0014-4754/95/080804-05$1.50 + 0.20

Sr^{2+} can become incorporated into an agonist-sensitive, cytoplasmic Ca^{2+} store in a cell line derived from the equine sweat gland epithelium

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Received 8 November 1994; received after revision 26 January 1995; accepted 8 February 1995

Abstract. We have explored the properties of a Ca^{2+}-dependent cell-signalling pathway that becomes active when cultured equine sweat gland cells are stimulated with ATP. The ATP-regulated, Ca^{2+}-influx pathway allowed Sr^{2+} to enter the cytoplasm but permitted only a minimal influx of Ba^{2+}. Experiments in which cells were repeatedly stimulated with ATP suggested that Sr^{2+}, but not Ba^{2+}, could become incorporated into the agonist-sensitive, cytoplasmic Ca^{2+} store. Further evidence for this was provided by experiments using ionomycin, a Ca^{2+} ionophore which has no affinity for Sr^{2+}.

Key words. Ca^{2+} mobilisation; purinergic receptors; nucleotide receptors; sweat gland; epithelial cells; ionomycin; Fura-2; horse.

Purinergic receptors belonging to the P_{2U} subclass[1,2] allow ATP to increase intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in cultured equine sweat gland epithelial cells. This response is initiated by the release of Ca^{2+} from a cytoplasmic store and subsequently sustained by Ca^{2+} influx[3]. Such regulated changes in $[Ca^{2+}]_i$ constitute one of the central important mechanisms that allow hormones and neurotransmitters to exert regulatory control over cellular metabolism[4]. In order to investigate further the properties of the Ca^{2+}-dependent signal transduction pathway in this cell line, we have now monitored the changes in the cytoplasmic concentrations of Sr^{2+} and Ba^{2+} that occur when cells are stimulated with ATP in the presence of these cations; this approach has given insights into the Ca^{2+}-dependent cell signalling pathway in a number of other cell types[5–7]. Some of these data have been presented to the Physiological Society[8].

Materials and methods

Standard techniques were used to maintain a spontaneously-transformed epithelial cell line derived from the equine sweat gland[9]. Coverslips bearing growing cells were incubated (20–30 min, 37 °C) in medium containing the acetoxmethylester form of the Ca^{2+}-sensitive, fluorescent dye Fura-2 and mounted in a small chamber attached to the stage of an inverted microscope. Here the cells were superfused with physiological salt solution containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) and 10 mM D-glucose; the pH was adjusted to 7.4 with NaOH. Nominally Ca^{2+}-free solutions were prepared simply by omitting $CaCl_2$ from the standard saline whereas Sr^{2+} saline and Ba^{2+} saline were prepared by replacing $CaCl_2$ with $SrCl_2$ or $BaCl_2$ respectively. Fura-2 fluorescence ratios were obtained (0.9 Hz) from groups of 2–5 cells as detailed elsewhere[3,9]. Experimental results are presented as fluorescence ratios. All responses were quantified by measuring the fluorescence ratio at the peak of the response and subtracting from it the ratio measured immediately before the solution change. Data are presented as means ± SEM (n = number of experiments) and the significance of any difference between mean values was tested using Student's t-test.

Results

ATP evoked a transient increase in fluorescence ratio in cells that were superfused with the nominally Ca^{2+}-free saline (fig. 1). This response has been described previously and can be attributed to receptor-regulated release of Ca^{2+} from an internal store[3]. In the present experiments, cells were subsequently exposed to Sr^{2+} saline in the continued presence of ATP. This caused a second rise in fluorescence ratio which became apparent after a latency of ~30 seconds, and which was sustained until Sr^{2+} was withdrawn (fig. 1). A third increase in fluorescence ratio occurred when the cells were exposed to Ca^{2+}; this caused the Fura-2 signal to rise to a clearly defined peak. There was subsequently an initial, rapid decline to a level that was still elevated, and which was maintained until Ca^{2+} was removed (fig. 1). The first, transient part of this response to Ca^{2+} was not seen[3] in cells that had never been exposed to Sr^{2+}. Separate

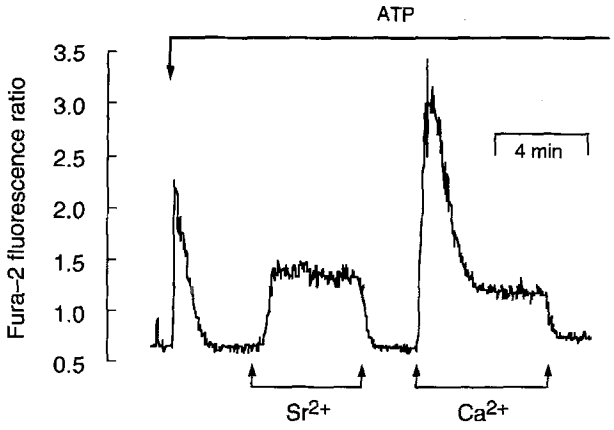

Figure 1. Cells were initially superfused with nominally Ca^{2+}-free solution to which 100 µM ATP was added as indicated. The cells were then exposed to Sr^{2+} saline and to the standard, Ca^{2+}-containing saline for each of the indicated periods. Essentially identical records were obtained in 5 experiments.

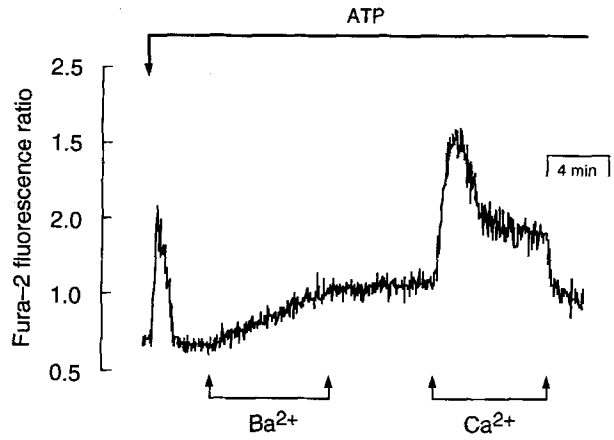

Figure 2. Cells were initially superfused with a nominally Ca^{2+}-free solution and exposed to 100 µM ATP as indicated. The cells were subsequently exposed to Ba^{2+} saline and then to the standard, Ca^{2+}-containing saline. Essentially identical data were obtained in a total of 5 experiments.

experiments showed that Sr^{2+} did not affect the Fura-2 signal recorded from cells that had not been exposed to ATP ($n = 3$). It thus appears that ATP can permit a large influx of Sr^{2+}. A directly analogous protocol was used to explore the effects of Ba^{2+}. This cation increased the Fura-2 fluorescence ratio recorded from ATP-stimulated cells but, in contrast to the response to Sr^{2+}, this increase in fluorescence ratio developed slowly (0.053 ± 0.006 unit per min, $n = 5$) and the fluorescence ratio did not return to control values once Ba^{2+} was removed (fig. 2). Subsequent application of Ca^{2+} evoked an essentially normal response (fig. 2). Further experiments showed that Ba^{2+} increased the Fura-2 ratio in cells which had not been exposed to ATP. The rate at which the fluorescence ratio rose under these conditions (0.036 ± 0.004 unit per minute, $n = 5$) was slower ($p < 0.05$) than the rate seen in the presence of ATP. ATP thus appears to cause only a small acceleration of the rate of Ba^{2+}-entry.

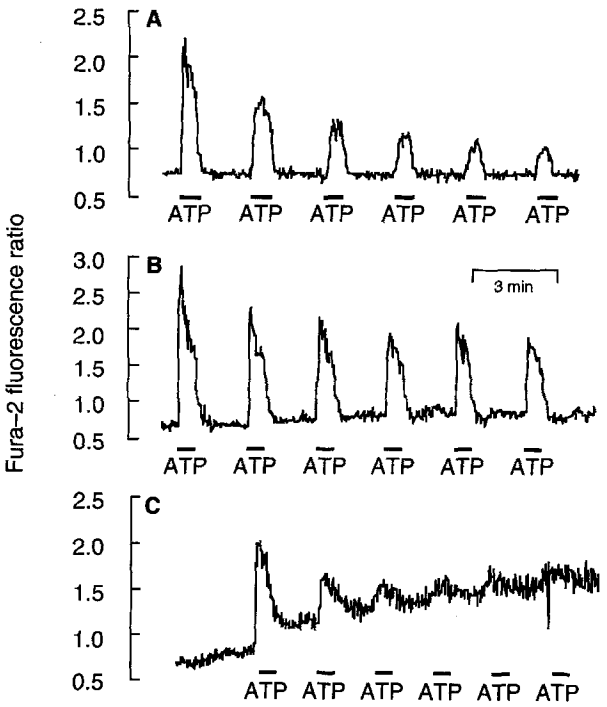

Figure 3. Cells were repeatedly stimulated with a series of 30-second pulses of 100 µM ATP that were delivered as indicated by the horizontal bars. *A)* shows a typical series of responses that were obtained during superfusion with nominally Ca^{2+}-free saline whereas *B)* and *C)* show results obtained in analogous experiments in which the cells were superfused with Sr^{2+} saline or Ba^{2+} saline respectively.

A series of brief pulses of ATP evoked a corresponding series of increases in the Fura-2 fluorescence ratio when

Table. Desensitization of the response to ATP.

Superfusing solution	Number	R_0 (ratio units)	Desensitization (%)
Ca^{2+}-free	6	0.91 ± 0.17	70 ± 6
Sr^{2+}-saline	5	1.15 ± 0.23	36 ± 8*
Ba^{2+}-saline	6	0.92 ± 0.09	78 ± 4

Cells superfused with the listed solutions were repeatedly stimulated with a series of 30-second pulses of 100 µM ATP that were delivered at two minute intervals. The extent to which sensitivity to ATP was lost during these experiments was quantified using the equation: % Desensitisation $= (1 - R_{3,4,5}/R_0) \times 100$, where R_0 is the increase in Fura-2 fluorescence ratio evoked by the initial pulse of ATP and $R_{3,4,5}$ is the mean response evoked by the final three pulses. The estimates of values of R_0 and of the desensitization that developed under the different experimental conditions are tabulated.

* $p < 0.05$ with respect to the data obtained during superfusion with the nominally Ca^{2+}-free solution.

cells were superfused with the nominally Ca^{2+}-free saline solution, although sensitivity to ATP was rapidly lost during these experiments (fig. 3A, table). Cells also responded to ATP during superfusion with Sr^{2+} saline. Under these conditions the response to the initial pulse of ATP was normal but the loss of sensitivity was not as large as that seen under Ca^{2+}-free conditions (fig. 3B, table). A steady increase in the fluorescence ratio developed when cells were superfused with Ba^{2+}-saline, but responses to ATP could be clearly discerned in the experimental results (fig. 3C). Analysis of these data showed that both the magnitude of the initial response and the loss of sensitivity to ATP were normal (table). It thus appears that Sr^{2+}, but not Ba^{2+}, is able to maintain sensitivity to ATP during repeated stimulation, and further experiments were undertaken to explore the physiological basis of this effect. Cells were exposed to ATP under nominally Ca^{2+}-free conditions in order to release Ca^{2+} from the agonist-sensitive internal store. Stimulation was continued until the Fura-2 fluorescence ratio had returned to basal levels which was assumed to indicate that this store had become completely depleted. Subsequent application of ionomycin, a Ca^{2+} ionophore, evoked a second, transient increase in fluorescence ratio. The peak of this response was $43 \pm 6\%$ ($n = 10$) of the corresponding response to ATP (fig. 4A) but it had a slower onset and decline; this second response was attributed to the ionophore-evoked release of Ca^{2+} from stores within the cytoplasm which had not become empty during stimulation with ATP. A further application of ATP elicited no response, confirming that Ca^{2+} cannot be retained by the agonist-sensitive store under these conditions (fig. 4A). In further experiments this store was again emptied by prolonged stimulation with ATP. These cells were exposed to Sr^{2+}-saline once ATP had been withdrawn (fig. 4B). This had no discernible effect upon the fluorescent ratio and subsequent application of ionomycin evoked an essentially normal response. The Sr^{2+}-treated cells, however, responded to the second application of ATP with a clear increase in fluorescence ratio (fig. 4B) that was not seen in control cells (fig. 4A). Ionomycin prevents the sequestration of Ca^{2+} but does not transport Sr^{2+} across cell membranes[10]. The response to the second application of ATP must therefore, reflect the agonist-evoked mobilisation of Sr^{2+} that had become sequestered within the cytoplasm.

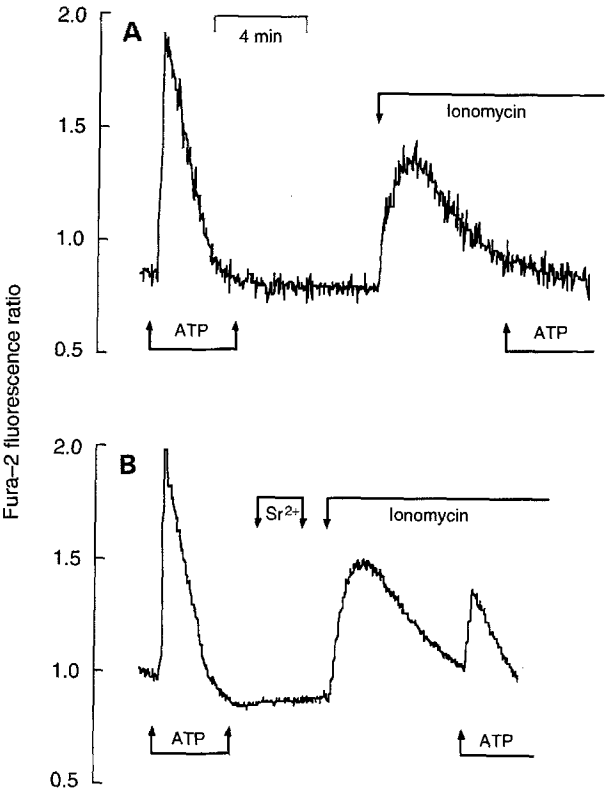

Figure 4. *A)* Cells were superfused with the Ca^{2+}-free solution and exposed to 100 μM ATP as indicated. Once $[Ca^{2+}]_i$ returned to basal levels ATP was withdrawn and the cells were subsequently exposed to 0.1 μM ionomycin and then again exposed to 100 μM ATP in the continued presence of this ionophore. *B)* Data from a directly analogous experiment in which the cells were also exposed to Sr^{2+} saline for the period indicated.

Discussion

Fura-2 chelates certain metal ions and this binding reaction causes a characteristic shift in the emission spectrum of the dye. This property is often exploited to allow changes in $[Ca^{2+}]_i$ to be monitored[11] but certain other metals, including Sr^{2+} and Ba^{2+}, also cause such a shift in emission spectrum. Other workers have demonstrated that Fura-2 can also be used to detect changes in the cytoplasmic concentrations of these ions[5–7].

The present experiments thus show that the P_{2U}-receptor[1,2] regulated, Ca^{2+}-influx pathway in cultured equine sweat gland epithelial cells[3] can allow a rapid influx of Sr^{2+}, but suggest strongly that this pathway has only a low permeability to Ba^{2+}. Studies of bovine endothelial cells[7] and rodent lacrimal acinar cells[5], in contrast, showed that Ca^{2+}-mobilising agonists allow a large influx of either of these exogenous cations. This discrepancy with earlier work suggests that Ca^{2+}-mobilising agonists do not activate a common, Ca^{2+}-influx pathway in all cell types. Comparison of the Ca^{2+}-signalling mechanisms in human platelets and in rodent parotid acini provided direct evidence of this; the Ca^{2+}-influx pathway in platelets appears to allow Mn^{2+} to enter the cytoplasm whereas the equivalent pathway in acinar cells does not[12]. Increases in the cytoplasmic concentration of Sr^{2+} reversed once this ion was withdrawn from the external solution whereas, over the time scale of the present experiments, increases in the concentration of Ba^{2+} were irreversible. A slow accumulation of Ba^{2+} occurred whenever cells were exposed to this cation and this has been observed in other cell

types[5,7,13]. It thus appears that the mechanism that normally maintains low $[Ca^{2+}]_i$ can remove Sr^{2+}, but not Ba^{2+}, from the cytoplasm. This is in good accord with data from biochemical studies of the Ca^{2+}-dependent ATPases in the plasma and microsomal membrane fractions[14,15]. These enzymes, which make an important contribution to the maintenance of low $[Ca^{2+}]_i$ by actively extruding Ca^{2+} across the plasma membrane and by sequestering it into intracellular organelles, have a much higher affinity for Sr^{2+} than for Ba^{2+}.

Earlier experiments showed that repeated applications of ATP could evoke a series of increases in the Fura-2 fluorescence ratio and that ~12% of the original sensitivity to ATP is normally lost during these experiments; this desensitization was attributable[3] to receptor-mediated activation of protein kinase C. The present data show that a more extensive (~70%) loss of sensitivity developed in cells that were repeatedly stimulated in the absence of external Ca^{2+}. The responses seen under these conditions can be attributed, almost exclusively, to the mobilisation of Ca^{2+} from an internal store[3]. Ca^{2+} ions may be re-accumulated by this store once ATP is withdrawn, but there would also be a continual extrusion of Ca^{2+} across the plasma membrane. We therefore attributed the additional loss of sensitivity seen in these experiments to the progressive depletion of the agonist-sensitive, internal store.

This loss of sensitivity to ATP proceded normally when the extracellular solution contained Ba^{2+}, demonstrating that this cation cannot substitute for Ca^{2+} in the agonist-sensitive store. Sensitivity to ATP was, however, maintained by cells that were superfused with Sr^{2+}-saline. There is evidence for the sequestration of Sr^{2+} by intracellular organelles in many cell types[5,6,16] and the Ca^{2+}-pumping systems of the microsomal membrane fraction can transport this cation[15]. Incorporation of Sr^{2+} into the agonist-sensitive store could, therefore, account for the present data. The responses obtained in the presence of Sr^{2+} would, however, reflect Sr^{2+} influx from the external fluid and Ca^{2+}-mobilisation from the cytoplasmic store and the maintenance of sensitivity to ATP may, therefore, be explained simply by Sr^{2+} influx.

We tested the possibility that Sr^{2+} could become incorporated into the agonist-sensitive pool using a more direct experimental protocol[17] that relies upon the observation[10] that ionomycin cannot prevent the cytoplasmic sequestration of Sr^{2+}. These experiments showed that ATP could increase the Fura-2 signal recorded from ionomycin-treated cells provided that they had previously been exposed to Sr^{2+}. The presence of ionomycin precluded the possibility of Ca^{2+} sequestration[10] and so the second response to ATP seen in these experiments must reflect the mobilisation of Sr^{2+} that had become incorporated into the agonist-sensitive store. This result is in excellent accord with data from a recent study of bovine endothelial cells[17]. Sr^{2+} thus appears able to traverse the receptor-regulated Ca^{2+}-influx pathway and become incorporated into the agonist-sensitive cytoplasmic Ca^{2+} depot. Furthermore, Sr^{2+} appears able to enter the agonist sensitive pool without evoking a discernible rise in the Fura-2 signal (fig. 4B). This accords well with the results of a study of secretory cells acutely dissociated from avian salt glands[18] where the agonist-sensitive Ca^{2+} store can be replenished, in the absence of agonist, without a large increase in $[Ca^{2+}]_i$. It thus appears that a receptor-regulated pathway allows Ca^{2+} to enter the cytoplasm but that a second pathway communicates more directly with the agonist-sensitive store, allowing it to be refilled in the absence of agonist[18]. The equine cells used in the present experiments thus appear to express both pathways. In the murine epididymal epithelium, however, ATP mobilises internal Ca^{2+} without evoking any obvious Ca^{2+} influx. A Sr^{2+}-permeable pathway does, however, allow this cation to enter the agonist-sensitive store in these cells and so they may be an interesting example of a tissue that expresses only the pathway communicating directly with the internal store[6].

Fluid secretion from most mammalian exocrine cells is regulated primarily[21] via changes in $[Ca^{2+}]_i$. It has been proposed that the secretory epithelium of the equine sweat gland is an unusual example of an exocrine tissue where secretory activity is regulated, almost exclusively, via cyclic AMP-dependent mechanisms[19,20]. However, the present experiments, together with our earlier work[3,21], show that cell lines derived from the equine sweat gland express a $[Ca^{2+}]_i$-dependent signal transduction pathway which is very similar to the pathway found in essentially all other mammalian exocrine cells[5,22–25] and which allows external ATP, and certain related compounds, to regulate membrane permeability[3,21]. These data thus raise the possibility that ATP released from periglandular nerve terminals[2] may contribute to the neural control of sweating in the Equidae[3]. It is now important to determine to what extent membrane permeability can be regulated via changes in $[Ca^{2+}]_i$ in isolated, equine sweat glands and in secretory cells acutely dissociated from these organs.

Acknowledgements. This work was supported by a grant from the Royal Hong Kong Jockey Club that was awarded to S. M. Wilson in conjunction with Hugh Elder and David McEwan Jenkinson, whose help and encouragement are gratefully acknowledged. We also thank Jane Grant for preparing the figures, and Godfrey Smith for his help with some of the experiments and his many helpful discussions and comments.

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