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Characteristics of the signal transduction system activated by ATP receptors in the hepatoma cell line N1S1-67

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Abstract

The transmembrane transduction mechanism coupled to purinergic receptors has been studied in a rat hepatoma cell line (N1S1) at the single cell level by a combination of microfluorimetric and electrophysiological techniques. ATP in the micromolar range causes release of Ca^{2+} from internal stores and consequent opening of Ca^{2+}-activated K^+ channels, leading to membrane hyperpolarization. The order of potency of the various nucleotides tested is UTP = ATP = ADP $\gg$ AMP, and ATP > β,γ-CH_2 ATP, indicating that these receptors belong to the P_{2U} subtype. The Ca^{2+} rise induced by various amounts of ATP exhibits an all-or-none behaviour already observable at 10 μM ATP. Intracellular injection of (10–20 μM) $InsP_3$ or of its non-metabolizable analogue 3-F-$InsP_3$ through the patch pipette, does not always result in a Ca^{2+} rise. These results may be interpreted assuming that the $InsP_3$ receptors-Ca^{2+} release channels involved in the purinergic/pyrimidinergic stimulation are located in a subcellular compartment not easily accessible from the bulk cytosol and that a positive feedback loop occurs in this restricted space.

Keywords: ATP receptor; Potassium ion channel, Ca^{2+}-activated ; Inositol 1,4,5-trisphosphate; Calcium ion release

1. Introduction

The transmembrane transduction mechanisms leading to cytosolic Ca^{2+} elevations are ubiquitously distributed in a variety of tissues and are involved in a large number of physiological processes, ranging from cell proliferation to secretion or to the regulation of catalytic activities. It is now well established that the turnover of the phosphoinositides plays a central role in this process [1], linking the membrane events to the release of Ca^{2+} from internal stores. However, several aspects of the Ca^{2+} signalling systems deserve further investigation: for example often the Ca^{2+} changes occur in an oscillatory or 'spiking' form [2,3], and these transient elevations can also travel as waves across the cell [4–6]. These behaviours generally require the presence of a positive feedback loop at some stage [7]. Also the role of receptor-operated or second messenger-operated channels is not always clear, the activation and regulation of this kind of channels being particularly difficult to detect with direct methods [8–11]. Furthermore, changes in membrane potential may occur, and their role has not been fully investigated.

We have decided to approach this series of problems by selecting a model system that could allow the coupling of fluorimetric Ca^{2+} measurements in single cells with electrophysiological methods (whole-cell patch-clamp). In this way Ca^{2+} changes and membrane currents may be monitored directly under controlled voltage conditions and, in addition, it is possible to introduce into the cytosol through the whole-cell pipette substances that could affect the process under study.

We have chosen, as biological model, the rat hepatoma line N1S1 (Novikoff hepatoma). The origin of the N1S1 line is unclear, as they are not actually believed to be hepatocytes, but an origin from Kupffer or endothelial cells has been proposed [12]. The reason of this choice is based on the fact that, because of their almost spherical shape, these cells are ideal for voltage-clamping and for cytosolic diffusion of molecules.

As will be shown in detail in the Results, they possess receptor for ATP linked to Ca^{2+} signalling.

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2. Materials and methods

2.1. Cells culture

N1S1 cells (Novikoff hepatoma) were cultured in S210 medium (Gibco) supplemented with F68 Pluronic (Sigma) 2.12 g/l, $NaHCO_3$ 2.2 g/l, Hepes 2.14 g/l and new-born bovine serum 10% (NBS, Irvine Scientific), and grown at 37°C in a sealed flask. These cells grow mainly in suspension forming aggregates of 100–200 cells; they need an everyday division and changing of the medium.

2.2. Electrophysiology

Classical patch-clamp methodology in whole-cell configuration was used. Currents were measured with pipettes having 2–4 MΩ resistance filled with (in mM): 143 KCl, 2 $MgCl_2$, 0.1 EGTA, 10 Hepes-KOH; the pH was set at 7.1. When simultaneously performing microfluorimetric measurements, EGTA was omitted and replaced by the same concentration of Fura-2. The extracellular control solution contained (in mM): 125 NaCl, 5 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 6 glucose, 25 Hepes-NaOH at pH 7.3; in the 'high K^+' external solution, K^+ replaced Na^+ equimolarly; in the 'Ca^{2+}-free' external solution $CaCl_2$ was absent and EGTA 1 mM was added.

2.3. Ca^{2+} measurement

Cells were loaded with Fura-2 penta-K^+ (100 μM) by diffusion through a patch pipette when in whole-cell condition. A special spectrophotometer (Cairn Research) for double excitation and decoding of the emitted light was used as explained in detail previously [13]. Ca^{2+} concentrations are expressed as values of the emission ratio R [14].

Agonists were applied through a small tip placed close to the cell under examination using the system developed by Murase et al. [15]; with this method agonists, and also the solution in which they were dissolved, reached the cell in 200 to 400 ms.

The experiments were performed at room temperature (22–25°C).

3. Results

3.1. ATP induces the opening of Ca^{2+}-activated K^+ channels in the membrane of N1S1 cells

The application of ATP at 10 to 100 μM concentration to a Novikoff hepatoma cell kept in current clamp conditions causes a sudden and transient hyperpolarization of the membrane potential from its rather low resting level (average values were: $V_{rest} = -26.3 \pm 3.9$ mV and $V_{hyper} = -69.9 \pm 3.9$ mV; S.E., $n = 15$; Fig. 1).

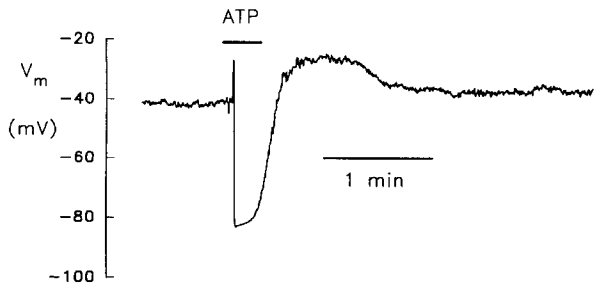

Fig. 1. Hyperpolarization induced by application of 10 μM ATP to a cell kept in current-clamp condition. The subsequent depolarizing phase was not always seen.

Since it is known that purinergic agonists induce Ca^{2+} transients in hepatocytes [16,17], it is likely that the hyperpolarization induced by ATP could be due to opening of Ca^{2+}-activated K^+ channels. To check the validity of this idea we performed some experiments coupling the measurement of membrane current in whole-cell condition with the measurement of the cytosolic Ca^{2+} concentration using Fura-2 microfluorimetry. Fig. 2A shows the result obtained from a cell in which Fura-2 was loaded through the whole-cell pipette.

The upper trace shows the changes in Ca^{2+} concentration while the lower trace represents the whole-cell current at a V_h of -20 mV. It can be seen that after a short lag from the beginning of ATP application (dissolved in the high external K^+ solution) the Ca^{2+} level begins to rise followed almost immediately by the development of an inward current. When the stimulation was repeated in

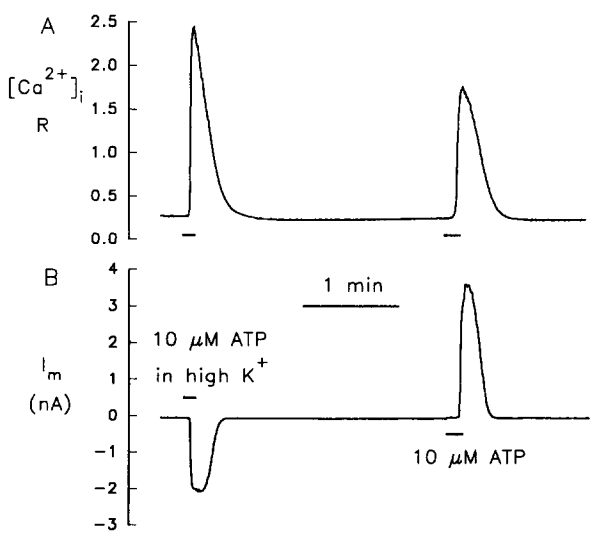

Fig. 2. Simultaneous recordings of cytosolic Ca^{2+} (A, expressed in term of fluorescence ratio, R) and (B) of membrane current in a cell kept in voltage-clamp at $V_h = -20$ mV; ATP 10 μM was applied twice for the periods indicated by the bars: the first time in a solution containing 140 mM K^+, the second time in a solution containing 5 mM K^+ (control solution). The two Ca^{2+} elevations are accompanied by currents in opposite directions.

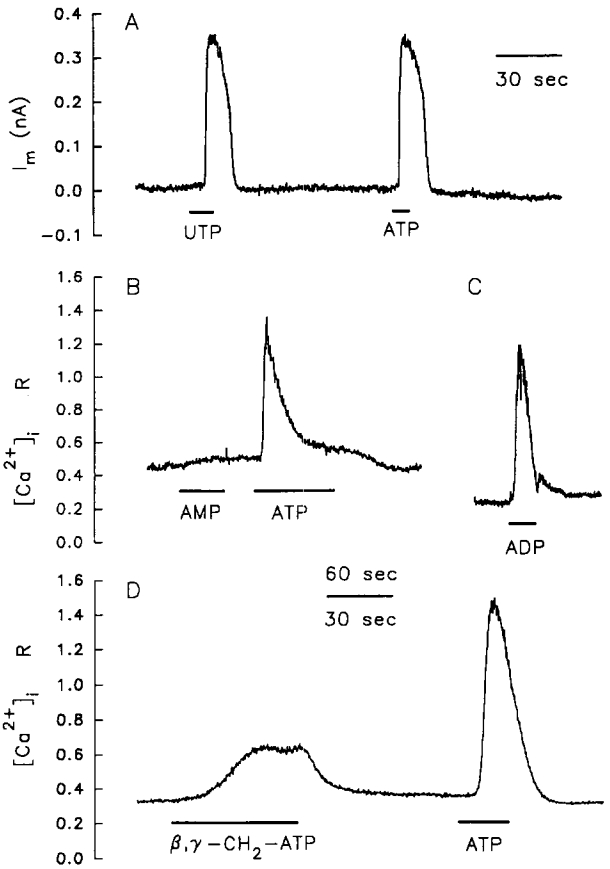

Fig. 3. Pharmacology of the receptor. (A) Ca^{2+}-activated K^+ current in response to 10 μM UTP and to 10 μM ATP in the same cell. (B) Stimulation with 100 μM AMP was almost completely ineffective in a cell that responded normally to a subsequent stimulation with 100 μM ATP. (C) In another cell ADP 100 μM was able to elicit a normal Ca^{2+} response. (D) A fourth cell was challenged first with 100 μM β,γ-CH_2-ATP and subsequently with 10 μM ATP.

normal external K^+, the current corresponding to the Ca^{2+} increase became outward.

This result indicates that a Ca^{2+} rise is triggered by a transmembrane transduction mechanism activated by ATP receptors and as a consequence Ca^{2+}-activated K^+ channels are opened. Indeed the K^+ channel may be used to monitor the Ca^{2+} changes using only electrophysiological measurements.

We have measured the reversal potential (E_{rev}) of the Ca^{2+}-activated current by applying voltage ramps from -100 to $+80$ mV and lasting 250 ms to cells bathed in normal or high external K^+. The average values were -73.2 ± 2.7 mV (S.E., $n = 10$) in normal K^+ and -6.0 ± 1.8 mV (S.E., $n = 5$) in high K^+. Therefore, both the membrane potential value reached during the hyperpolarization and the value of E_{rev} are close to the calculated K^+ equilibrium potential (E_K) and indicate good selectivity for K^+. In conclusion, this series of experiments shows that in N1S1 cells ATP stimulation causes the opening of Ca^{2+}-activated K^+ channels.

3.2. Classification of the receptor

The various subtypes of P_2 purinergic receptors activate different transduction mechanisms [18], leading to Ca^{2+} elevations both from influx and from internal release. To identify the class of ATP receptors present in N1S1 cells we have performed a series of experiments comparing the efficacy of various nucleotide agonists and also testing whether the source of the Ca^{2+} rise was intra- or extracellular.

The upper part of Fig. 3 shows that UTP is equipotent to ATP in eliciting a Ca^{2+}-activated K^+ current. In the

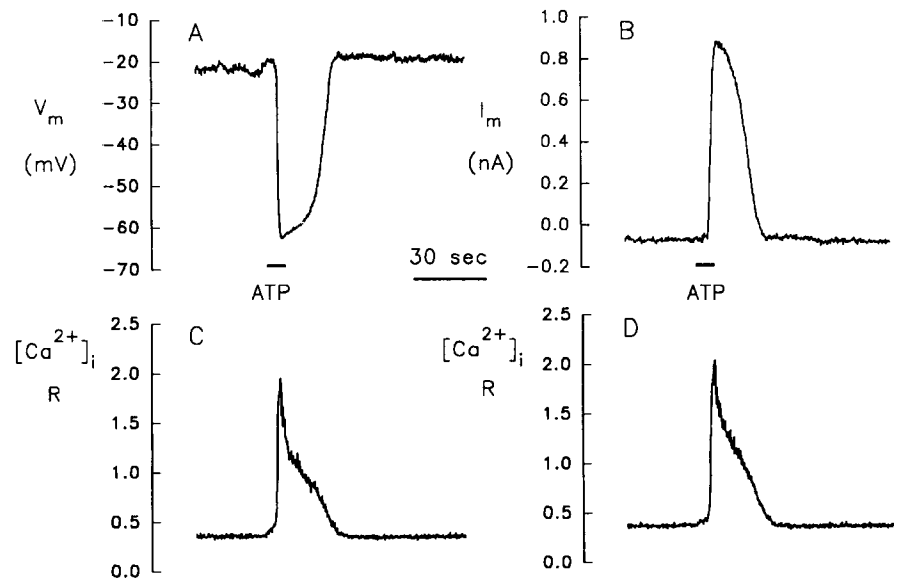

Fig. 4. Lack of effect of membrane potential on the ATP-induced Ca^{2+} transient. The same cell was stimulated with 10 μM ATP first in current-clamp conditions (A, C) and later in voltage-clamp conditions at $V_h = -20$ mV (B, D). In spite of the large voltage swing visible in A, the shape of the Ca^{2+} change was not significantly different (compare C and D).

central row, AMP 100 μM was unable to induce a Ca^{2+} elevation, while ADP 100 μM appears to be as potent as ATP. In the lower part of Fig. 3 a cell was tested with the derivative β,γ-CH_2 ATP which appears to be much less potent than ATP. The order of potency of the nucleotides is therefore ATP = UTP = ADP $\gg$ AMP, and ATP > β,γ-CH_2 ATP. Consequently the receptors present in the hepatoma line N1S1 can be classified as P_{2U} [18,19].

In order to investigate a possible role of Ca^{2+} influx, we have also performed some experiments in the absence of external Ca^{2+}. ATP stimulation is able to induce the Ca^{2+}-induced K^+ current even in these conditions (not shown), indicating an intracellular source for the Ca^{2+} changes.

Another indication that the rise in Ca^{2+} concentration is essentially an intracellular phenomenon and that it is not strongly affected by membrane fluxes nor membrane potential comes from Fig. 4. In this experiment the same cell was stimulated twice with ATP, first under current-clamp conditions, leaving therefore the membrane potential free to hyperpolarize, and after recovery, under voltage-clamp at $V_h = -20$ mV.

Although the calcium transients recorded in the two conditions are not strictly identical, no significant differences can be observed in peak value, duration and general time course between them.

3.3. All-or-none behaviour of the Ca^{2+} transient

The Ca^{2+} transients induced by ATP stimulation occur normally with a fast rise followed by a slower decline. It is possible to distinguish a delay between agonist application and onset of the Ca^{2+} increase. Control experiments show that in our system the agonist-containing solution reaches the cell in 200–400 ms. It is interesting to compare the rate of rise of cytosolic Ca^{2+} caused by application of 10 μM ATP to that induced by 2 μM of the Ca^{2+} ionophore Ionomycin. Two such traces obtained in the same cell are plotted in Fig. 5A where they have been aligned in order to compare the kinetics.

Clearly, Ca^{2+} begins to increase earlier following ionomycin stimulation, and its rise proceeds in a slow, quasi-exponential way, while the ATP-induced transient starts with a delay of few seconds, but then it is much faster, suggesting an autocatalytic process. Fig. 5B shows, as control, the Ca^{2+} rise induced by 2 μM ionomycin in another cell not previously exposed to ATP. We have calculated the maximum rate of rise of the Ca^{2+} concentration (in terms of R per second) for the various conditions, obtaining average values of 0.69 ± 0.12 R/s (S.E., $n = 8$) for the cells stimulated with ATP 10 μM, of 0.17 ± 0.03 R/s (S.E., $n = 6$) for the cells exposed to 2 μM ionomycin as first agonist and of 0.13 ± 0.03 R/s (S.E., $n = 4$) for the cells exposed to 2 μM ionomycin after a first exposure to ATP.

The existence of an autocatalytic process is confirmed

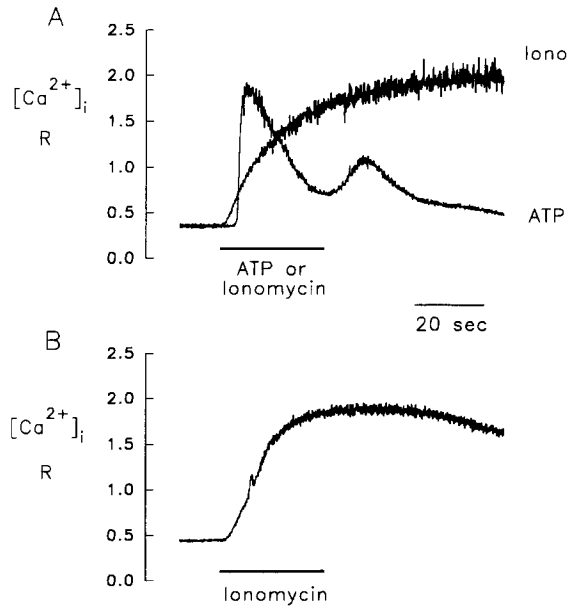

Fig. 5. (A) The two superimposed traces show the kinetics of the Ca^{2+} rise in the same cell upon stimulation with ATP 10 μM and, subsequently, with ionomycin 2 μM. The second hump visible in the ATP trace was occasionally observed in other cells, although N1S1-67 do not generally show oscillations of Ca^{2+} concentrations after ATP stimulation. (B) Ca^{2+} changes in another cell exposed to ionomycin 2 μM as first agonist.

by the following experiments, in which application of various amounts of ATP to a single N1S1 cell produced elevations of cytosolic Ca^{2+} which exhibited an all-or-none behaviour. This is exemplified in Fig. 6 where the responses to three successive stimulations with ATP 1, 10 and 100 μM to the same cell are shown. The lower ATP concentration caused only a very small increase that never gave rise to a Ca^{2+} spike; on the contrary the Ca^{2+} changes in response to 10 and 100 μM ATP show a lag phase of slow Ca^{2+} rise which gives rise to a fast up-

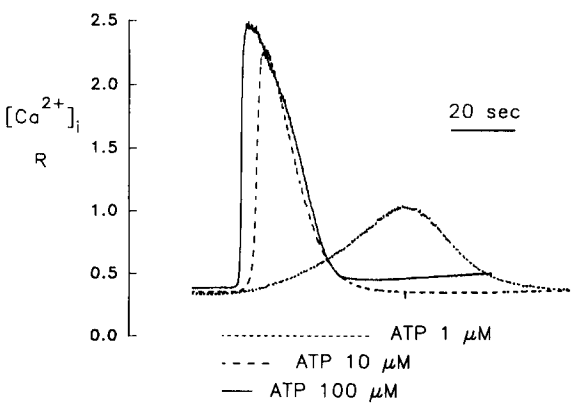

Fig. 6. All-or-none behaviour of the Ca^{2+} response to ATP. The same cell was subsequently stimulated with three increasing doses of ATP as indicated by the bars. The lowest dose (1 μM) caused a continuous and slow increase in Ca^{2+}. Both the 10 and 100 μM doses triggered a Ca^{2+} spike with similar peak amplitude and shape but with shorter latency for the stronger stimulation.

stroke, occurring earlier at the higher concentration. Furthermore, the peak Ca^{2+} levels reached after the two stronger stimulations were not very much different.

The threshold behaviour is suggestive of the presence of a positive feedback loop at some stages of the transmembrane transduction system and several possibilities have been proposed in models in which $InsP_3$ production occupies a central role [7].

3.4. Intracellular $InsP_3$ injections

We have directly investigated the role of $InsP_3$ in the Ca^{2+} response to ATP by introducing 10 μM $InsP_3$ or 10 μM of its non metabolizable analogue D-3-deoxy-3-fluoro-myoinositol 1,4,5-trisphosphate (3-F-$InsP_3$) inside the cells through the whole-cell pipettes. This compound has been shown to be equipotent to 1,4,5 $InsP_3$ to mobilise intracellular Ca^{2+}, but it cannot be phosphorylated by the 3-kinase to give the 1,3,4,5 inositol tetrakisphosphate [20].

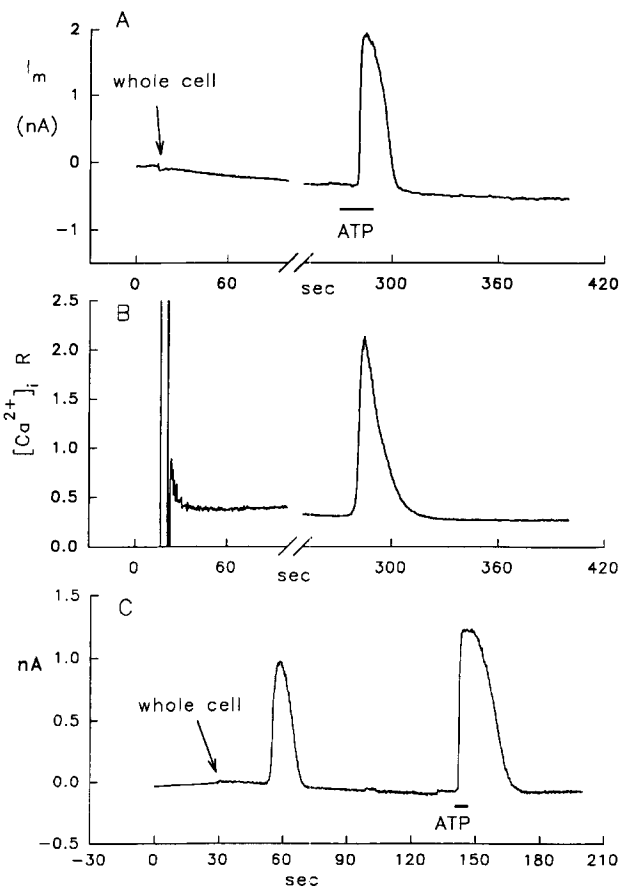

Fig. 7. Variable effects of 3-F-$InsP_3$. (A, B) Following patch breaking, as indicated in the upper (current) trace, 10 μM 3-F-$InsP_3$ began diffusing inside the cell from the pipette. The Ca^{2+} level (lower trace) becomes measurable some seconds later as Fura-2 diffusing from the pipette reaches a sufficient concentration to give a useful signal. No signs of a Ca^{2+} transient are visible for more than 4 min. The cell was anyway able to generate a normal Ca^{2+} and current transient when stimulated with 10 μM external ATP. (C) In another cell a Ca^{2+}- induced outward current developed about 25 s after patch breaking; the cell was subsequently stimulated extracellularly with 100 μM ATP.

The presence of Ca^{2+}-activated K^+ channels in N1S1 cells is particularly useful because the channels act as an endogenous Ca^{2+} indicator which can signal Ca^{2+} increases close to the membrane immediately after the passage from the cell-attached to the whole-cell condition. This is important because the exogenous indicator (Fura-2) becomes useful to detect Ca^{2+} changes with some delay needed to reach a sufficient intracellular concentration.

This kind of experiments gave variable results: over 13 cells tested, six gave rise to a Ca^{2+} transient smaller but comparable to that induced by ATP, two exhibited a weak Ca^{2+} increase and five did not show any response. This variable behaviour is shown in Fig. 7. In the upper and middle traces it can be seen that neither the membrane current nor the Ca^{2+} signal (from the moment when it became detectable) did change for several minutes after patch-breaking, although 10 μM 3-F-$InsP_3$ was certainly diffusing together with Fura-2 into the cell.

The capacity of this cell to release Ca^{2+} after appropriate stimulation was checked by applying 10 μM ATP after several minutes of internal perfusion with 3-F-$InsP_3$: as can be seen, the cell generated a Ca^{2+} spike and a consequent outward current whose amplitudes and time courses were not affected at all by the presence of 3-F-$InsP_3$.

The lower trace in Fig. 7 shows another cell in which a response to 3-F-$InsP_3$ was detectable. It can be seen that 20–30 s after rupturing the patch, a transient of Ca^{2+}-induced outward current occurred. It is worth noting that even in this case the response induced by the 3-F-$InsP_3$ diffusing from the pipette was smaller and slower than the subsequent transient caused by external application of ATP. The same kind of result has been obtained in cells internally perfused with 10 μM $InsP_3$.

4. Discussion

The results of our fluorimetric and electrophysiological experiments indicate that N1S1 cells express ATP receptors and Ca^{2+}-activated K^+ channels in the plasma membrane. In hepatocytes external ATP is able to influence glycogen metabolism through a cAMP-independent, Ca^{2+}-mediated, activation of glycogen phosphorylase [17,21]. However, the uncertain origin of the Novikoff hepatoma cell line [12] does not allow to make inferences on the physiological role of these receptors. Therefore we will discuss here only the relevance of our results on the Ca^{2+} signalling system.

4.1. Functional $InsP_3$ receptors are probably localised in restricted spaces near the plasma membrane

The most surprising results from our experiments were those involving intracellular perfusion of agonists of the $InsP_3/Ca^{2+}$ release system. As reported, not in all cells

internally perfused with $InsP_3$ or with 3-F-$InsP_3$ could a Ca^{2+} response be seen; furthermore these cells, although filled with a high concentration of the active and non-metabolizable 3-F-$InsP_3$, were still able to respond to a subsequent external ATP application, with no noticeable impairment of the Ca^{2+} transient.

We have checked very carefully that these results were not artefactual. $InsP_3$ and 3-F-$InsP_3$ from two sources (Sigma and Calbiochem) were used, with analogous results. The access resistance from the pipette to the cytosol and the time constant of diffusion of $InsP_3$ were checked according to Pusch and Neher [22], in order to be sure that the desired amount of agonist could be present in the cytosol. This was also confirmed directly in the cells in which Fura-2 was introduced from the pipette together with 3-F-$InsP_3$ (see Fig. 7B). All cells considered were in good conditions as demonstrated by the fact that they responded normally to extracellular ATP irrespective of a previous response to injected $InsP_3$.

We are then left with the difficult task of finding an explanation for the individual variability of these results. A possibility might be that these ATP receptors cause internal release of calcium without the involvement of inositol phosphates; however this possibility is in our opinion unlikely because the pharmacological experiments show that the receptors involved belong to the P_{2U} subtype, which are known to act through the breakdown of phosphoinositides [18,19]. Furthermore, our data also show that several cells indeed possess functional $InsP_3$ receptors.

We favour instead another possible interpretation of our experimental observations which is based on the idea that the $InsP_3$ receptors/Ca^{2+} release channels may not be readily accessible from the bulk cytosol, but may expose their $InsP_3$ binding sites in a secluded space in close apposition to the plasma membrane. This kind of situation, together with the threshold behaviour discussed in the next paragraph, might explain why some cells failed to generate a Ca^{2+} transient upon introduction of $InsP_3$ in the bulk cytosol.

4.2. Threshold behaviour

Another interesting result is the threshold behaviour of the Ca^{2+} rise in response to different amounts of ATP. This observation is indicative of the presence of a positive feedback loop which triggers a kind of all-or-none transient.

We had observed previously a similar behaviour of the Ca^{2+} transient in response to increasing amounts of internally applied $InsP_3$ in mouse oocytes [23,24]; in that case the experiments suggested the involvement of a Ca^{2+}-induced-Ca^{2+} release (CICR) process as responsible of the autocatalytic loop. Whether this mechanism is involved in N1S1-67 cells is doubtful because Ca^{2+} can be made to reach rather high levels, by ionomycin stimulation (see Fig. 5), without triggering a rapid transient. However, the ionophore stimulation may differ from receptor activation in generating a diffuse Ca^{2+} increase, in contrast to more localised events in a restricted compartment close to the surface receptors which may be essential in generating a positive feedback loop.

4.3. Role of membrane potential

The finding that the Ca^{2+} elevation activates K^+ selective channels in the plasma membrane may suggest that the ensuing strong hyperpolarization could play the role of increasing Ca^{2+} influx from the outside by increasing the electrochemical gradient. However, we have not found evidence supporting this idea: the traces in Fig. 4 actually show a slightly smaller increase in Ca^{2+} when the hyperpolarization was permitted, compared to that under voltage-clamp; such changes must be considered non-significant, as successive Ca^{2+} transient elicited in the same cell and in the same conditions were never exactly identical. Therefore the role played by the Ca^{2+}-activated K^+ channels remains elusive; this is an interesting point to investigate in future work, as Ca^{2+}-activated K^+ channels are quite often present together with ATP receptors of the P_{2Y} (or P_{2U}) subtype [25].

Thus far, we have not attempted to classify the Ca^{2+}-activated K^+ channels, but the good linearity of the I–V relationship may suggest that they belong to the SK channel class [26].

4.4. Receptor characterisation

All the five (or more) subtypes of the P_2-purinergic class lead to elevation of cytosolic Ca^{2+}, either by influx from the outside or by intracellular mobilization [18]. At least two subtypes, namely P_{2Y} and P_{2U}, appear to be coupled to the phosphoinositide pathway and recently the cDNAs encoding for two G-protein coupled proteins, resembling these two subtypes of purinergic/pyrimidinergic receptors, have been cloned [27,28]. Our results indicate that the receptors involved in this response are of the P_{2U} subtype.

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