THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 33, Issue of November 25, pp. 20351-20355, 1990
Printed in U.S.A.
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Receptor-mediated Calcium Influx in PC12 Cells

ATP AND BRADYKININ ACTIVATE TWO INDEPENDENT PATHWAYS*

(Received for publication, March 14, 1990)

Cristina Fasolato‡§, Paola Pizzo‡, and Tullio Pozzan¶

From the ‡Institute of General Pathology, Consiglio Nazionale delle Ricerche Center of Biomembranes, University of Padova, Padova, 35131 Italy, and the ¶Institute of General Pathology, University of Ferrara, Ferrara, Italy

In the neurosecretory cell line PC12 the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, and membrane potential were affected by both external ATP and the nonapeptide bradykinin, BK. The latter caused a rapid and large release of Ca^{2+} from intracellular stores (Ca^{2+} redistribution) and, in the presence of external Ca^{2+}, a long lasting, but moderate Ca^{2+} influx, which was insensitive to dihydropyridine blockers. On the contrary, ATP evoked a $[Ca^{2+}]_i$ rise which rapidly inactivated. At least three different mechanisms accounted for the ATP-induced increase in $[Ca^{2+}]_i$: less than 20% of the total response was due to intracellular Ca^{2+} redistribution, consistent with a small increase in inositol 1,4,5-trisphosphate level; the rest (over 80%) was equally accounted for by ATP-activated cation channels and voltage-gated Ca^{2+} channels. ATP and BK (the latter after K^+ channel blockade) caused plasma membrane depolarization. With both agonists the inward current was carried by both Na^+ and Ca^{2+}, although the BK-activated current appeared to be more selective for Ca^{2+}. Channels triggered by ATP and BK differed not only in their cation selectivity, but also in modulation by both $[Ca^{2+}]_i$ and drugs such as the phorbol ester phorbol 12-myristate 13-acetate and the new antagonist of ligand-activated Ca^{2+} influx, SK&F 96365.

In eukaryotic cells, the best characterized pathway of Ca^{2+} influx is that occurring through voltage-gated Ca^{2+} channels (Ca^{2+} VOCs)[1] (1). In the last few years, however, much interest has focused on other types of Ca^{2+} channels, the so called "receptor-operated channels" (ROCs), where the channel is part of the receptor itself and "second messenger-operated channels" (SMOCs) activated by a soluble second messenger (2, 3). Unlike the situation for ROCs, our understanding of Ca^{2+} SMOCs is rather primitive. In particular, whether one or more types of Ca^{2+} SMOCs coexist in the same cell and whether the definition of SMOCs is correct is still unclear. The demonstration that soluble second messengers are involved in regulating Ca^{2+} influx is based, in fact, on indirect evidence.

Ca^{2+} redistribution from intracellular stores, due to $Ins(1,4,5)P_3$, and Ca^{2+} influx are often simultaneously triggered by agonists of receptors linked to $PtdInsP_2$ hydrolysis (3). As far as Ca^{2+} influx is concerned, general consensus has only been reached on the fact that it is sustained by the activation of a Ca^{2+} inward current, independent of Ca^{2+} VOCs. In PC12 cells Ca^{2+} influx due to the natural nonapeptide BK has been shown to be due to a B_2 type receptor coupled to $PtdInsP_2$ hydrolysis and $Ins(1,4,5)P_3$ formation (4). Stimulation by BK is also accompanied by activation of an inward current and (after K^+ channel blockade) by plasma membrane depolarization whose dose dependence, pharmacological sensitivity, and kinetics correlate with the BK-stimulated Ca^{2+} influx.

In the last few years several reports have appeared indicating that, in a variety of eukaryotic cells, external ATP rises $[Ca^{2+}]_i$. In some cells triggering of the ATP receptors causes both Ca^{2+} redistribution and Ca^{2+} influx (5, 7). In other cells, however, particularly in smooth muscle, external ATP appears to control only the opening of cation channel(s), permeable to both monovalent and divalent cations (8, 10).

In this paper we demonstrate that in PC12 cells ATP and BK increase Ca^{2+} permeability through distinct mechanisms. The response to ATP seems to depend in part on indirect activation of Ca^{2+} VOCs and in part on a cation channel different from that modulated by BK in terms of cation selectivity and pharmacological sensitivity.

Some of the data characterizing the response to ATP in PC12 cells will be shown in miniprint form.

MATERIALS AND METHODS[2]

RESULTS

ATP and BK: Two Independent Pathways for Cation Fluxes—The natural nonapeptide BK (4) and ATP rapidly increase the $[Ca^{2+}]_i$ of PC12 cells. The data, reported below, stress the major differences between the two agonists. Further characterization of the ATP response in PC12 cells will be found in the Miniprint.

A) Ca^{2+} Redistribution and Influx—PC12 cells, loaded with the Ca^{2+} indicator fura-2, were challenged with an optimal dose of ATP (100 μM, Fig. 1, *a* and *b, continuous trace*) or BK (0.2 μM, Fig. 1, *a* and *b, dashed trace*). In the presence of 1 mM external Ca^{2+} (Fig. 1*a*), ATP evoked a rapid and short

* This work was supported by grants from the Italian Ministry of Education, by the National Research Council of Italy (Special Projects Biotechnology and Bioinstrumentation and Signal Transduction), and by the Associazione Italiana per la Ricerca sul Cancro. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: VOCs, voltage-gated Ca^{2+} channels; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; BK, bradykinin; PMA, phorbol 12-myristate 13-acetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ROCs, receptor-operated channels; SMOCs, second messenger-operated channels; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate.

[2] Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 5-8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

This is an Open Access article under the CC BY license.

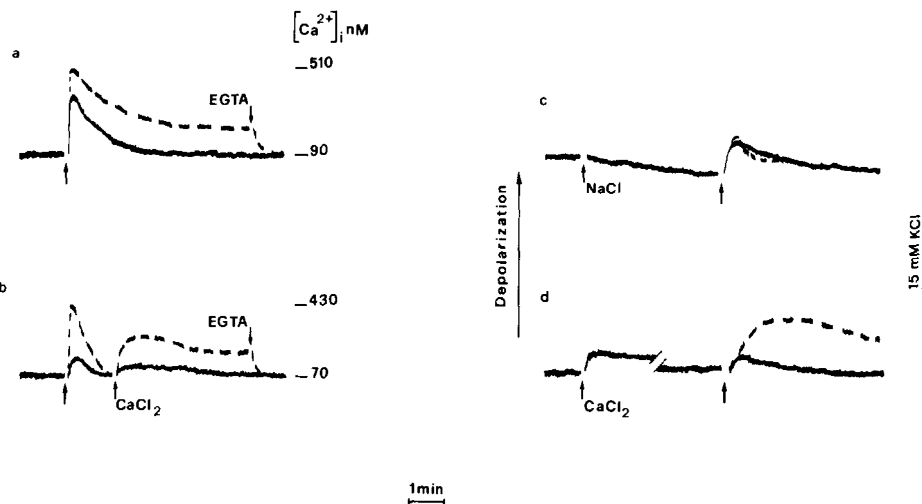

FIG. 1. **Effects of ATP and BK on $[Ca^{2+}]_i$ and cation influx.** *a* and *b*, effects of ATP and BK on $[Ca^{2+}]_i$. Conditions: fura-2-loaded cells (see "Materials and Methods") were incubated in Ca^{2+}-containing medium (*a*) or in Ca^{2+}-free, EGTA (100 μM)-containing medium (*b*). Cell number 0.5×10^6/ml. *Arrows* indicate agonist addition, 100 μM ATP (*continuous trace*) or 0.2 μM BK (*dashed trace*). Where indicated 1 mM EGTA and 1 mM $CaCl_2$ were added. In this and the following figures, the calibrated $[Ca^{2+}]_i$ is reported on the right-hand side. Traces shown are typical of results obtained in 12 (ATP) or eight (BK) experiments. Resting $[Ca^{2+}]_i$ varied in the range 91 ± 8 nM in Ca^{2+}-containing medium, and 72 ± 9 nM in Ca^{2+}-free, EGTA-containing medium. *c* and *d*, effects of ATP and BK on depolarization in sucrose-based medium. PC12 cells were pre-equilibrated with bisoxonol, apamin (0.4 μM), and tetraethylammonium (10 mM) in a sucrose-based medium (see "Materials and Methods"). Where indicated 30 mM NaCl (*c*) or 1 mM $CaCl_2$ were added (*d*). *Arrows* indicate 100 μM ATP (*continuous trace*) or 0.2 μM BK (*dashed trace*) addition. 4 min interruption (//).

lived $[Ca^{2+}]_i$ rise with a peak (329 ± 71 nM, n = 35) at 5–8 s and a half-life of less than 1 min. BK, on the contrary, elicited a larger and more sustained $[Ca^{2+}]_i$ rise (peak at 535 ± 84 nM, n = 30; half-life > 5 min). The most evident difference between the two agonists was thus the presence of a long lasting plateau with BK, but not with ATP.

The initial $[Ca^{2+}]_i$ rise evoked by BK was almost entirely due to Ca^{2+} release from internal stores as the peak $[Ca^{2+}]_i$ was little affected by removal of extracellular Ca^{2+} (Fig. 1*b*, *dashed trace*). As shown previously (4), the plateau phase, due to BK, depended on the presence of Ca^{2+} in the external medium. In contrast, a minor part of the ATP-induced $[Ca^{2+}]_i$ rise was due to Ca^{2+} redistribution (*cf.* Fig. 1, *a* and *b*, *continuous traces*). As measured in different cell batches, the contribution of Ca^{2+} redistribution accounted for 18 ± 8% (n = 12) of the total response to 100 μM ATP. Consistent with these findings, ATP induced only a small accumulation of $[^3H]Ins(1,4,5)P_3$ compared with that observed with BK (*cf.* Miniprint, Fig. 5, *inset*).

B) Membrane Potential—In Ca^{2+}-containing medium, both ATP and BK, at optimal doses, depolarized membrane potential (the latter only after K^+ channel blockade, see Ref. 4). When sucrose-based medium was used (devoid of permeant cations) neither BK nor ATP induced depolarization (not shown and see Ref. 4). However, when mM $CaCl_2$ or NaCl were added to the sucrose medium, both agonists did depolarize the cells (see Fig. 1, *c* and *d*; ATP, *continuous trace*; BK, *dashed trace*). The depolarization caused by BK was almost identical to that evoked by ATP in the presence of 30 mM NaCl. It was, however, three times bigger and more sustained when Ca^{2+} (1 mM) was the permeant cation (Fig. 1, *c* and *d*, *dashed traces*).

Additional evidence, for the existence of two distinct cation pathways, emerged from the following three different "pharmacological" treatments: lowering the $[Ca^{2+}]_i$, exposing the cells to the inhibitor of ligand-activated Ca^{2+} channels, SK&F 96365, and to the phorbol ester PMA.

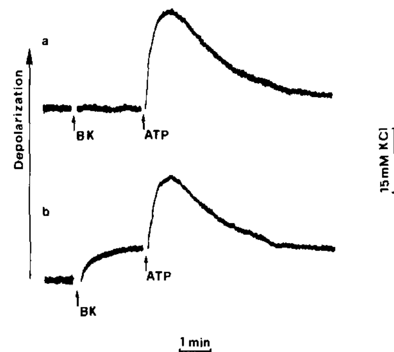

FIG. 2. **Effect of $[Ca^{2+}]_i$ on ATP- and BK-induced depolarization.** *a*, PC12 cells (10^7 ml) were incubated at 37 °C for 15 min with 30 μM quin2/AM in RPMI medium supplemented with 3% fetal calf serum and 1 mM EGTA. Cells were then diluted (1:5) with the same medium, left for a further 45 min at 37 °C, washed, and resuspended in Ca^{2+}-free, EGTA-containing medium with apamin and tetraethylammonium. Under these conditions the $[Ca^{2+}]_i$ was 15 nM. *b*, aliquots from the same quin2-loaded cells were incubated in Ca^{2+}-containing medium for 15 min until a stable level of $[Ca^{2+}]_i$ was reached (180 nM). Depolarization was checked in the Ca^{2+}-containing medium with apamin and tetraethylammonium as described in Fig. 7*b* (Miniprint). Where indicated 0.2 μM BK and 100 μM ATP were added.

C) Effect of Lowering the $[Ca^{2+}]_i$—Fig. 2 shows that when $[Ca^{2+}]_i$ was lowered to 10–15 nM (by loading cells with quin2/AM in EGTA-containing medium) the depolarization induced by BK was completely abolished, while that due to ATP was unaltered (Fig. 2*a*). Unspecific effects of quin2 treatment can be excluded since the same cells were capable of responding to BK after short recovery in the Ca^{2+}-containing medium (Fig. 2*b*).

D) Effect of SK&F 96365—Fig. 3 shows that the novel inhibitor of receptor-mediated Ca^{2+} entry, SK&F 96365 (12), largely prevented the prolonged $[Ca^{2+}]_i$ plateau caused by BK, while it had practically no effect on the peak rise caused by

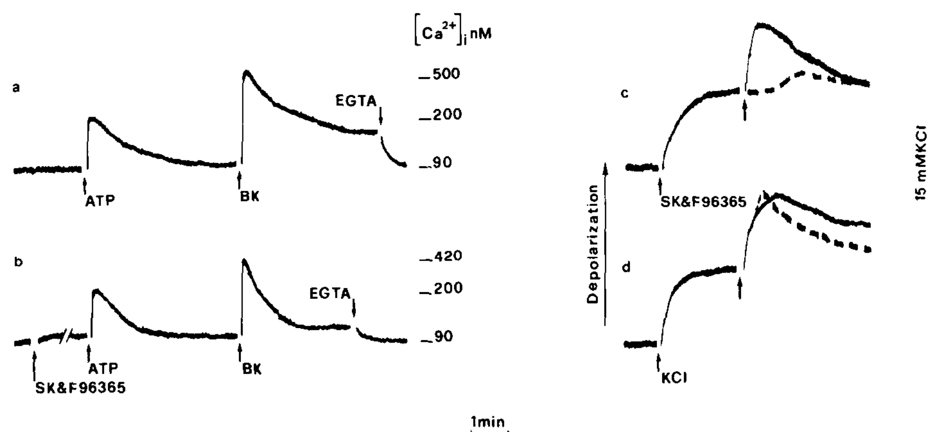

FIG. 3. **Effects of SK&F 96365 on $[Ca^{2+}]_i$ rise and depolarization due to ATP and BK.** *a* and *b*, effects on $[Ca^{2+}]_i$. Conditions as in Fig. 1. Ca^{2+}-containing medium. Where indicated 100 μM ATP, 0.2 μM BK, and 30 μM SK&F 96365 were added. *Trace b* was interrupted for 2 min (//). *c* and *d*, effects on membrane potential. Conditions as in Fig. 7*c* (Miniprint). Ca^{2+}-free, EGTA-containing medium supplemented with apamin and tetraethylammonium. Where indicated 30 μM SK&F 96365 or 15 mM KCl were added. *Arrows* indicate 100 μM ATP (*continuous trace*) or 0.2 μM BK (*dashed trace*) addition.

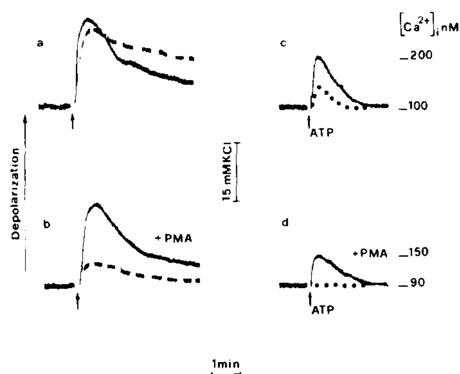

FIG. 4. **Effect of PMA on $[Ca^{2+}]_i$ rise and depolarization due to ATP and BK.** *a* and *b*, effects on depolarization. Conditions as in Fig. 7*c* (Miniprint). Ca^{2+}-free, EGTA-containing medium with apamin and tetraethylammonium. *b*, PMA-treated cells. PC12 cells were preincubated at 37 °C for 5 min with 100 nM PMA. *Arrows* indicate 100 μM ATP (*continuous trace*) or 0.2 μM BK (*dashed trace*). *c* and *d*, effects of PMA on $[Ca^{2+}]_i$ evoked by ATP. *Continuous trace,* Ca^{2+}-containing, sucrose-based medium. *Dotted trace,* Ca^{2+}-free, EGTA-containing, sucrose-based medium. *d*, PMA treatment as in *panel b*. Where indicated 100 μM ATP was added.

ATP (Fig. 3, *a* and *b*). Since SK&F 96365 is known to inhibit Ca^{2+} VOCs as well (12), in this experiment the cells were pretreated with verapamil to reduce the contribution of Ca^{2+} VOCs to the ATP response (see Miniprint). Fig. 3*c* shows that SK&F 96365 had an unexpected effect on membrane potential, causing a small depolarization. Control cells were thus treated with a dose of KCl that produced equivalent depolarizations. SK&F 96365 caused a marked inhibition of the BK-dependent depolarization (70–80%, *cf.* Fig. 3, *c* and *d*, *dashed traces*) while depolarization by ATP was hardly altered by the drug (Fig. 3, *c* and *d*, *continuous traces*).

E) Effect of PMA—Fig. 4 shows that a 5-min pretreatment with the potent protein kinase C activator PMA had a substantial inhibitory effect on the BK-induced depolarization (Fig. 4, *a* and *b*, *dashed traces*; see Ref. 4). On the contrary, PMA did not affect the ATP-induced depolarization (Fig. 4, *a* and *b*, *continuous traces*). The effect of PMA on $[Ca^{2+}]_i$ rises was complicated by the well-known inhibitory effect of PMA on Ca^{2+} VOCs (13). The effect of PMA on $[Ca^{2+}]_i$ rise due to ATP was therefore investigated under conditions where the contribution of Ca^{2+} VOCs was negligible. Fig. 4 (*c* and *d*) shows an experiment performed in sucrose-based, Ca^{2+}-containing medium. Under these conditions, PMA still reduced the total ATP response (*continuous traces*). The inhibition, however, appeared to be entirely due to the effect of PMA on Ca^{2+} redistribution (*dotted traces*). Similar results were obtained in Na^+-based medium in the presence of Ca^{2+} VOCs inhibitors (not shown).

On the other hand, pharmacological treatments known to affect receptors coupled to inhibition of adenylate cyclase, such as incubation with pertussis toxin (14), did not alter significantly the responses to the two agonists.

DISCUSSION

Extensive evidence suggests that agonists linked to $PtdInsP_2$ hydrolysis cause, in addition to intracellular Ca^{2+} release, also the opening of cation channels permeable to Ca^{2+} (2). The BK receptor (B_2) expressed in PC12 cells appears to be a typical example (4). In a number of systems, an ATP receptor, classified as P_{2y} (5), also appears to be coupled to $PtdInsP_2$ hydrolysis, Ca^{2+} redistribution, and stimulated influx. The relative importance of Ca^{2+} influx *versus* redistribution varies in different cell models (6, 10). The PC12 cells, available in our laboratory, are characterized by ATP receptors coupled to activation of a large Ca^{2+} influx, with little release of Ca^{2+} from internal stores.

Ca^{2+} influx induced by BK and ATP shows different characteristics, in particular duration, contribution of Ca^{2+} VOCs, and sensitivity to a number of pharmacological treatments. In this respect, the parallel investigation of agonist-induced $[Ca^{2+}]_i$ rises and membrane potential depolarization was particularly useful.

As far as time courses are concerned, Ca^{2+} influx due to BK lasted for several minutes and so did depolarization (after K^+ channel blockade), while Ca^{2+} influx due to ATP inactivated within 1 min. Interestingly, under parallel conditions, depolarization by ATP did not return to basal levels for several minutes. This latter observation indicates that ATP activates a long-lasting current which, however, sustains little or no net Ca^{2+} influx. The rapid inactivation of the ATP-triggered Ca^{2+} influx was not due to ATP hydrolysis nor to receptor down-regulation (see Miniprint).

A second major difference between BK and ATP was the contribution of Ca^{2+} VOCs to the overall Ca^{2+} influx. While BK-induced influx was highly insensitive to blockers of Ca^{2+}

VOCs (nitrendipine, ω-conotoxin, verapamil) (4, 15), that induced by ATP was partially sensitive (30–40%). Accordingly, in sucrose medium containing $CaCl_2$, Ca^{2+} influx due to BK was increased while that due to ATP was reduced; under the same conditions, ATP-evoked depolarization was in fact negligible.

About a third of the $[Ca^{2+}]_i$ rise caused by ATP was due to activation of a cation influx which is linked to plasma membrane depolarization. These data concur with those of Inoue *et al.* (11) indicating that in PC12 cells ATP activates an inward cation current. A number of results indicate that this pathway is different from that triggered by BK. (*a*) The depolarization due to ATP is stronger than that due to BK, as indicated by its activation even in the absence of K^+ channel blockers. (*b*) Although both ATP- and BK-activated currents can be sustained by $choline^+$ or N-$methylglucamine^+$, the BK-evoked depolarization appeared 3-fold larger than that activated by ATP when Ca^{2+} was the only permeant cation. (*c*) SK&F 96365 (12) blocked more than 70% of Ca^{2+} influx and depolarization caused by BK, while leaving the ATP-induced depolarization and VOCs-independent, Ca^{2+} influx clearly unaltered. (*d*) BK-induced depolarization was potently inhibited by reducing $[Ca^{2+}]_i$ below resting level, while the effect of ATP was unchanged. The same independence from $[Ca^{2+}]_i$ of the ATP-activated channel was also observed in smooth muscle cells by Benham and Tsien (8) in patch clamp studies. (*e*) Protein kinase C activation, through the phorbol ester PMA, inhibited both Ca^{2+} redistribution, Ca^{2+} influx and depolarization caused by BK (4), but did not alter ATP-evoked depolarization and VOCs-independent Ca^{2+} influx.

As far as the mechanism by which BK and ATP activated the cation channels, the present data allow us only to exclude the involvement of a few obvious candidates, such as pertussis toxin-sensitive G protein(s), protein kinase C, and $[Ca^{2+}]_i$. In the case of BK, we have previously demonstrated that InsPs levels are not the moment-to-moment regulators of Ca^{2+} influx (4). ATP caused only a minor accumulation of $Ins(1,4,5)P_3$, without significantly altering the concentration of either inositol 1,3,4-trisphosphate or inositol 1,3,4,5-tetrakisphosphate. Concentrations of BK which caused InsPs increases, similar to those of ATP, induced negligible stimulation of Ca^{2+} influx or depolarization. Thus the involvement of InsPs in ATP-induced Ca^{2+} influx depolarization seems unlikely. This conclusion is perfectly consistent with the results of Benham and Tsien (8) in smooth muscle cells. In that model clear evidence demonstrated that ATP-activated current is not mediated by a soluble second messenger. This channel-linked ATP receptor may be regarded as a true ROC, for which we propose the name of P_{2C} (C, for channel).

Acknowledgments—We are indebted to Dr. E. Sher for the gift of ω-conotoxin and Dr. J. Merritt for SK&F 96365, Drs. F. Di Virgilio, S. Treves, and J. Meldolesi for their suggestions, G. Ronconi for skillful technical assistance, and K. Marchioro for typing.

REFERENCES

1. Hess, P. (1990) *Annu. Rev. Neurosci.* **13,** 337–356
2. Meldolesi, J. & Pozzan, T. (1987) *Exp. Cell Res.* **171,** 271–283
3. Berridge, M. T. & Irvine, R. F. (1989) *Nature* **341,** 197–205
4. Fasolato, C., Pandiella, A., Meldolesi, J. & Pozzan, T. (1988) *J. Biol. Chem.* **263,** 17350–17359
5. Gordon, J. L. (1986) *Biochem. J.* **233,** 309–319
6. Greenberg, S., Di Virgilio, F., Steinberg, T. H. & Silverstein, S. C. (1988) *J. Biol. Chem.* **263,** 10337–10343
7. Kim, K. T. & Westhead, E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9881–9885
8. Benham, C. D. & Tsien, R. W. (1987) *Nature* **328,** 275–278
9. Bjornsson, O. G., Monck, J. & Williamson, J. R. (1989) *Eur. J. Biochem.* **186,** 395–404
10. Geschwind, J. F., Hiriart, M., Glennon, M. C., Najafi, H., Corkey, B. E., Matschinsky, K. M. & Prentki, M. (1989) *Biochim. Biophys. Acta* **1012,** 107–115
11. Inoue, K., Nakazawa, K., Fujimori, K. & Takanaka, A. (1989) *Neurosci. Lett.* **106,** 294–299
12. Merritt, J. E., Armstrong, W. P., Hallam, T. J., Jaxa-Chemiec, A., Leigh, B. K., Moores, K. E. & Rink, T. J. (1989) *Br. J. Pharmacol.* **98,** 674P
13. Di Virgilio, F., Pozzan, T., Wollheim, C. B., Vicentini, L. M. & Meldolesi, J. (1986) *J. Biol. Chem.* **261,** 32–35
14. Gatti, G., Madeddu, L., Pandiella, A., Pozzan, T. & Meldolesi, J. (1988) *Biochem. J.* **255,** 753–760
15. Sher, E., Pandiella, A. & Clementi, F. (1988) *FEBS Lett.* **235,** 178–182
16. Pozzan, T., Gatti, G., Dozio, M., Vicentini, L. M. & Meldolesi, J. (1984) *J. Cell Biol.* **99,** 628–638
17. Malgaroli, A., Milani, D., Meldolesi, J. & Pozzan, T. (1987) *J. Cell Biol.* **105,** 2145–2153
18. Di Virgilio, F., Fasolato, C. & Steinberg, T. H. (1988) *Biochem. J.* **256,** 959–963
19. Di Virgilio, F., Milani, D., Leon, A., Meldolesi, J. & Pozzan, T. (1987) *J. Biol. Chem.* **262,** 9189–9195
20. Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203,** 169–177
21. Gomperts, B. D. (1983) *Nature* **306,** 64–66
22. Gould, G. W., East, M., Froud, R. J., McWhirter, J. M., Stefanova, H. I. & Lee, A. G. (1986) *Biochem. J.* **237,** 217–227

Supplementary Material to

Receptor mediated calcium influx in PC12 cells : ATP and BK activated two indipendent pathways

Cristina Fasolato, Paola Pizzo and Tullio Pozzan

MATERIALS AND METHODS

Abbreviations - $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; AM, acetoxymethyl ester; BK, bradykinin; ATPγS, adenosin 5' - O - (3-thiotriphosphate); GTP S, guanosin 5' - O - (3-thiotriphosphate; AMP-PNP, adenosine 5' (β,γ-imido)triphosphate ; AMP-PCP, adenosine 5' (β,γ - methylene)triphosphate; Ap4A, adenosin(5')tetraphospho(5') adenosine; $PtdInsP_2$, phosphatydilinositol (4,5)bisphosphate; InsPs, inositol phosphates; $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$ and $Ins(1,3,4,5)P_4$, inositol tris- and tetrakisphosphate; HPLC, high performance liquid chromatography; EGTA, (ethylenebis(oxyethylenenitrilo)tetracetic acid; Hepes, 4-(2-hydroxyethyl) -1- piperazineethane sulfonic acid; Ap, apamin; TEA, tetraethylammonium chloride; ω-CgTx, ω-conotoxin; PMA, phorbol 12-myristate-13-acetate; Me, divalent cation.

Cells and Chemicals - PC12 cells, obtained initially from Dr. Calissano (Rome), were subcultured in our laboratory as described (16). The standard medium contained in mmol/liter: 125 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 5 glucose, 1 $CaCl_2$ and 20 Hepes (ph 7.4, 37°C), alternatively $CaCl_2$ was omitted and and 0.1 EGTA was added instead. In some experiments NaCl was replaced by equimolar amounts of either choline-HCl (choline-based medium) or N-methylglucamine (N-methylglucamine-based medium), or sucrose (sucrose-based medium). In these latter media, Na_2HPO_4 was replaced by K_2HPO_4 and the pH adjusted to 7.4 with Tris-HCl. BK, PMA, Ap, nitrendipine and sulfinpyrazone were purchased from SIGMA, St. Louis, MO, USA; quin2/AM and bisoxonol from Molecular Probes, Eugene, OR, USA; fura-2/AM, adenosine, ATP and all other nucleotides from Boehringer Mannheim, West Germany; RPMI-1640 from Flow Labs, Milano, Italy; inositol-free RPMI, fetal calf serum and horse serum from Seromed, Berlin, West Germany; ^3H-$Ins(1,4,5)P_3$, ^3H-$Ins(1,3,4,5)P_4$ and ^3H-myoinositol from Amersham International, U.K.; ω-CgTx was a kind gift of Dr. E. Sher, Milano, Italy; pertussis toxin was a kind gift of Dr. R. Rappuoli, Siena, Italy; SK&F 96365 was a kind gift of Dr. J. Merritt (SK&F Welwyn, England). All other materials were analitical or highest available grade.

Measurement of $[Ca^{2+}]_i$ - Loading with fura-2 was performed essentially as described (17). Briefly PC12 cells suspensions (10^7 x cells/ml) were incubated for 30 min at 37°C, under continuous stirring in RPMI-1640 medium supplemented with 3% fetal calf serum, 20 mM Hepes (pH 7.4 at 37°C) and 2 μM fura-2/AM. The cells were then washed with fresh medium and kept in RPMI medium containing 1% fetal serum and left at room temperature until use. Before each experiment, cell aliquots (2 x 10^6) were centrifuged; the pellets were resuspended in different, prewarmed media, and mantained, under continuous stirring, in a thermostated (37°C) cuvette. Excitation and emission wavelengths were 339 and 505 nm, respectively. Sulfinpyrazone (250 μM) was added at the beginning of each experiment to prevent fura-2 leakage (18). The calibration of the fluorescent signal in term of $[Ca^{2+}]_i$ was performed as described (17).

Membrane Potential - Measurements of membrane potential with the fluorescent probe bisoxonol (540-580 nm) were performed as described in detail by Di Virgilio *et al.* (19). The fluorescent signal was calibrated by adding 0.5 μM gramicidin D or 60 mM KCl at the end of each experiment.

Inositol Phosphate Extraction and Separation - Nearly confluent PC12 cells were incubated for 48 h in RPMI inositol-free medium, supplemented with 20 mM Hepes, 1% fetal calf serum and 6 μCi/ml of ^3H-myoinositol. The cells were then washed twice and resuspended in appropriate media. Cell aliquots (about 5 x 10^6 cells) were used for each sample. Inositol phosphate extraction and separation was performed as previously described (4) with a Partisil SAX HPLC column (Technicol, U.K.). Identification of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ was based on commerciable ^3H-InsPs standards. The ^3H-$Ins(1,3,4)P_3$ standard was obtained from ^3H-$Ins(1,3,4,5)P_4$ as described by Downes *et al.* (20).

ω-Conotoxin Treatment - ω-CgTx treatment was performed as described by Sher *et al.* (15) with a slight modification. PC12 cells (10^7 cells/ml), previously loaded with fura-2, were incubated for 30 min at 37°C in RPMI supplemented with 1% fetal calf serum, 250 μM sulfinpirazone and 2 μM ω-CgTx. Control cells received the same treatment without w-CgTx. Cells were then washed and immediately used.

Pertussis Toxin Treatment - Semi-confluent PC12 cells were incubated for 15 h in culture medium supplemented with pertussis toxin (200 ng/ml). Cells were then washed and loaded with fura-2 as described above.

Permeabilization Test - Membrane integrity was checked by testing the ability of the cells to exclude the fluorescent dye ethidium bromide (365-580 nm) (21). Calibration was performed by adding 10 µM digitonin at the end of each experiment.

Data Analysis - The ATP response in PC12 cells varied significantly from one batch of cells to another, especially in term of absolute $[Ca^{2+}]_i$ peak values (from 200 to 400 nM) and extent of depolarization (1-2 fold the depolarization caused by 15 mM KCl). The reason of this variability, which was only quantitative and not qualitative, is not known, and is now under investigation. Each experiment presented was performed at least on three different batches of cells. When effects on $[Ca^{2+}]_i$ and membrane potential were compared, the experiments were performed on aliquots of the same batch of cells. ATP^{4-} concentrations, in different media, were calculated by using the binding constants for Mg^{2+}, Ca^{2+} and H^+ to EGTA and ATP as given by Gould et al. (22).

RESULTS

Desensitization of the ATP-dependent $[Ca^{2+}]_i$ rise. Fig 5 (panels a and d) shows that the response to ATPγS (a slowly hydrolyzable ATP analogue) was almost identical, in size and duration, to that induced by ATP. Furthermore PC12 cells became unresponsive to high ATP concentrations (up to 500 µM) after being challenged with optimal doses of ATP or ATPγS (100 µM) (fig 5 a and d). This desensitization required the continuous presence of the nucleotide. In fact, when the cells were first treated with saturating concentrations of ATP for 5 min and then washed, they responded normally to a second dose of the stimulant (not shown). In contrast, if PC12 cells were first stimulated with ATP and then with BK (or viceversa), they did not reveal cross desensitization (see fig 5 b and e). Combination of the two stimuli resulted in a partially additive effect (fig 5c). These findings suggest that inactivation of the ATP-triggered Ca^{2+} influx, was not dependent on ATP hydrolysis, since i) inactivation with the same time course was observed even with ATP concentrations 10 times higher than those eliciting maximal response and ii) similar inactivation was obtained with ATPγS.

The inset of fig 5 shows that a short (10 s) stimulation with ATP (500 µM; similar results in the 5µM-5mM concentration range) induced a small accumulation of ^3H-Ins(1,4,5)P_3 compared to that observed with BK (0.2 µM) (120% and 310% of control cells, respectively). Moreover, in contrast to the results obtained with BK, stimulation with ATP (500 uM), from 10 s to 5 min, was unable to modify significantly the levels of both ^3H-Ins(1,3,4)P_3 and ^3H-Ins(1,3,4,5)P_4 (not shown; see ref. 4).

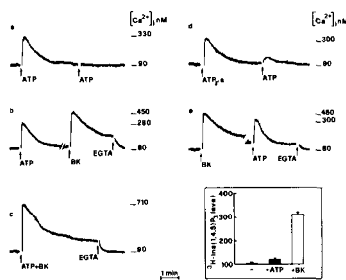

FIGURE 5. *Desensitization of the ATP-dependent $[Ca^{2+}]_i$ rise.* Conditions as in fig 1. a-e: Ca^{2+} -containing medium. a and d: first addition of ATP or ATPγS, 100 µM, second ATP addition, 500 µM. b: PC12 cells were first challenged with 100 µM ATP and then with 0.2 µM BK (// trace interrupted for 4 min). e: Same conditions as in panel b with BK added first and ATP second. c: 100 µM ATP and 0.2 µM BK were added together. Inset: Cells were labeled with ^3H-myo-inositol for 48h (see *Exp. Proc.*) and stimulated for 10 s with 500 µM ATP (hatched bar) or 0.2 µM BK (open bar) in Ca^{2+} -containing medium. ^3H-Ins(1,4,5)P_3 was isolated by HPLC, with a Partisil SAX column and measured as percent of that found in unstimulated cells (solid bar).

Different nucleotides were tested for their ability to mimick the response to ATP (fig 6). AMP-PCP, ADP, AMP and adenosine, at concentrations up to 500 µM, were completely uneffective. At 100 µM CTP and dATP were about ten times less effective than ATP. At higher doses (500 µM), however, they evoked about 50% of the response obtained with ATP. All the other compounds tested between 5 µM - 500 µM caused less than 30% of the $[Ca^{2+}]_i$ rise induced by 500 µM ATP.

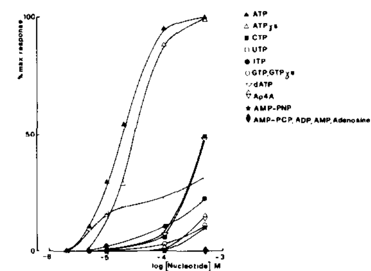

FIGURE 6. *Dose-response curve of $[Ca^{2+}]_i$ rises caused by different nucleotides.* Conditions as in fig 1. $[Ca^{2+}]_i$ rises due to different nucleotides (listed on the right side of the figure), tested at concentrations ranging from 5 to 500 µM, were measured as percent of the maximal $[Ca^{2+}]_i$ increase induced by 500 µM ATP. The results shown are from a typical experiment, which was repeated in 3 different PC12 batches.

Data obtained in recent years suggest that the molecular form of ATP active at the level of Ca^{2+} mobilizing receptors is ATP^{4-} instead of $MgATP^{2-}$ or $MeATP^{2-}$ (6). In order to investigate this point, experiments were carried out in Mg^{2+} -free medium. The results obtained show that in PC12 cells the $[Ca^{2+}]_i$ rise evoked by ATP did not require Mg^{2+}, but rather was enhanced in Mg^{2+}-free medium. Under the latter conditions, the dose-response curve for ATP-induced $[Ca^{2+}]_i$ rise was shifted to the left (see below). Absence of Mg^{2+}, on the other hand, failed to modify the BK responses (not shown).

Effect of ATP on Membrane Potential. In Ca^{2+} -containing medium, ATP (100 µM)-induced depolarization dissipated, although only in part, within 2 min (fig 7a). Such a depolarization was enhanced in the presence of the Ca^{2+}-activated K^+ channel inhibitors, apamin (0.4 µM) and TEA (10 mM) (fig 7 b,c). Under the latter conditions, the dose response curve of the ATP-induced depolarization in Ca^{2+}-free, EGTA containing medium, was slightly shifted to the left compared to that obtained for the $[Ca^{2+}]_i$ rises in the Ca^{2+} -containing medium (fig 7 inset A). The differences between the two curves disappeared when the results were normalized to $[ATP^{4-}]$ rather than total [ATP] (fig 7 inset B).

Similarly to BK (4), ATP still induced a small depolarization when choline$^+$ and N-methylglucamine$^+$ were used to replace Na^+ in the incubation medium (not shown; see ref. 4) suggesting that cation channels of low selectivity are activated by both agonists.
ATP-induced depolarization was also observed in a Ca^{2+} and Mg^{2+} -free, Na^+ -containing medium. Under those conditions 2 µM total ATP, a subthreshold dose under standard conditions, was already maximally effective (cfr. fig 7 inset B). The depolarization observed with 2 µM ATP in Ca^{2+} and Mg^{2+} - free medium was not due to cellular damage since, under the latter conditions, permeabilization to ethidium bromide was not observed even after 10 min incubation (not shown).

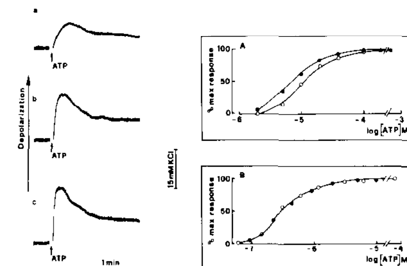

FIGURE 7. *Effects of ATP on membrane potential and $[Ca^{2+}]_i$.* a: Ca^{2+} -containing medium. b: Ca^{2+} -containing medium supplemented with Ap (0.4 uM) and TEA (10 mM). c: Ca^{2+} -free, EGTA (0.1 mM)-containing medium, with Ap and TEA as in panel b. Cells (0,5 x 10^6/ml) were pre-equilibrated with 100 nM bisoxonol for 5-10 min at 37°C. Depolarization is indicated by an increase in the bisoxonol fluorescence. On the right hand side the increase in fluorescence caused by 15 mM KCl is shown. Inset A: dose response curves of the $[Ca^{2+}]_i$ rises, in Ca^{2+} medium, (open circle) and the depolarization, in Ca^{2+}-free, EGTA-containing medium with Ap and TEA, (closed circle). The percent of maximal ATP response is plotted as a function of the total ATP concentration. Data are from a typical experiment which was repeated in 3 different PC12 batches. Inset B: the same data of inset A were plotted as a function of the ATP^{4-} concentration.

Voltage Operated Ca^{2+} channels (VOCs) involvement in the effects of ATP on $[Ca^{2+}]_i$. In standard medium, the BK induced $[Ca^{2+}]_i$ rise was unaffected by blockers of Ca^{2+} VOCs (4,15). On the contrary, nitrendipine (0.4 µM) a specific inhibitor of L-type Ca^{2+} VOCs, induced a partial inhibition, ranging from 15 to 25%, of the total $[Ca^{2+}]_i$ rise evoked by 100 µM ATP. Under the same conditions, nitrendipine treatment resulted in a 60% inhibition of the $[Ca^{2+}]_i$ peak and totally prevented the plateau induced by 60 mM KCl (not shown and see below). The $[Ca^{2+}]_i$ rise due to KCl was greatly reduced (> 85%) when nitrendipine and ω-CgTx (an inhibitor of N-type Ca^{2+} VOCs) were used together cfr. fig 8 a and b). In contrast, under the latter conditions, the ATP-induced $[Ca^{2+}]_i$ response was still observed (above 55% of control cells, cfr. fig 8 a,b). Such an ATP-induced, nitrendipine- and ω-CgTx-insensitive, $[Ca^{2+}]_i$ rise was sustained only in small part by intracellular Ca^{2+} release (fig 8, dashed traces). Verapamil (20 µM) a widely used, but not very specific, inhibitor of Ca^{2+} VOCs was also used and the inhibition by this drug was greater than that obtained with nitrendipine alone and similar to that of nitrendipine plus ω-CgTx (45% inhibition of a maximal ATP response, not shown).

In the sucrose-based medium, supplemented with 1 mM external $CaCl_2$, the ATP-induced depolarization was very small compared to that obtained in standard medium (cfr. fig 1d and 7b). Under these conditions, the ATP-induced $[Ca^{2+}]_i$ rise was similar to that observed in standard medium in the presence of nitrendipine plus ω-CgTx (cfr. fig 8 b and c), and it was insensitive to the Ca^{2+} VOCs blockers (not shown).

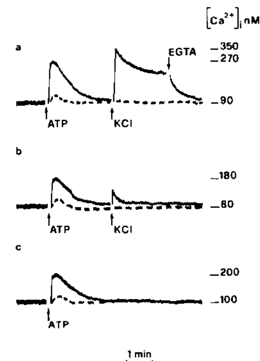

FIGURE 8. *Ca^{2+} VOCs contribution to ATP effects on $[Ca^{2+}]_i$.* Conditions as in fig 1. a-c: Continuous traces, Ca^{2+} -containing medium, dashed traces, Ca^{2+}-free, EGTA-containing medium. a: Control cells. b: Cells treated for 30 min at 37°C with 2 µM ω-CgTx, then washed and resuspended in media containing 0.4 µM nitrendipine. c: Sucrose-based medium. Where indicated 100 µM ATP, 60 mM KCl and 1 mM EGTA were added.

Our data suggest that ATP-induced Ca^{2+} redistribution is due to different receptors than those causing Ca^{2+} influx. In fact pretreatment of PC12 cells with low UTP concentrations completely desensitized the ATP-induced Ca^{2+} redistribution, leaving ATP-dependent Ca^{2+} influx and plasma membrane depolarization unaffected (Fasolato unpublished observation). Of interest, Kim *et al.* (7) showed that, in bovine chromaffin cells, Ca^{2+} redistribution can be activated by either ATP or UTP, while ATP only triggers Ca^{2+} influx and cathecolamine secretion. Thus although in our PC12 cells, a P_{2Y} subtype of ATP receptor (5), coupled to PtdInsP_2 hydrolysis, is probably expressed, the main response to the nucleotide depends on the activation of a type of ATP receptor closely resembly that described by Benham and Tsien (8) in smooth muscle cells and by Geschwind *et al.* (10) in HIT cells. On the other hand this receptor differs in pharmacological specificity from that expressed in rat heart myocytes (9). In fact the ATP receptor of our PC12 cells appears to be sensitive to ATP^{4-} while that expressed in myocytes is activated by $MgATP^{2-}$. In addition, unlike the cardiac myocyte receptor, in our model, the sugar residue, the purinic base and the *gamma* phosphate appear essential for effective stimulation.