signalling. Undoubtedly some of the delay following stimulation depends upon the time to generate $\text{Ins}(1,4,5)\text{P}_3$ (Miledi and Parker, 1989; Lipinsky et al., 1993) but there may be additional delays required for this messenger to set the stage for the regenerative release of calcium, especially at low levels of stimulation. A significant part of this preparation seems to require the entry of external calcium, which is then taken up by the internal stores to increase their sensitivity to the ambient level of $\text{Ins}(1,4,5)\text{P}_3$. Just how this loading of the stores alters the sensitivity of $\text{Ins}(1,4,5)\text{P}_3$ receptor is still unclear. It may result from a direct effect of the increase in luminal calcium (Oldershaw and Taylor, 1993; Parys et al., 1993) or it may occur indirectly due to a build up of cytosolic calcium resulting from the saturation of the internal buffering systems (Petersen et al., 1993). Since the $\text{Ins}(1,4,5)\text{P}_3$ receptor is sensitive to both $\text{Ins}(1,4,5)\text{P}_3$ and calcium (Berridge, 1993), any elevation of cytosolic calcium will help to sensitize the receptor and set the stage for the onset of the regenerative process of CICR responsible for the rapid upstroke of the calcium spike. This sensitizing effect of elevating the cytosolic level of calcium will be particularly important at low agonist concentrations where the level of $\text{Ins}(1,4,5)\text{P}_3$ is limiting. Indeed a pacemaker elevation of calcium precedes the onset of calcium spikes in endothelial cells (Jacob et al., 1988), sympathetic ganglion neurons (Friel and Tsien, 1992), hepatocytes (Somogyi et al., 1992), hamster eggs (Miyazaki et al., 1992), mouse eggs (Peres, 1990; Cheek et al., 1993) and in smooth-muscle cells (Iino et al., 1993). Evidence of a pacemaker depolarization preceding the rapid upstroke of the transepithelial responses in the blowfly salivary gland are clearly evident in Figures 3(b) and 6(A). In smooth-muscle cells, the onset of regenerative spikes also displayed two phases (Nicholls et al., 1993; Iino et al., 1993). Nicholls et al. (1993) have argued that these two phases may depend upon the existence of stores having different sensitivities to $\text{Ins}(1,4,5)\text{P}_3$. The most sensitive stores would respond first to give the pacemaker elevation of calcium followed by the all-or-none regenerative process of CICR. It is argued, therefore, that variations in L and P are determined by the time taken for the pacemaker to raise calcium to the threshold for the onset of CICR. The steeper the pacemaker

elevation of calcium the sooner the spike will be initiated. The entry of external calcium, together with the state of filling of the intracellular stores, seem to be major factors in determining the kinetics of the calcium pacemaker and hence the onset of a calcium spike.

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