British Journal of Pharmacology (1997) 121, 497–502

The effect of the neuropeptide substance P on desensitization of ATP receptors of PC12 cells

L. Khiroug, *R. Giniatullin, *M. Talantova and [1]A. Nistri

Biophysics Sector and INFM Unit, International School for Advanced Studies (SISSA), 34013 Trieste, Italy and *Department of Physiology, Kazan Medical University, Kazan, Tatarstan, Russia

1 Patch clamp recording (whole cell configuration) was employed to investigate the modulatory action of substance P on inward currents elicited by adenosine 5′-triphosphate (ATP, focally applied via a pressure pipette) from phaechromocytoma (PC12) cells usually held at −70 mV.

2 Bath-applied substance P (0.2–20 μM) had no effect on baseline membrane current but reversibly reduced ATP peak currents in a concentration-dependent fashion. The depressant effect was not associated with a change in the ATP current reversal potential.

3 Equiamplitude peak responses induced by 50 μM or 5 mM ATP were differentially affected by substance P which preferentially reduced currents evoked by 5 mM ATP. In the presence of substance P a conditioning pulse of ATP evoked a stronger depression to subsequent test pulses of the same agonist.

4 Combined patch clamping and confocal laser imaging of intracellular Ca^{2+} ($[Ca^{2+}]_i$) of single PC12 cells showed that substance P (applied by a pressure pipette) *per se* had no effect on $[Ca^{2+}]_i$ or current baseline, although it reduced the inward current and associated $[Ca^{2+}]_i$ rise elicited by ATP.

5 These results are interpreted as due to facilitation by substance P of desensitization of ATP-gated P_{2X_2} receptors of PC12 cells. It is proposed that the novel modulation by this peptide of ATP responses may serve as a model for further studies aimed at elucidating the action of substance P on purinergic neurotransmission.

Keywords: Substance P; ATP; desensitization; P_{2X_2} receptor; confocal microscopy; intracellular calcium

Introduction

Neuropeptides are often colocalized with classical transmitters within the same nerve terminals from which they are released to modulate the efficiency of transmitter action on the postsynaptic membrane (for a review see Sneddon, 1995). One of the most widely investigated neuropeptides is substance P which belongs to the tachykinin peptide family (Otsuka & Yoshioka, 1993) and is capable of modulating synaptic responses through two distinct mechanisms: (a) tachykinin-receptor mediated closure of certain K^+ channels (Adams *et al*., 1983; Morita & Katayama, 1992; Phenna *et al*., 1996) with consequent increase in membrane resistance and amplification of signals generated by other transmitters such as acetylcholine (Adams *et al*., 1983), γ-aminobutyric acid (GABA, Yamada & Akasu, 1996) or glutamate (Urban *et al*., 1994); (b) allosteric binding of substance P to a receptor for a neurotransmitter such as acetylcholine. This latter phenomenon, which does not involve any of the known tachykinin receptors (Stafford *et al*., 1994), has typically been studied on nicotinic receptors of peripheral neurones (Livett *et al*., 1979; Role *et al*., 1981; Akasu *et al*., 1983) including phaeochromocytoma (PC12) cells (Simasko *et al*., 1987). Such an allosteric modulation of receptor function by substance P is somewhat intriguing, particularly since it consists of a transient down-regulation of nicotinic receptor activity. On chromaffin cells or the closely related PC12 cells the modulatory action of substance P is brought about by it promoting desensitization of nicotinic receptors (Clapham & Neher, 1984; Boyd, 1987; Valenta *et al*., 1993). By recording from cell-attached patches of ganglion cells Simmons *et al*. (1990) have confirmed the depressant effect of bath-applied substance P, but proposed that it is brought about by an intracellular second messenger system (perhaps related to protein kinase C). Curiously though, this effect is unaltered by intracellular dialysis when the whole-cell configuration is attained and is not mimicked by selective agonists of distinct tachykinin receptors (Simmons *et al*., 1990). A different proposal has been put forward by Blanton *et al*. (1994) who have shown the incorporation of a substance P derivative into a certain subunit of the nicotinic receptor which appears to correspond to the lining of the ionic channel. These authors have therefore suggested that the downregulation by substance P of nicotinic channels involves a degree of channel block. In summary then, the mechanism responsible for the action of substance P on nicotinic channels is not fully clarified.

On PC12 cells the endogenously occurring nucleotide adenosine 5′-triphosphate (ATP) evokes responses similar to those due to nicotinic receptor activation (Inoue *et al*., 1989) via its own receptors which seemingly belong to the P_{2X_2} subclass (Collo *et al*., 1996) of the P_{2X} receptor family (Burnstock & Kennedy, 1985). P_{2X_2} receptors of PC12 cells display fast and slow desensitization (Giniatullin *et al*., 1996). Like acetylcholine (ACh), ATP induces a rapid inward current via channels non-selective for monovalent cations (Nakazawa *et al*., 1990) and displaying high permeability to Ca^{2+} (Rogers & Dani, 1995). In view of the analogies between the actions of ACh and ATP on PC12 cells, one might ask whether, against a background of depression of ACh-induced responses by substance P, these cells could still respond to ATP. In other words, is the modulatory role of substance P encompassing ATP receptors? This issue was examined by electrophysiological recording of ATP-induced membrane currents and by confocal laser imaging of associated intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients.

Methods

Experiments were carried out in accordance with recently published methods (Giniatullin *et al*., 1996; Khiroug *et al*., 1997). In brief, PC12 cells were superfused at a rate of 10 ml min^{-1} with control solution containing (in mM): NaCl 132, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10; pH was adjusted to 7.4 with NaOH. Tight-seal whole cell recordings were obtained with patch pipettes which had resistance of 1.5–

[1] Author for correspondence.

3 MΩ when filled with (in mM): CsCl 120, HEPES 20, $MgCl_2$ 1, Mg_2ATP_3 3 and EGTA 5 (pH = 7.2 adjusted with CsOH). For confocal $[Ca^{2+}]_i$ imaging experiments, 25 μM of the Ca^{2+}-sensitive dye fluo-3 was added to the patch pipette and EGTA was omitted. ATP was diluted in control solution and delivered to the cell by pressure application (10–20 p.s.i.) from glass pipettes (positioned 15–20 μm away). Substance P was usually bath-applied although in some experiments it was pressure-applied via a second pipette positioned closely to the ATP pipette. In order to compare ATP-induced responses in the absence with those in the presence of substance P, the same cell was continuously recorded under the various experimental treatments; from individual cells ATP currents (and $[Ca^{2+}]_i$ transients) in the presence of substance P were expressed as % of their controls and pooled together. Membrane currents were amplified with a List L/M-PC amplifier and measured in terms of amplitude and exponential decay. When confocal Ca^{2+} imaging was performed in combination with patch clamp recording, fluo-3 emission was induced by the Ar-Kr laser of the MultiProbe 2001 confocal laser scanning microscope and analysed over the whole optical section of the cell by use of the 32-line rapid scan mode (320 ms per scan). The fluorescent signals are expressed as relative amplitude ($\Delta F/F_0$) over baseline (F_0). Data are presented as mean ± s.e.mean. Statistical significance was assessed with Student's t test for normally distributed data and with the one-way ANOVA test for non-parametric data.

Results

The data base of the present study comprised 34 PC12 cells usually clamped at −70 mV holding potential. Figure 1a shows an example of the inward current induced by a brief (20 ms) pulse of ATP (5 mM; arrows point to pressure application) which, under these conditions, is known to elicit half-maximal responses (Giniatullin *et al*., 1996). Bath-applied substance P (20 μM; 5 min) had no effect on baseline current but it strongly depressed the ATP peak current (by 73%; Figure 1a), an effect which was reversible after 7 min washout. The depressant action of substance P was concentration-dependent as indicated in Figure 1b, although even at 20 μM a residual response to ATP persisted. On average substance P (4 μM) reversibly decreased the current amplitude induced by 20 ms pulses of ATP from 185 ± 44 to 90 ± 25 pA (47 ± 6% reduction; $n = 6$; $P < 0.05$). Figure 1c shows a typical example of the time profile of the depressant action of substance P which developed after 3 min superfusion, reached a maximum at 7 min and gradually recovered upon washout over the next 10 min. Subsequent applications of substance P usually led to tachyphylaxis of its depressant action: for this reason bath-applied substance P was routinely administered only once to each culture dish. The action of substance P was not due to a change in the reversal potential of the ATP-induced current as shown by the superimposed current-voltage tracings of Figure 1d, obtained by applying voltage ramps (400 mV s^{-1}) from −110 to +70 mV before and during application of substance P (4 μM). Since the two traces are leak-subtracted, it is possible to observe that there was an equivalent degree of reduction in ATP-induced responses throughout the test potential range. This finding was confirmed in 6 experiments by normalization and scaling of the two current-voltage curves which became virtually identical, indicating that the depressant action of substance P was voltage-independent.

Longer pulses (2 s) of ATP (5 mM) evoked inward currents which first peaked to a value smaller then the maximum, rapidly faded and were followed by a large current rebound immediately after the end of ATP delivery (see example in left-

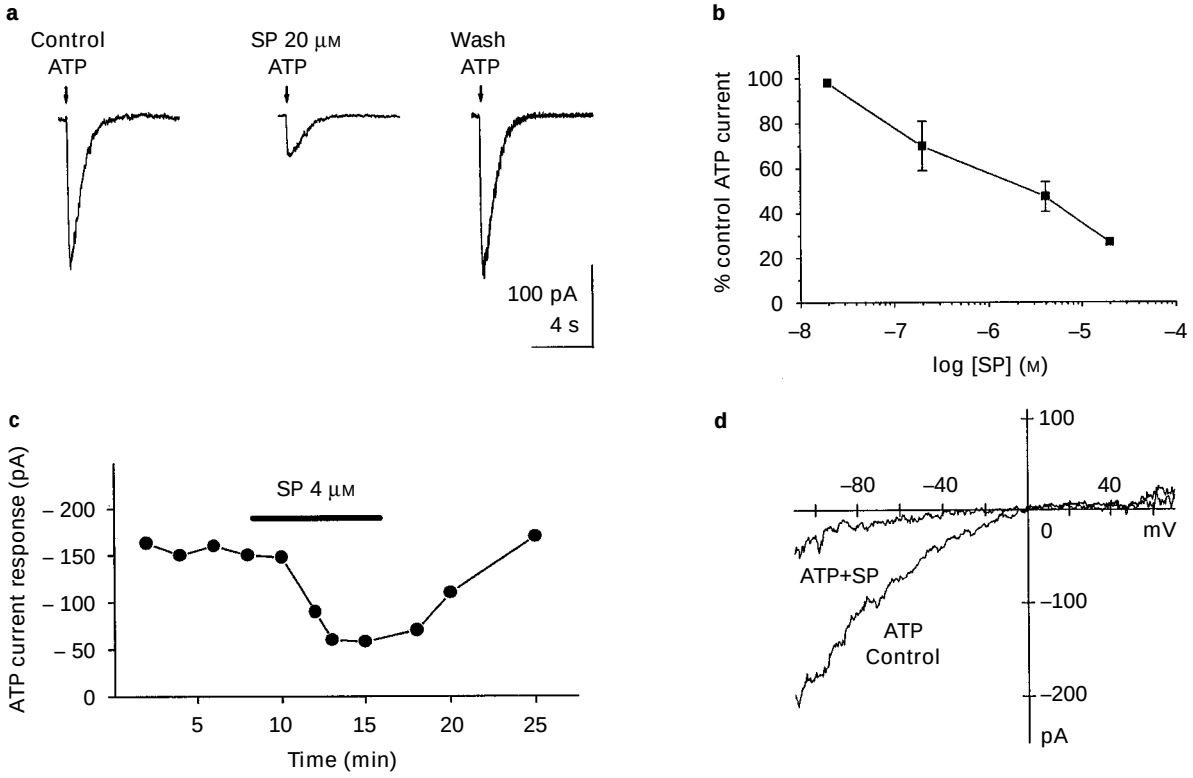

Figure 1 Depression of ATP-induced currents by bath-applied substance P (SP). (a) Effect of substance P (20 μM) on currents evoked by a 20 ms pressure pulse of 5 mM ATP (indicated by arrows). (b) Plot of substance P depressant effect (measured as % of control ATP current, ordinate scale) vs log substance P concentration (abscissa scale). Vertical lines indicate s.e.mean for 4–6 cells. (c) Plot showing the timecourse of the depression of the ATP current induced by bath application of 4 μM substance P and subsequent recovery after washout. (d) Current-voltage relationship for ATP currents in control conditions or in the presence of 4 μM substance P. Membrane potential was changed with a continuous voltage ramp (400 mV s^{-1}) from −130 to +40 mV at the peak of ATP-induced currents.

hand side of Figure 2a; cf Giniatullin *et al*., 1996). The initial peak amplitude was reduced by 4 μM substance P from 151 ± 30 to 65 ± 13 pA ($n=6$; $P>0.0001$), corresponding to a 56% reduction comparable to the one found when 20 ms applications were used. This effect is exemplified in Figure 2a which also shows that the rebound current was similarly depressed in a reversible manner. The rapid fade of the inward current during long pulses of ATP has been interpreted as due to fast receptor desensitization (Giniatullin *et al*., 1996) and may be quantified by measuring its decay time constant which was reduced from 726 ± 339 to 408 ± 225 ms ($n=6$) by substance P (see inset to Figure 2a). On a separate group of cells, when inward currents were evoked by 2 s long pulses of rather diluted ATP concentrations (50 μM), as depicted in Figure 2b, 4 μM substance P had minimal depressant effect (15 ± 5%) even if the peak amplitude of the ATP current was comparable (128 ± 12pA; $n=7$) to the one induced by 5 mM ATP.

Previous experiments have suggested that a slowly developing form of P_{2X_2} receptor desensitization is manifested as a transient reduction in currents elicited by 20 ms ATP (5 mM) after a conditioning 2 s pulse of the same agonist (Giniatullin *et al*., 1996): since currents are usually restored after 30 s if the patch pipette contains strong Ca^{2+} buffers (Khiroug *et al*., 1997), this time can be taken as an end-point for apparent recovery from desensitization on the assumption that responses to 20 ms ATP had no intrinsic desensitization. Figure 3Aa shows that the inward current produced by 20 ms ATP had recovered to 83% of its initial value 30 s after a conditioning pulse of 2 s ATP, with complete recovery 2 min later. On a sample of 3 cells, full recovery to test pulses of ATP in control solution took 47 ± 22 s. When the same protocol was repeated on the same cell in the presence of 4 μM substance P (Figure 3Ab) the initial test response to ATP was inhibited by 29%. Thirty seconds after the conditioning pulse of ATP the responses were more severely depressed (by 92%) with partial recovery 2 min later. In fact, full recovery to test pulses was only observed 150 ± 13 s later. Figure 3B shows the intensification by three concentrations of substance P of the depression of test ATP responses by conditioning pulses of ATP. In particular, when compared with controls, the ATP response was 58 ± 11% ($n=4$), 19 ± 6% ($n=3$; $P>0.05$) or 5% ($n=1$) in the presence of 0.2, 4 or 20 μM substance P, respectively.

A previous study has shown that ATP induces a transient rise in $[Ca^{2+}]_i$ proportional to the charge transfer operated by P_{2X_2} receptor activation and capable of promoting slow receptor desensitization (Khiroug *et al*., 1997). In the present experiments combined patch clamp recording and $[Ca^{2+}]_i$ imaging were thus performed in order to ascertain if substance P itself was changing baseline $[Ca^{2+}]_i$ and if it interfered with the increases in $[Ca^{2+}]_i$ produced by ATP. Figure 4 shows

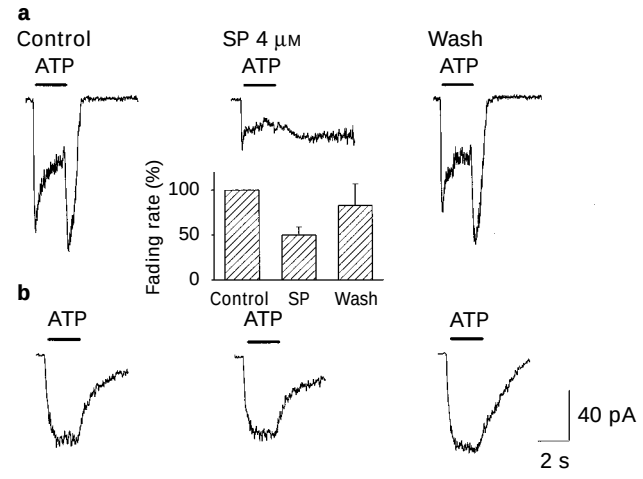

Figure 2 Effects of bath-applied substance P (SP) on membrane currents induced by long (2 s) pulses of ATP. (a) In control solution (left) a 2 s pulse of 5 mM ATP evoked a biphasic response with initial peak followed by fade and rebound currents (period of pressure application is indicated by the horizontal bars). All components of the response were reversibly depressed by substance P (see centre panel) with recovery observed after 12 min; inset shows histograms for the changes in the time-constant of peak inward current decay (fading rate expressed as % of control one) in the presence of substance P ($n=6$). (b) A 2 s pulse of 0.05 mM ATP (horizonal bars) evoked non-desensitizing inward currents which were virtually insensitive to substance P (middle panel).

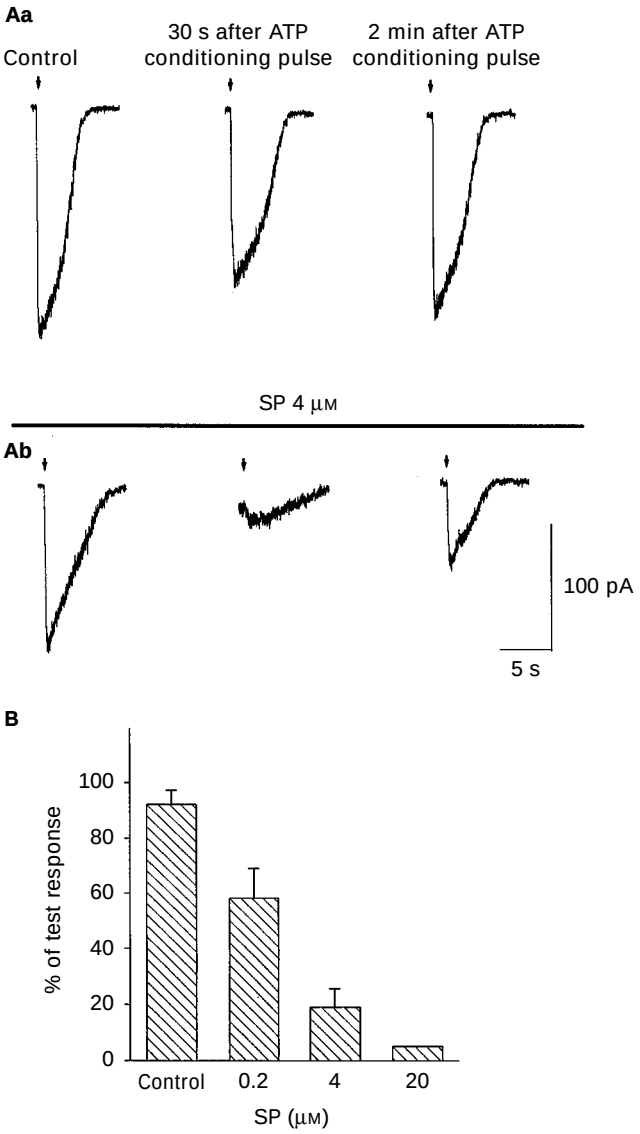

Figure 3 Effect of bath-applied substance P (SP) on recovery from desensitization induced by 2 s conditioning pulse of 5 mM ATP. (A) Brief (20 ms) pressure application of 5 mM ATP (arrows) evoked an inward current in control solution (a; left). After a 2 s conditioning pulse of the same pipette concentration of ATP which produced P_{2X_2} receptor desensitization, the response to the last application of ATP recovered to 83% of control amplitude (centre; 30 s later) with complete recovery after 2 min (right). (b) Addition of 4 μM substance P to the superfusion solution (horizontal bar) led to a 30% decrease in the test response amplitude (left; compared with control trace in Aa) and slowed down recovery of ATP current from the desensitization induced by ATP conditioning pulse, so that after 30 s the response was only 8% of control and even 2 min later it was 55%. (B) Dose-dependence of the substance P effect on recovery of ATP current from desensitization (measured as % of test response) 30 s after the conditioning pulse in control solution or in the presence of increasing concentrations of substance P (error bars represent s.e.mean for 3–4 cells).

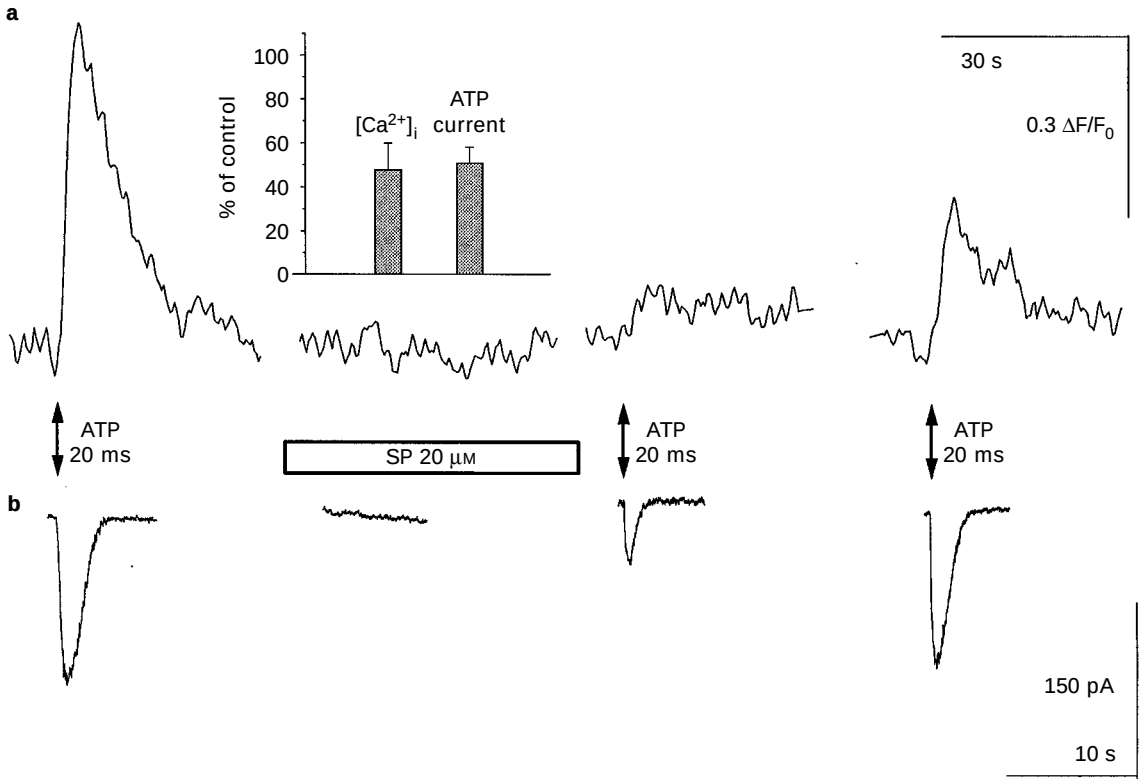

Figure 4 Pressure application of substance P (SP) produced depression of simultaneously recorded ATP-evoked membrane currents and $[Ca^{2+}]_i$ transients. (a) and (b) Responses to brief (20 ms; doubleheaded arrows) pulses of 5 mM ATP recorded as $[Ca^{2+}]_i$ increases (a) and membrane currents (b). Note different time calibration for (a) and (b). Pressure application of 20 μM substance P for 1 min (open bar) did not change either $[Ca^{2+}]_i$ level or baseline current. Test pulse (20 ms) of the same concentration of ATP applied after the substance P washout elicited largely depressed responses both in terms of $[Ca^{2+}]_i$ transient amplitude and membrane current. Upon 5 min washout current amplitude to 20 ms ATP recovered substantially while the $[Ca^{2+}]_i$ response had incomplete recovery. Inset shows the histograms for depression (measured as % of control) of $[Ca^{2+}]_i$ transient and current amplitude induced by pressure application of substance P ($n=6$; $P<0.005$ for both sets of data).

simultaneous increases in $[Ca^{2+}]_i$ (a) and membrane inward current (b; note faster timebase) during 20 ms applications of ATP (5 mM). Substance P (20 μM; pressure-applied via a separate pipette for 1 min) did not change $[Ca^{2+}]_i$ or current baseline but strongly depressed the rise in $[Ca^{2+}]_i$ and inward current elicited by ATP (81% and 69% depression, respectively). After 5 min wash recovery of the ATP current was complete while the $[Ca^{2+}]_i$ transient was 54%. The inset to Figure 4 shows the reduced size of the $[Ca^{2+}]_i$ and current amplitude following pressure application of substance P to six cells. No direct action of substance P on $[Ca^{2+}]_i$ or current baseline was observed on the same group of cells.

Discussion

The principal finding of the present study on PC12 cells was the novel demonstration that substance P, without exerting any measurable action on baseline current or $[Ca^{2+}]_i$, strongly depressed only those responses which were produced by high concentrations of ATP. These results are interpreted as due to facilitation by substance P of ATP-induced receptor desensitization.

Characteristics of substance P depression of ATP responses

Substance P *per se* had no effects on PC12 cells in terms of changes in either baseline current or $[Ca^{2+}]_i$. This observation confirms that PC12 cells do not possess conventional tachykinin receptors (Simasko *et al*., 1987; Simmons *et al*., 1990). Previous experiments have indicated that brief applications of ATP to PC12 cells induce membrane currents and an associated increase in $[Ca^{2+}]_i$ via activation of a homogeneous population of P_{2X_2} receptors (Giniatullin *et al*., 1996; Khiroug *et al*., 1997). The depressant action of substance P on ATP-induced currents was dependent on the concentration of the peptide, had slow onset and offset and was unaffected by changes in membrane potential. Likewise, substance P reduced the $[Ca^{2+}]_i$ transients evoked by ATP in parallel with the decrease in membrane currents. It is worth noting that the ability of substance P to depress ATP-induced currents was clearly related to the concentration of ATP. Short applications (20 ms) of 5 mM ATP evoked rapid inward currents which were readily reduced by substance P: an analogous reduction was also apparent when the application of ATP induced strong fading of the response and a rebound response. However, currents induced by small doses of ATP (0.05 mM for 2 s) were minimally affected by substance P even if their amplitude was comparable to that of currents observed after larger doses of ATP. Furthermore, substance P significantly slowed down recovery of ATP currents after a conditioning application of ATP.

Possible mechanisms underlying the substance P-induced depression of ATP currents

Although Simmons *et al*. (1990), working on chick sympathetic ganglion cells, found that substance P induced downregulation of nicotinic receptors through a second messenger system, it seems unlikely that a similar interpretation can be applied to the present results obtained with ATP-mediated responses of PC12 cells. In fact, there was no evidence (either in terms of current or $[Ca^{2+}]_i$ changes) for a direct action of substance P on PC12 cells, although it should be realized that in theory

substance P might have acted via an unidentified receptor system which does not translate its activation either in terms of membrane channel activity or changes in $[Ca^{2+}]_i$. Nevertheless, it seems more likely that substance P exerted a modulatory role on P_{2X_2} receptor activity, either by block of P_{2X_2} receptors (or of their open channels), or facilitation of their desensitization. If substance P were a competitive antagonist of P_{2X_2} receptors, its inhibitory effect should have been proportionally the same regardless of the ATP concentration. Against this interpretation, it was observed that substance P preferentially blocked ATP responses induced by large doses of this agonist. Such a depressant activity was unlikely to be due to an open channel block process. In fact, while open channel block is usually a voltage-dependent phenomenon (see Clapham & Neher, 1984) the action of substance P was the same despite wide variations in membrane potential and did not involve any change in the reversal potential for the ATP currents. Since these findings make improbable open channel block as the mechanism of action of substance P (see also analogous conclusions for the effect of substance P on nicotinic receptors; Stafford *et al*., 1994), it seems worth considering the possibility of facilitation by the peptide of P_{2X_2} receptor desensitization. In the case of nicotinic receptors, Blanton *et al*. (1994) proposed that part of the effect of substance P may be due to its interaction with a receptor region lining the ionic channel. However, those experiments were performed on *Torpedo* isolated membranes exposed for 30 min to 3 mM carbachol, which probably elicited a virtually complete desensitization of nicotinic receptors. The present experiments used comparatively less intense receptor desensitization, since even 5 mM ATP did not normally evoke complete fading of the inward current back to baseline.

The present results differ from those of a recent study (Wildman *et al*., 1997) describing potentiation by substance P of ATP-mediated responses on *Xenopus* oocytes expressing recombinant P_{2X_2} receptors. It should be first noted that the concentration of substance P used by Wildman *et al*. (1997) was 100 μM (i.e. much higher than the ones normally tested in the present experiments) while the ATP concentration (3 μM) was low enough to avoid any apparent desensitization. On native receptors of PC12 cells moderately low concentrations of ATP (50 μM from a pressure pipette) elicited non-desensitizing responses little sensitive to substance P. An interesting possibility is that recombinant P_{2X_2} receptors expressed by oocytes lack the membrane domain necessary for expressing the depressant action of substance P.

How might substance P interfere with P_{2X_2} receptor desensitization?

In the case of nicotinic acetylcholine receptors substance P is known to potentiate desensitization via allosteric site modulation (Stafford *et al*., 1994) without interfering with channel activation or ion permeation (Clapham & Neher, 1984). The precise molecular mechanism is still uncertain although it appears to require the presence of the β receptor subunit (Stafford *et al*., 1994). One possibility is that substance P allosterically binds to the nicotinic receptor and stabilizes its desensitized state, which may be present even in the absence of substance P (Clapham & Neher, 1984). As far as P_{2X_2} receptors are concerned, single channel recordings of their activity during substance P application will be necessary to cast light on this phenomenon. However, in analogy with the interpretation applied to nicotinic receptor data, the present results may be explained by facilitation of P_{2X_2} receptor desensitization by substance P, presumably bound to an unidentified subunit of the P_{2X_2} receptor. In favour of this interpretation is the observation that desensitized responses were largely depressed by substance P which also delayed recovery of ATP responses from desensitization.

Our previous experiments have suggested that on PC12 cells P_{2X_2} receptor desensitization is a heterogeneous process, which includes distinct fast and slow states (Giniatullin *et al*., 1996). According to this notion fast desensitization is typically manifested during ATP application as rapid current fade, which in the present study was significantly reduced by substance P. Slow desensitization is observed as a persistent depression of current peak amplitude after exposing the cell membrane to sustained application of ATP and is correlated with a rise in $[Ca^{2+}]_i$. Also in this case substance P enhanced slow desensitization and prolonged recovery from it.

Nevertheless, the view that substance P facilitated P_{2X_2} receptor desensitization is apparently in contrast with some observations of the present study. For instance, the response peak to short pulses of high concentrations of ATP was depressed even if there was no manifest desensitization. Perhaps these control responses were already limited by a degree of desensitization which developed very rapidly (Werman, 1976; Nistri & Constanti, 1979) beyond the resolution of the present measurements, and this was enhanced by the peptide application. It seems less likely that substance P merely converted closed channels to a desensitized state, since relatively large amplitude currents induced by low doses of ATP were unaffected.

Even if the present study does not identify the precise mechanism responsible for the substance P/ATP interaction, these novel data raise the interesting possibility that this peptide might control the neurotransmitter action of ATP. Future studies might explore any modulation by substance P of the neurotransmitter function of ATP for example on the dorsal horn neurones of the spinal cord (Burnstock & Wood, 1996) or on chromaffin cells of the adrenal gland (Nakazawa *et al*., 1990), since both tissues contain endogenous substance P (Otsuka & Yoshioka, 1993).

This work was supported by grants from CNR, INFM and MURST to A.N. R.G. and M.T. thank the Russian Foundation for Fundamental Research for financial support. L.K. is a predoctoral fellow of ICGEB. We would like to thank Massimo Righi for cell culture support.

References

ADAMS, P.R., BROWN, D.A. & JONES, S.W. (1983). Substance P inhibits the M-current in bullfrog sympathetic neurons. *Br. J. Pharmacol*., **79,** 330–333.

AKASU, T., KOJIMA, M. & KOKETSU, K. (1983). Substance P modulates the sensitivity of the nicotinic receptor in amphibian cholinergic transmission. *Br. J. Pharmacol*., **80,** 123–131.

BLANTON, M.P., LI, Y.-M., STIMSON, E.R., MAGGIO, J.E. & COHEN, J.B. (1994). Agonist-induced photoincorporation of a *p*-benzoylphenylalanine derivative of substance P into membrane-spanning region 2 of the *Torpedo* nicotinic acetylcholine receptor δ subunit. *Mol. Pharmacol*., **46,** 1048–1055.

BOYD, N.D. (1987). Two distinct kinetic phases of desensitization of acetylcholine receptors of cloned rat PC12 cells. *J. Physiol*., **389,** 45–67.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P2-purinoceptors? *Gen. Pharmacol*., **16,** 433–440.

BURNSTOCK, G. & WOOD, J.N. (1996). Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Curr. Opin. Neurobiol*., **6,** 526–532.

CLAPHAM, D.E. & NEHER, E. (1984). Substance P reduces acetylcholine-induced currents in isolated bovine chromaffin cells. *J. Physiol*., **347,** 255–277.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, C., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and the properties of an extended family of ATP-gated ion channels. *J. Neurosci*., **16,** 2495–2507.

GINIATULLIN, R., KHIROUG, L., TALANTOVA, M. & NISTRI, A. (1996). Fading and rebound of currents induced by ATP on PC12 cells. *Br. J. Pharmacol.*, **119,** 1045–1053.

INOUE, K., NAKAZAWA, K., FUJIMORI, K. & TAKANAKA, A. (1989). Extracellular adenosine 5′-triphosphate-evoked norepinephrine secretion not related to voltage-gated Ca^{2+} channels in pheochromocytoma PC12 cells. *Neurosci. Lett.*, **106,** 294–299.

KHIROUG, L., GINIATULLIN, R., TALANTOVA, M. & NISTRI, A. (1997). Role of intracellular calcium in fast and slow desensitization of P2-receptors in PC12 cells. *Br. J. Pharmacol.*, **120,** 1552–1560.

LIVETT, B.G., KOZOUZEK, V., MIZOBE, F. & DEAN, D.M. (1979). Substance P inhibits nicotinic activation of chromaffin cells. *Nature*, **278,** 256–257.

MORITA, K. & KATAYAMA, Y. (1992). Substance P inhibits activation of calcium-dependent potassium conductances in guinea-pig myenteric neurones. *J. Physiol.*, **447,** 293–308.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *J. Physiol.*, **428,** 257–272.

NISTRI, A. & CONSTANTI, A. (1979). Pharmacological characterization of different types of GABA and glutamate receptors in vertebrates and invertebrates. *Prog. Neurobiol.*, **13,** 117–235.

OTSUKA, M. & YOSHIOKA, K. (1993). Neurotransmitter functions of mammalian tachykinins. *Physiol. Rev.*, **73,** 229–307.

PHENNA, S., CARPENTER, E., PEERS, C., MAUDSLEY, S. & GENT, J.P. (1996). Inhibition of Ca^{2+}-sensitive K^+ currents in NG 108-15 cells by substance P and related tachykinins. *Br. J. Pharmacol.*, **119,** 315–320.

ROGERS, M. & DANI, J.A. (1995). Comparison of quantitative calcium flux through NMDA, ATP, and ACh receptor channels. *Biophys. J.*, **68,** 501–506.

ROLE, L.W., LEEMAN, S.E. & PERLMAN, R.L. (1981). Somatostatin and substance P inhibit catecholamine secretion from isolated cells of guinea-pig adrenal medulla. *Neuroscience*, **6,** 1813–1821.

SIMASKO, S.M., DURKIN, J.A. & WEILAND, G.A. (1987). Effects of substance P on nicotinic acetylcholine receptor function in PC12 cells. *J. Neurochem.*, **49,** 253–260.

SIMMONS, L.K., SHUETZE, S.M. & ROLE, L.W. (1990). Substance P modulates single-channel properties of neuronal nicotinic acetylcholine receptors. *Neuron*, **2,** 393–403.

SNEDDON, P. (1995). Co-transmission. In *Neurotransmitter Release and its Modulation*, ed. Powis, P.A. & Bunn, S.J. pp. 22–37. Cambridge: Cambridge University Press.

STAFFORD, G.A., OSWALD, R.E. & WEILAND, G.A. (1994). The β subunit of neuronal nicotinic acetylcholine receptors is a determinant of the affinity for substance P inhibition. *Mol. Pharmacol.*, **45,** 758–762.

URBAN, L., THOMPSON, S.W.N. & DRAY, A. (1994). Modulation of spinal excitability: co-operation between neurokinin and excitatory amino acid neurotransmitters. *Trends Neurosci.*, **17,** 432–438.

VALENTA, D.C., DOWNING, J.E. & ROLE, L.W. (1993). Peptide modulation of ACh receptor desensitization controls neurotransmitter release from chicken sympathetic neurons. *J. Neurophysiol.*, **69,** 928–942.

WERMAN, R. (1976). Desensitization and stoichiometry of transmitter-receptor interactions. In *Electrobiology of Nerve, Synapse and Muscle*. ed. Reuben, J.P., Purpura, D.P., Bennett, M.V.L. & Kandel, E.R. pp. 45–60. New York: Raven Press.

WILDMAN, S.S., KING, B.F. & BURNSTOCK, G. (1997). Potentiation of ATP-responses at a recombinant P_{2X_2} receptor by neurotransmitter and related substances. *Br. J. Pharmacol.*, **120,** 221–224.

YAMADA, K. & AKASU, T. (1996). Substance P suppresses $GABA_A$ receptor function via protein kinase C in primary sensory neurones of bullfrogs. *J. Physiol.*, **496,** 439–449.

(Received December 9, 1996
Revised February 24, 1997
Accepted March 4, 1997)