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SALICYLATE-INDUCED CATION FLUXES ACROSS BIOLOGICAL MEMBRANES

A STUDY OF THE UNDERLYING MECHANISM

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Abstract—The effect of salicylate on cation transport by lactating rat mammary tissue has been examined. Consistent with previous results, salicylate increased the unidirectional efflux of K^+ (Rb^+): this increase was dependent on extracellular Ca^{2+}. Lowering the temperature of the incubation medium from 37° to 4° did not attenuate the effect of salicylate on K^+ (Rb^+). Ca^{2+} uptake by mammary tissue was stimulated by salicylate, whereas Ca^{2+} efflux was not greatly affected. Salicylate reduced the ouabain-insensitive uptake of K^+ (Rb^+). The net K^+ content of mammary tissue was reduced by incubating tissue in a medium containing salicylate, whereas net Na^+ content was increased. The results are consistent with the hypothesis that salicylate activated a calcium-dependent K^+ efflux channel and/or a calcium-dependent non-selective cation channel.

Salicylate can affect the permeability of biological membranes to both anions and cations. For example, Wieth [1] demonstrated that salicylate inhibits chloride and sulphate transport by human erythrocytes and Shennan and Russell [2] have shown that it can block sulphate fluxes via an anion exchanger in human placental brush-border membrane vesicles. The action of salicylate on cation transport seems to be more complex. The permeability of human red cells to K^+ and Na^+ is markedly enhanced when extracellular chloride is replaced by salicylate: the influx and efflux of both cations is increased, resulting in a net loss of K^+ and a net gain of Na^+ [3]. Moreover, Wieth [3] showed that the effect of salicylate on red cell cation permeability varies in an unusual way with temperature. Thus, K^+ efflux and Na influx fell as the temperature of the incubation medium was raised from 0° to 18° and then increased with temperature between 18° and 38°. The effect of salicylate on cation fluxes is not limited to human erythrocytes. Shennan [4] showed that salicylate can increase the rate of K^+ efflux from rat mammary tissue. Replacing extracellular Cl^- with salicylate leads to a reversible increase in unidirectional K^+ efflux (measured using ^{86}Rb) from preloaded lactating mammary tissue slices.

The mechanism responsible for the action of salicylate on cation transport has not been elucidated, and the present study was therefore undertaken in an attempt to characterize the action of salicylate on cation fluxes in lactating mammary tissue.

MATERIALS AND METHODS

Lactating rats 10–15 days post-partum were lightly anaesthetized with ether and killed by cervical dislocation. The abdominal mammary glands were immediately removed and placed in an ice-cold solution containing (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–BES* pH 7.4. The tissue was finely chopped to produce fragments weighing between 5 and 20 mg.

Measurement of K^+ *transport.* Potassium transport by mammary tissue fragments was assayed using ^{86}Rb as a tracer; it is now well established that this isotope is suitable for this purpose in a variety of tissues including mammary gland (e.g. Ref. 5). However, whilst Rb^+ and K^+ fluxes via systems such as the Na^+:K^+ pump, and the ($Na^+ + K^+ + Cl^-$) cotransporter have been shown to be similar it must be borne in mind that this may not be the case for Rb^+ fluxes via K^+ channels.

Measurement of K^+ (Rb^+) *uptake.* Mammary tissue segments were incubated at 37° in the appropriate buffer for 20 min before the addition of ^{86}Rb. Uptake was assayed over a period of 60 min. At pre-determined times tissue was removed and immediately washed with 4 mL of an ice-cold solution identical in composition to that described above. The tissue fragments were then lightly blotted and placed in pre-weighed vials. After the tissue had been weighed 4 mL of distilled H_2O was added to each vial. The tissue fragments were left to stand in the water for at least 16 hr in order to allow the isotope to leak out.

In each experiment extracellular tissue water was measured by following the distribution of [^{14}C]-sucrose (see Ref. 6) and total tissue water was obtained by weighing tissue fragments before and after drying overnight at 110°. K^+ (Rb^+) uptake was calculated from the following equation:

$$[K^+]_c = \frac{[K^+]_t - (F \cdot [K^+]_m)}{1 - (F + A/B)}$$

where $[K^+]_c$ is the concentration of K^+ in the cells in mmol/kg of cell water, $[K]_t$ is the concentration of K^+ in whole tissue in mmol/kg, $[K]_m$ is the

* Abbreviations: BES, *N*,*N*-bis (2-hydroxyethyl)-2-aminothane-sulphonic acid; Tris, tris (hydroxymethyl) amino-methane.

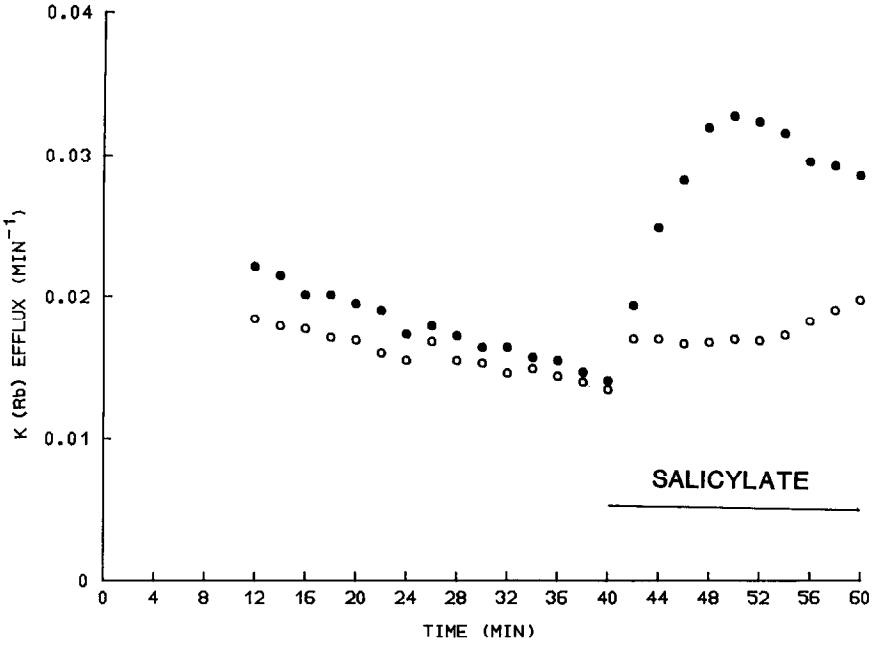

Fig. 1. The effect of Cl^- replacement by salicylate in the absence (○) and presence (●) of external Ca^{2+} on K^+ efflux from lactating rat mammary tissue. The incubation medium contained (mM): 135 NaX (where X = Cl^- or salicylate), 5 KCl, 1 $MgSO_4$, 10 glucose and 10 Tris–BES pH 7.4. When Ca^{2+} was required, 2 mM $CaCl_2$ was added to the incubation medium. The period during which salicylate was present is indicated by the bar. Efflux was assayed at 37°. Each point represents the mean of six determinations.

concentration of K^+ in the medium, F is the extracellular space, and A and B are respectively the tissue dry and wet weight.

Measurement of K^+ (Rb^+) *efflux*. Mammary tissue fragments were pre-loaded with ^{86}Rb by a 1 hr incubation at 37° in a medium containing (mM):135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–BES pH 7.4 (+10 μCi/mL ^{86}Rb). Following the loading period the tissue was transferred through a series of tubes containing 2.0 mL of non-radioactive solutions at 2 min intervals. The radioactivity remaining in the tissue at the end of the incubation was determined by placing the tissue slice in water (4 mL) and leaving for at least 16 hr. The fractional efflux of K^+ (Rb^+) was estimated for each collection period by calculating the ratio of the radioactivity lost from the tissue per minute to the arithmetic mean content of radioactivity. Efflux rate constants quoted in the text were calculated from plots of ln (fraction of isotope remaining in tissue) as a function of time [4].

Ca^{2+} *flux measurements*. Calcium uptake and efflux from lactating rat mammary tissue slices was assayed using ^{45}Ca by the methods described above for measuring K^+ (Rb^+) transport. Ca^{2+} uptake was corrected for tissue extracellular space and expressed as mmol/kg cell H_2O.

^{86}Rb (as RbCl) and ^{45}Ca (as $CaCl_2$) were obtained from Amersham International plc (Amersham, U.K.). All other chemicals including sodium salicylate, were obtained from the Sigma Chemical Co. (Poole, U.K.).

Statistics. Data are expressed as means ± SEM. Differences were assessed by Student's paired or unpaired t-tests as appropriate and were considered significant when $P < 0.05$.

RESULTS

The fractional release of K^+ (Rb^+) from preloaded lactating rat mammary tissue slices maintained at 37° initially fell before reaching a relatively constant rate after about 20 min (Fig. 1). Replacement of Cl^- in the medium with salicylate at t = 40 min caused an increase in the fractional release of K^+ (Rb^+) in accordance with previous results [4]. The calculated efflux rate constant was increased from a value of $0.0159 \pm 0.0018\ min^{-1}$ in a Cl^- medium to $0.0322 \pm 0.0032\ min^{-1}$ in a salicylate medium ($N = 6$; $P < 0.01$ paired t-test). Attempts to quantify the effect of varying the concentration of salicylate on K^+ (Rb^+) efflux gave equivocal results: in some experiments a salicylate concentration as low as 5 mM caused a significant increase in K^+ (Rb^+) efflux, whereas in others 20 mM failed to stimulate K^+ (Rb^+) loss (results not shown). The stimulation of K^+ (Rb^+) efflux by salicylate from lactating mammary tissue cannot be attributed to the ($Na^+ + K^+ + Cl^-$) cotransport pathway because furosemide, an inhibitor of ($Na^+ + K^+ + Cl^-$) [7] cotransport failed to attenuate the salicylate-induced increase in K^+ (Rb^+) efflux [4]. However, one candidate pathway could be Ca^{2+}-activated K^+ efflux. Therefore, the effect of salicylate was tested

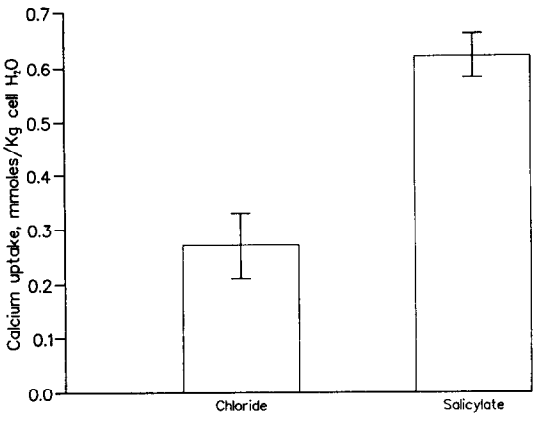

Fig. 2. The effect of salicylate on Ca^{2+} uptake by lactating rat mammary tissue. The composition of the medium was the same as that described in the legend to Fig. 1. The data shown represents the mean ± SEM from four experiments. Uptake was assayed at 37°.

under conditions in which extracellular Ca^{2+} was removed (Fig. 1). In this series of experiments the tissue was pre-loaded with ^{86}Rb in a Ca^{2+}-free medium. The rate constants in Cl^- and salicylate media (in the absence of Ca^{2+}) were, respectively, 0.0144 ± 0.008 and 0.0180 ± 0.0021 min^{-1} (N = 6; not significant, paired *t*-test). Thus, salicylate failed to stimulate K^+ (Rb^+) efflux in the absence of extracellular Ca^{2+}. Note that Ca^{2+} removal did not affect the rate of K^+ (Rb^+) release from mammary tissue suspended in a Cl^- medium (Fig. 1).

The finding that the salicylate-induced K^+ (Rb^+) efflux depends on the presence of extracellular Ca^{2+} suggested that an increase in the Ca^{2+} permeability of mammary cells may have to occur before an increase in K^+ (Rb^+) efflux. Therefore, the effect of salicylate on the movement of radiolabelled Ca^{2+} into and from lactating mammary tissue was studied. Figure 2 illustrates the amount of Ca^{2+} uptake found with Cl^- and salicylate media measured over a period of 20 min. It is evident that salicylate markedly increases the rate of Ca^{2+} accumulation beyond that

Table 2. The effect of salicylate on the Na^+ and K^+ content of lactating mammary tissue

	Cation concentration (mmol/kg cell H_2O)	
	$[Na^+]$	$[K^+]$
Chloride	81.6 ± 2.8	79.3 ± 2.4
Salicylate	159.2 ± 15.2*	28.7 ± 1.9†

Mammary tissue was incubated in a medium containing (mM):135 NaX (where X = Cl^- or salicylate), 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–BES pH 7.4 at 37° for 60 min. Na^+ and K^+ concentrations were determined by flame photometry.

Values shown (± SEM) are data from three separate experiments.

* $P < 0.01$, † $P < 0.001$.

found with Cl^- (0.62 ± 0.04 vs 0.27 ± 0.06 mmol/kg cell H_2O, N = 4; $P < 0.01$, paired *t*-test). Figure 3 shows the effect of replacing Cl^- with salicylate on the efflux of Ca^{2+} from preloaded tissue. The rate constant in Cl^- and salicylate medium was, respectively, 0.0284 ± 0.0023 and 0.0305 ± 0.0060 (N = 3; not significant, paired *t*-test). The efflux of Ca^{2+} measured over the entire time course into a medium with Cl as the major anion is also shown in Fig. 3. The rate constant of Ca^{2+} efflux in these experiments was 0.0334 ± 0.0014 (± SD, N = 2). It is therefore apparent that Cl removal and replacement by salicylate has no effect on Ca^{2+} efflux.

Wieth [3] demonstrated that salicylate is capable of stimulating K^+ efflux from human red blood corpuscles at temperatures as low as 4°. In view of this, the effect of salicylate on K^+ (Rb^+) loss from lactating mammary tissue maintained at 4° was examined. The results (Table 1) show that salicylate increases the rate constant of K^+ (Rb^+) efflux to the same extent irrespective of temperature. However, K^+ (Rb^+) loss is reduced with temperature when measured into a Cl^- medium; this probably reflects the temperature sensitivity of the ($Na^+ + K^+ + Cl^-$) cotransport system. Figure 4a and b shows the effect of salicylate over the range of 1–135 mM on K^+

Table 1. The effect of temperature on salicylate-induced K^+ efflux from mammary tissue

Temperature (°C)	K^+ efflux rate constants (min^{-1})		
	Chloride	Salicylate	Difference
37	0.0135 ± 0.0006	0.0303 ± 0.0022	*0.0168 ± 0.0022
4	0.0060 ± 0.0005	0.0179 ± 0.0009	*0.0119 ± 0.0004

K^+ efflux was measured from mammary tissue suspended in a medium containing (mM):135 NaX (where X = Cl or salicylate), 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–BES pH 7.4.

The data shown are the means ± SEM for eight experiments at 37° and four experiments at 4°.

* $P < 0.001$.

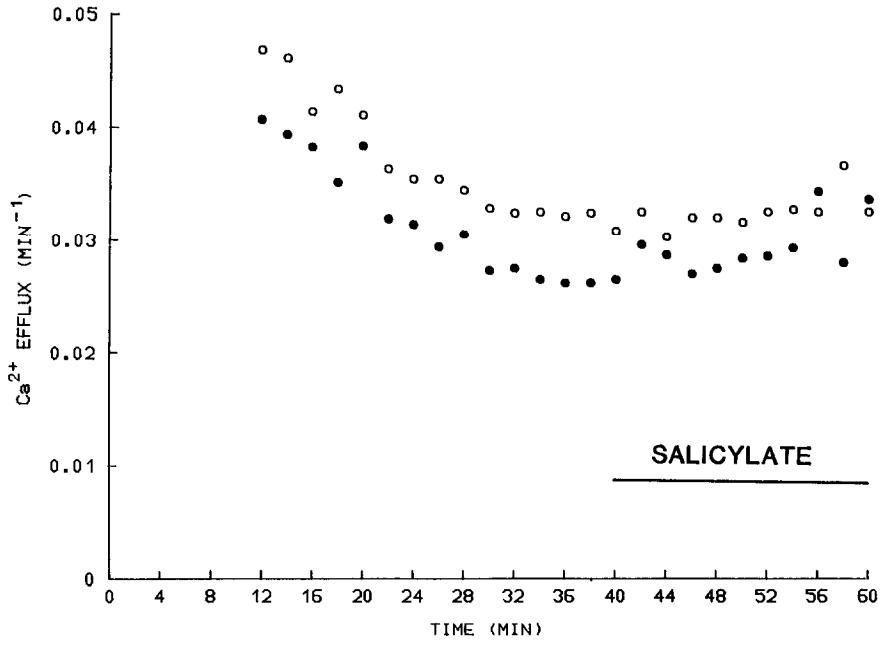

Fig. 3. The effect of Cl^- replacement by salicylate on Ca^{2+} efflux from lactating rat mammary tissue. In the experiments denoted by filled circles the efflux of Ca^{2+} was measured into a medium containing (mM):135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–BES pH 7.4 before being transferred into a medium in which NaCl was replaced by an equimolar amount of Na salicylate (indicated by bar). Each point represents the mean of these three determinations. Efflux was assayed at 37°. The open circles represents the fractional efflux of Ca^{2+} measured into a medium containing NaCl throughout. Each point represents the mean of two determinations. Efflux was assayed at 37°.

(Rb^+) efflux measured at 4°. It is apparent that the stimulatory effect of salicylate is dose-dependent.

The effect of salicylate on the ouabain-insensitive moiety of K^+ (Rb^+) uptake by lactating rat mammary tissue was also examined. K^+ (Rb^+) uptake was measured from media containing ouabain with either Cl^- or salicylate as the principal anion. The fraction of K^+ (Rb^+) uptake via the ($Na^+ + K^+ + Cl^-$) cotransporter was assessed using the loop-diuretic furosemide. Figure 5 shows that furosemide was effective at blocking K^+ (Rb^+) uptake from tissue suspended in a medium containing Cl^-. This is in accordance with previous results [4]. In contrast, furosemide had no effect when Cl^- was replaced with salicylate. This suggests that salicylate cannot substitute for Cl^- in the 'cotransport' pathway. It is evident that salicylate reduced the component of K^+ (Rb^+) uptake which was inhibited by neither ouabain nor furosemide compared with that found in a Cl^- medium.

Salicylate inhibits K^+ (Rb^+) uptake by mammary tissue whilst concomitantly stimulating K^+ (Rb^+) efflux. It was therefore predicted that it should induce a net loss of K^+. To test this prediction mammary tissue was incubated for 60 min in a medium containing either Cl^- or salicylate as the major anion. Following this, the tissue was disrupted with trichloroacetic acid and the ion content (i.e. Na^+ and K^+) was analysed by flame photometry. The results of these experiments are shown in Table 2. As predicted, replacing Cl^- with salicylate gave rise to a net loss of K^+. In addition salicylate also caused a net gain of sodium ions.

DISCUSSION

These experiments were conducted to investigate the effect of salicylate on cation permeability of biological membranes. The results confirm and extend the earlier findings of Shennan [4] that salicylate can induce a large increase in the fractional efflux of K^+ (Rb^+) from lactating rat mammary tissue. This is in accordance with the finding of Wieth [3], who showed that salicylate can stimulate a massive loss of K^+ from human erythrocytes. A new finding is that salicylate-induced K^+ (Rb^+) efflux from mammary tissue depends on the presence of extracellular Ca^{2+}. Moreover, it is apparent that salicylate can stimulate Ca^{2+} uptake by mammary tissue. Therefore, the salicylate-dependent increase in K^+ (Rb^+) efflux is consistent with the activation of a Ca^{2+}-dependent K^+ pathway. It appears that this pathway is a channel rather than a carrier given that K^+ (Rb^+) efflux was stimulated by salicylate at 4°, since carrier-mediated cation transport would be expected to be inactivated at this low temperature. Calcium-activated K^+ channels form a ubiquitous class of transporters; indeed Furuya *et al.* [8] have evidence for such channels in cultured mammary epithelial cells. However, Wieth [3] found that salicylate could also increase the permeability of red membranes to Na^+. The data shown in Table 2

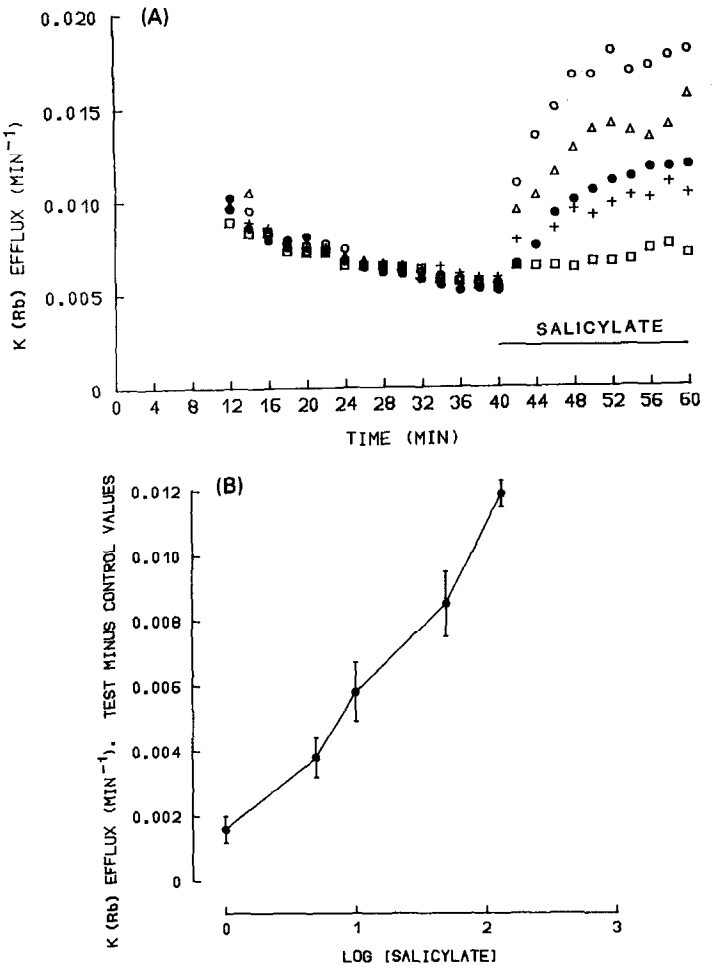

Fig. 4. (A) The effect of varying concentrations (1–135 mM) of salicylate on the fractional efflux of K^+ from lactating rat mammary tissue maintained at 4°. The salicylate concentrations used were 1 mM (□), 5 mM (+), 10 mM (●), 50 mM (△) and 135 mM (○). (B) Dose–response curve showing the effect of salicylate on K^+ efflux. The points were calculated from the data shown in (A). Each point represents the difference in rate constant values found between Cl^- and salicylate media.

suggest that mammary tissue Na^+ permeability is also increased by salicylate. Another explanation for the action of salicylate is that it induces Ca^{2+}-activated non-selective cation channels. In this connection there is a large body of evidence for non-selective cation channels in a number of cell types (e.g. Refs 9–12), including mammary tissue [13].

The question arises as to how salicylate increases Ca^{2+} uptake by mammary tissue? There are several possible explanations for this. Firstly, salicylate could increase Ca^{2+} uptake by forming a complex with Ca^{2+}. Salicylate is known to cross biological membranes rapidly [14], and if Ca^{2+} formed a complex with salicylate it could lead to a rise in intracellular Ca^{2+}. Nishihata *et al.* [15] have shown that Ca^{2+} forms a complex with salicylate and that the complex is more lipophilic than salicylate alone. However, the finding that salicylate does not greatly affect Ca^{2+} efflux from mammary tissue suggests that the movement of a salicylate–Ca^{2+} conjugate does not greatly contribute to Ca^{2+} movements. Secondly, salicylate may act as a Ca^{2+}-channel former (i.e. an ionophore). Thirdly, salicylate may activate endogenous quiescent Ca^{2+} channels.

The effect of salicylate on K^+ (Rb^+) efflux from mammary tissue was found to be dose-dependent at 4° and a concentration as low as 1 mM increased efflux. In contrast, the effect of low concentrations of the drug on K^+ (Rb^+) efflux measured at 37° was variable. This difference may be related to the Ca^{2+} extrusion mechanisms within mammary cells. At 4° and 37° it is likely that salicylate induces the uptake of similar amounts of Ca^{2+}. However, at 37° Ca^{2+} will be extruded via Ca^{2+} pumps situated in the plasma membranes, whereas at 4° such mechanisms will be inactivated. This would mean that a low concentration of salicylate would be more effective at raising the intracellular [Ca^{2+}] at 4° than at 37°. A similar explanation may account for the complex

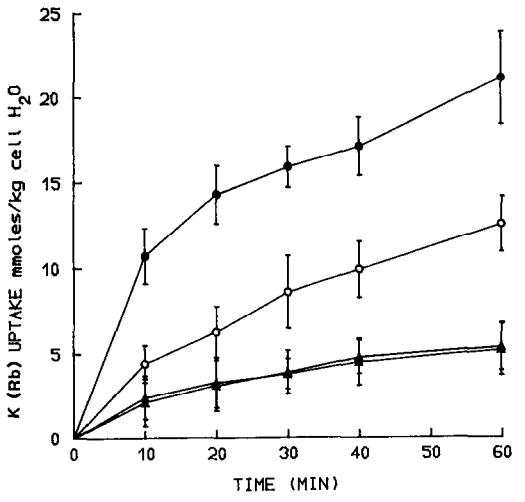

Fig. 5. The effect of salicylate on K^+ uptake by lactating rat mammary tissue. The composition of the incubation medium was the same as that given in the legend to Fig. 1. Uptake was measured at 37° from media containing Cl^- + 1 mM ouabain (●), Cl^- + 1 mM ouabain + 1 mM furosemide (○), salicylate + 1 mM ouabain (▲) and salicylate + 1 mM ouabain + 1 mM furosemide (△). Each point represents the mean ± SEM of three experiments.

effect of temperature on salicylate-induced cation fluxes in red cells found by Wieth [3].

Whilst there are many similarities between the response of red cells and mammary tissue to salicylate several differences were noted. For example, Wieth [3] found that salicylate increased passive K^+ uptake by red cells whereas K^+ accumulation by mammary tissue was inhibited. This result may reflect differences in the cell membrane potential. If salicylate increases Na^+ fluxes as well as K^+ fluxes (as appears to be the case) cell depolarization will result, which in turn will reduce K^+ uptake. The reason that a decrease in K^+ uptake not seen by Wieth [3] may be related to the fact that the red cell membrane potential (approximately −8 mV) is determined by the Cl^- permeability (which is several orders of magnitude greater than the cation permeability) [16]. Wieth [3] also found that thiocyanate increased red cell cation permeability, although to a lesser extent than salicylate. In contrast, SCN^- does not stimulate K^+ (Rb^+) efflux from mammary tissue, either at 37° [4] or at 4° (Shennan, unpublished).

On the basis that salicylate can effect cation transport in tissues diverse as red cells and mammary gland it is probable that the action of this drug may be widespread. In this connection it is interesting to note that salicylate can induce an increase in the unidirectional efflux of K^+ (Rb^+) from human placental tissue fragments (Shennan, unpublished). It is tempting to suggest that salicylate may be able to induce cation fluxes in some cell types at therapeutic (<2 mM) concentrations.

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